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PREPARATION OF POLYMERIC NANOPARTICLES FOR TOPICAL ANTI-INFLAMMATORY APPLICATIONS

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Résumé

L'objectif de cette thèse est d'encapsuler l'indométacine et l'huile essentielle de *Nigella Sativa* L. dans des nanoparticules polymériques. Cette huile est extraite à partir de ses graines afin d'optimiser son utilisation par voie cutanée et potentialiser son activité anti-inflammatoire.

Pour ce faire, des nanoparticules à base de poly- ϵ -caprolactone ont été préparées par nanoprécipitation. Une étude systématique a été menée pour comprendre l'effet de la variation des paramètres de préparation sur les propriétés colloïdales des nanoparticules obtenues. Une fois les différents paramètres optimisés, l'indométacine et l'huile essentielle de *Nigella Sativa* L. ont été encapsulées séparément dans les nanoparticules polymériques. Puis, l'ensemble, indométacine et huile essentielle de *Nigella Sativa* L. a été encapsulé. Les nanoparticules préparées ont été caractérisées en termes de taille, potentiel zêta, stabilité et performance d'encapsulation. Ensuite, nous avons mené une étude *ex-vivo* et *in-vivo* des nanoparticules obtenues afin d'évaluer le potentiel de pénétration cutanée d'une part, et le potentiel clinique dans la prise en charge de l'inflammation.

Summary

The objective of this PhD thesis was to extract the *Nigella Sativa* L. Seeds Essential Oil and its encapsulation together with indomethacin within polymeric nanoparticles in order to reduce taken amount and to enhance indomethacin cutaneous penetration, and anti-inflammatory activity. To this direction poly- ϵ -caprolactone based nanoparticles were designed using nanoprecipitation method. A systematic study was performed to figure out the effect of process and formulation parameters on the characteristics of obtained nanoparticles. Once the effects of all parameters were studied, then indomethacin and *Nigella Sativa* L. Seeds Essential Oil was encapsulated separately. Consequently, both together indomethacin and *Nigella Sativa* L. Seeds Essential Oil was encapsulated. Then prepared nanoparticles were characterized in terms of size, zeta potential, DSC, FTIR, stability, and encapsulation efficiency. In addition, *ex vivo* skin penetration and *in vivo* anti-inflammatory activity of designed nanoparticles was investigated.

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Part I

GENERAL INTRODUCTION

Introduction

The word “Inflammation” is derived from the latin “inflammare” word that means to set on fire. Inflammation is implicated in diseases such as arthritis, cancer, stroke, neurodegenerative and cardiovascular disorders (Ricciotti and FitzGerald, 2011). Host can be protected from the aggressive agents like irritants, infection, or damaged cells and other injuries by a natural defense cascade that is named inflammation and comprises different interactions between cells and mediators, in other words inflammation is an immunity system reaction to an infection or injury. The raised blood flow, amplified cellular metabolism, cellular influx, soluble mediators release, vasodilation, fluids extravasation, on site heat, redness, swelling and pain are the signs of inflammation. Acute inflammation is the first reaction that is identified through the raised plasma movement and indigenous immune cells as neutrophils and macrophage from the blood into the damaged tissues (Ricciotti and FitzGerald, 2011) (Ferrero-Miliani et al., 2007) (Medzhitov, 2008) (Scott et al., 2004). In fact, inflammatory agents application cause cell membranes phospholipase A₂ activation that would trigger the release of arachidonic acid and inflammatory mediators (cytokines, serotonin, histamine, prostaglandin and leukotrienes) that make easy leukocytes migration to the inflamed site (Sarkhel, 2016). Moreover, release of products such as histamine, bradykinin, serotonin, and cyclooxygenase (COX) is linked with the first phase of inflammation (0-1 h), while prostaglandins release, oxygen-derived free radicals production and polymorphonuclear leukocytes (PMN) infiltration is related to the late phase of edema (Sadeghi et al., 2014). Non-Steroidal Anti-inflammatory Drugs (NSAIDs) are being used in a huge quantity all over the world. It is considered that above 90 % of patients aged higher than 65 years are using NSAIDs (Sostres et al., 2010). In NSAIDs class, heterogeneous drug molecules having different structures and various benefit/risk profiles without steroid core within their chemical formula are included. Indeed, NSAIDs are mostly prescribed in primary health care (Jones, 2001). Indomethacin (IND) is a gold standard within NSAIDs drugs category which lessens fever, swelling, stiffness, and pain. Since indomethacin inhibits cyclooxygenase (COX) enzymes to exert its pharmacological activity therefore indomethacin action is associated with the presence of gastrointestinal side effects (Sostres et al., 2010) (Cordero et al., 2001). Indicated side effects can be reduced by the reduction of taking amount and target drug delivery design of indomethacin (Závišová et al., 2007). Therefore, to control indomethacin delivery ratio and to decline its dosage, skin can be used for the administration of indomethacin. Despite that indomethacin is a potent anti-inflammatory drug and shown an

acceptable efficiency for the external application, is a poorly water-soluble drug (~ 0.01 mg/ml) and weak acid in nature like majority of NSAIDs (Prausnitz and Langer, 2008). Consequently, indomethacin this aqueous insolubility in skin as a drawback leads to poor bioavailability. From the other hand, indomethacin is not stable under UV light (Lin et al., 1994). These reasons together make indomethacin a good drug candidate for the encapsulation within polymeric nanoparticles. However, in spite of skin crucial function of body protection, permeability of digestive tube mucous membrane towards drugs is comparably higher than skin (stratum corneum). This fact can act as a barrier for the design of dosage forms administered via this route. Thus, to deal with such challenge a number of chemical and physical strategies have been known to improve skin penetration that can face skin to irritation, damage, etc. It is highly motivating to keep the skin barrier function normal and deliver drug. Hence, skin drug delivery using nanoparticles may grasp this objective (Tomoda et al., 2012). Limitations attributed to number of drugs in term of low bioavailability, poor stability, and unpleasant organoleptic properties are possible to be solved by encapsulation (Iqbal et al., 2015). A special attention has focused on the encapsulation due to its ability of removing barriers that faced conventional dosage forms. The encapsulation method mostly selecting based on the hydrophobicity or hydrophilicity nature of drug molecules (Jelvehgari and Montazam, 2012). *Nigella Sativa* (Ranunculaceae family) is the native and annual herbaceous plant of Southwest Asia, North Africa, and Southern Europe. *Nigella Sativa* as medicinal plant is considered to be safer than modern medicines for the treatment of different diseases. Since centuries *Nigella Sativa* Seeds Oil was used for treatment of diverse diseases. The biological activities of *Nigella Sativa* Seeds Essential Oil (NSSEO) such as: anti-inflammatory, analgesic, immunomodulatory, spasmolytic, anti-oxidant and etc., has been well studied. Since NSSEO including thymoquinone prevent cytokines production, consequently different diseases as rheumatism, bronchitis, asthma and etc., are treated by the seeds of NSSEO (Ahmad et al., 2013). The principal constituents of NSSEO encompassing thymoquinone, p-cymene, α -pinene, thymohydroquinone, dithymoquinone, linoleic acid and nigellone (Al Juhaimi et al., 2013) (Aljabre et al., 2015). As thymoquinone is the most abundant component of NSSEO therefore, it may boost indomethacin anti-inflammatory activity (Aljabre et al., 2015). In order to encapsulate drug molecules several techniques can be used. Nanoprecipitation is an encapsulation method also called solvent displacement or interfacial deposition.

Nanoprecipitation method has firstly developed by Fessi et al (Fessi et al., 1989), and this technique has been commonly used for the encapsulation of hydrophobic drug molecules (nanocapsule and nanosphere forms). The organic phase or solvent phase principally containing film-forming materials, drug molecule, lipophilic surfactant, oil and solvent of drug molecule or if required oil solvent. Indeed, in this method natural, synthetic or semi-synthetic polymers can be employed; surfactants should be added as well in order to avoid the aggregation of nanoparticles (Miladi et al., 2016). Here in this method certain polymers particularly biodegradable polyesters like polylactide (PLA), polylactide-co-glycolide (PLGA) and polycaprolactone (PCL), have been used. To design nanoparticles by nanoprecipitation method, solvent and nonsolvent phases are necessary (Fessi et al., 1989). In addition, ethanol, acetone, hexane, methylene chloride and dioxane are from the frequently used solvents. Normally, solvent and nonsolvent phases are named respectively organic and aqueous phases (Miladi et al., 2016) (Mora-Huertas et al., 2010). Polycaprolactone (PCL) is a biocompatible and biodegradable polymer that is employed to encapsulate certain drugs for the purposes of bioavailability improvement, targeting and sustained delivery. For biodegradable polymers do not need to be removed from the body after application (Park et al., 2005). Furthermore, PCL has been commonly used for drug delivery purposes (Dash and Konkimalla, 2012). Quantum dots are defined as tiny particles or nanocrystals of a semiconducting material with diameters in the range of 2-10 nanometers (10-50 atoms) (Ekimov and Onushchenko, 1981). Quantum dots have unique electronic properties, intermediate between those of bulk semiconductors and discrete molecules, which are partly the result of the unusually high surface-to-volume ratios for these particles (Marc, 1993) (Ashoori, 1996) (Collier et al., 1998). The most noticeable result of this is fluorescence, wherein the nanocrystals can produce distinctive colours determined by the size of the particles. The discrete, quantized energy levels of quantum dots relate them more closely to atoms than bulk materials and have resulted in quantum dots being nicknamed 'artificial atoms'. Generally, as the size of the crystal decreases, the difference in energy between the highest valence band and the lowest conduction band increases. Indeed, more energy is then needed to excite the dot, and concurrently, more energy is released when the crystal returns to its ground state, resulting in a colour shift from red to blue in the emitted light. Quantum dots can emit any colour of light from the same material simply through changing the size of dot accordingly. Furthermore, due to the high level of control possible over the size of the nanocrystals produced, quantum dots can be tuned during manufacturing to emit any colour of light (Yoffe, 2001). Quantum dots can be classified based on their composition and structure

into different types of Core-Type Quantum Dots, Core-Shell Quantum Dots, and Alloyed Quantum Dots. The unique size and composition tunable electronic property of these very small, semiconducting quantum dots make them very attractive for a variety of applications and new technologies (Vahala, 2003). Quantum dots are predominantly important for optical applications owing to their bright, pure colours along with their ability to emit rainbow of colours coupled with their high efficiencies, longer lifetimes and high extinction coefficient (Yoffe, 2001) (Nirmal and Brus, 1999) (Sargent, 2012). Quantum dots small sizes allow them to go anywhere in the body making them suitable for different bio-medical applications like medical imaging, biosensors, etc. Currently, fluorescence based biosensors depend on organic dyes with a broad spectral width that limits their effectiveness to a small number of colours and shorter lifetimes to tag the agents. In biomedical applications, the fact that made quantum dots superior to traditional organic dyes is that they can emit the whole spectrum, are brighter and have little degradation over time (Medintz et al., 2005). In fact, quantum dots nanocrystals are available in both aqueous and organic formulations suitable for use in different applications.

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Part II
BIBLIOGRAPHY

Summary

In this part of thesis, a profound analysis of state of art has been done that can provide us the deep knowledge and better understanding of previously performed studies by different researchers before our experimental study. It is also helpful to us for making a good decision to select the best approach to grasp our goal and to bring the novelty to our study. An important feature of this work is the introduction of employed techniques for the encapsulation of natural extracts in terms of importance and limitations. Therefore, to initiate the state of art, the maximum amount of available date regarding plant extracts encapsulation were collected.

The most critical section of our literature review is the need of initial knowledge concerning Non-steroidal Anti-Inflammatory Drugs (NSAIDs) polymeric encapsulation. In the first part, Inflammation, NSAIDs mechanism of action, classification, side effects, and their physicochemical properties are explained. Moreover, the encapsulation objectives, used methods for the encapsulation, challenges and opportunities for the encapsulation of NSAIDs are described. Basic information relating to the structure, nature, properties, and medical usage of plant extracts are provided. The polymeric encapsulation of plant extracts, their characterization, and the goal of plant extracts encapsulation are the issue on which a special focus has been made. The methods such as: Emulsions solvent evaporation, nanoprecipitation, emulsion solvent diffusion, and ionic gelation (IG) were included. Encapsulation role of plant extracts for their stability and delivery have also brought to light. In addition, the results obtained from studies related to biological activities *in vitro* and *in vivo* models were highlighted. The next section is associated with the description of nanoprecipitation technique. It will explain the formation mechanism, *in vitro* release profile, marketed products containing nanoparticles prepared by nanoprecipitation method and application of this method in medicine, food and agriculture technologies. Furthermore, nanoprecipitation method advantages, limitations and industrial scale-up are also highlighted.

II.1. Encapsulation of NSAIDs for inflammation management: Overview, progress, challenges and prospects

Summary

NSAIDs as the mostly prescribed drugs are a class of heterogeneous drugs having different structural groups and various profiles of benefit/risk that do not contain steroid core in their chemical formula. Approximately 30 million people are taking NSAIDs as over-the-counter (OTC) medication daily in the world. The design of nanocarriers can be a good approach for the target drug delivery in the treatment of cancer, cardiovascular and inflammatory diseases. Molecules arrangements that sizes range between 1 and 100 nm in at least one dimension called nanoparticles (NPs). NPs can possess nanospheres (NS) or nanocapsules (NC) forms, respectively represents metrical and vesicular organization. Inflammation is taken from the latin word of *inflammare* that depict set on fire and can be defined by its common signs of heat on site, redness, swelling and pain. In fact, infection and injury produce an inflammation as an answer by immunity system response. NSAIDs inhibit cyclooxygenase enzymes, which play roles in both physiologic and pathologic cases (COX-1 is constitutive and COX-2 is inducible by inflammation that found abundantly at the site of inflammation). Indeed, COX-1 plays a significant role in normal physiologic function of gastrointestinal (GI) system, platelets, kidneys and vascular endothelium. The therapeutic effect of NSAIDs is associated from their action on COX-2 whereas their side effects are because of the COX-1 inhibition. Selective NSAIDs have a higher affinity for COX-2 than COX-1. NSAIDs are classified into 8 groups of oxicams, phenylpropionic (arylpropionic) acid derivatives, phenylacetic derivatives, sulfo-nanilides (nimesulide), indoleacetic acid derivatives, pyrazolone derivatives, para-aminophenol derivatives and salicylates based on their chemical structure. Furthermore, plasma half-life can be another criterion for NSAIDs classification. Generally, NSAIDs are taken by oral route. Nevertheless, NSAIDs oral administration would not provide merely therapeutic properties (anti-inflammatory, analgesic and antipyretic activities) but also gastrointestinal side effects. Furthermore, NSAIDs can be administered topically or through intramuscular injection as well. The dermal delivery of NSAIDs is dealt with these limitations attributing to the oral administration by reduced systemic exposure of drug molecule. Generally, NSAIDs are weak acids with pKa values ranged between 3 and 5. Encapsulation can reduces systemic toxicity, protects unstable molecules from degradation in the gastrointestinal tract, provides controlled release properties and covers drug disagreeable taste. Furthermore, encapsulation of NSAIDs could decrease their mucosal contacts. Since NSAIDs are commonly weak acids, as a result encapsulation can decline their toxicity. Different polymers are used for the preparation of particulate carriers. The physicochemical properties of these polymers are possibly various while they are commonly biodegradable and biocompatible. Particulates can be prepared by different methods that are relied on the usage of preformed polymers and methods based on the polymerization of monomers. Techniques using the preformed polymers are including emulsion coacervation, nanoprecipitation, salting out, dialysis, spray drying, emulsion

solvent diffusion and solvent evaporation. Nanoprecipitation is the most used technique for the drugs encapsulation. The criteria for selection of encapsulation method are the drug physicochemical characteristics. Liposomes among lipid based carriers were firstly developed. The most *in vivo* investigated drug molecules were flurbiprofen, diclofenac, indomethacin and oxicams and it has been also found that conventional dosage forms are less efficient than encapsulated NSAIDs. Indeed, the used method for the preparation of drug vehicle could exert an effect on designed nanocapsules. Thus, it has to be taken into account that under the operating condition the selected method should not disrupt the active ingredient stability. In this review the efforts has been made to describe the theories and detailed researches about NSAIDs encapsulation in biodegradable polymers and lipid based structures. The inflammation, NSAIDs mechanism of action, side effects, classification, routes of administration, physicochemical properties are described in details. In addition, drugs encapsulation methods within biodegradable polymers and lipids with examples are discussed. The most relevant *in vivo* applications of encapsulated NSAIDs are also reviewed and analyzed. Furthermore, most of recently performed researches on the encapsulation of NSAIDs, especially indomethacin are compiled as well.



Review

Encapsulation of NSAIDs for inflammation management: Overview, progress, challenges and prospects



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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed drugs. Debilitating diseases such as rheumatoid arthritis and osteoarthritis are commonly managed by NSAIDs. However, NSAIDs pharmacological mechanism is often associated with the presence of gastrointestinal side effects. NSAIDs encapsulation is performed in order to overcome some of the drawbacks linked to their clinical use. To fulfill this purpose, various vectors like polymer-based nanoparticles, liposomes and solid lipid nanoparticles have been proposed. Such vehicles could have advantages but some limitations as well. This manuscript highlights current NSAIDs encapsulation approaches based on either preformed polymers or lipids. Moreover, properties of the prepared carriers and their applications are also discussed. Many factors are taken into account for selecting carrier type and encapsulation method. It was concluded that different vehicles and preparation methods have been employed for NSAIDs encapsulation. Mostly, vehicles sizes ranged within the nanoscale. Main advantages that have been confirmed by *in vitro* and *in vivo* studies include promoted stability, sustained release and bioavailability enhancement.

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1. Introduction

Globally, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most consumed drug substances. As rheumatic diseases are increasing, the usage of NSAIDs is expected to be boosted as well. The patients above 65 years of age are considered to account for more than 90% of NSAIDs users (Sostres et al., 2010). NSAIDs are a group of heterogeneous molecules with various structural classes and variable benefit/risk profiles, which do not comprise steroid core in their chemical structure. Diclofenac and ibuprofen are the most widely used NSAIDs in the world (Araújo et al., 2009; Patrono and Rocca, 2009). They are followed by naproxen (22%), indomethacin (5%), piroxicam (3%), ketoprofen (2%), and benorilate (1%). The majority of the NSAIDs are prescribed in primary healthcare (Jones, 2001). It was reported that Alzheimer's disease would result in from brain low-grade inflammation. Therefore, NSAIDs intake decreases the risk of Alzheimer's disease (de Villiers et al., 2011; McGeer and McGeer, 2007; Lehrer, 2014).

Advances in scientific technologies are currently being directed towards evolution of new pharmaceutical products. By exploration of these advanced and diversified technologies, conventional drug delivery approaches are bit by bit replaced *via* more adaptable and well-refined drug delivery systems. Indeed, special focus is paid on tackling the constraints associated with conventional drug delivery (Iqbal et al., 2015).

Nanocarriers are designed to deliver active molecules to the right site of action, at the right time, and in accurate amount. Nanoparticles appear to be a useful platform for treatment of cancer, cardiovascular and inflammatory diseases (Barua and Mitragotri, 2014). It is one of the most attractive sides of drug delivery researches that have attracted considerable attention (Ibrahim et al., 2013). Pharmaceutical and biotechnological industries have been extremely influenced by nanotechnology (Zhu et al., 2014). Nanoparticles (NPs) are arrangements of molecules where sizes range between 1 and 100 nm in at least one dimension. This increases surface area and consequently, changes their biological activity with comparison to the original bulk substances (Yildirimer et al., 2011). In the past two decades, the use of nanoparticles (NPs) has risen exponentially in experimental and clinical settings due to their wide range of biomedical applications, for example in drug delivery, imaging and cell tracking technologies. Different materials e.g. polymers, lipids and metals can be used for nanoparticle fabrication and a large number of active molecules can be hosted. The majority of encompassed nanoscale particles in clinical experiments are liposomes and polymer based nanoparticles (Chung et al., 2015). NPs could be nanospheres (NS) or nanocapsules (NC), respectively representing matricial and vesicular organization. Biodegradable polymers could undergo large physicochemical modification in order to improve biopharmaceutical activity after *in vivo* administration. Therefore, biopolymeric nanoparticles are becoming a promising approach in the treatment of several diseases. Moreover, in the area of nanomedicine, nanoparticles can be combined with smart components to permit their delivery through biological

barriers, such as, skin, mucus, blood, extracellular matrix, cellular and subcellular organelles (Elsababy and Wooleya, 2012). For efficient encapsulation, chemical solubility in solvents must be taken into account during nanoparticles preparation (Mora-Huertas et al., 2010). In this review, we mainly focused on the theories and detailed researches concerning encapsulation of NSAIDs in biodegradable polymers and lipid structures. In addition, the most relevant *in vivo* applications of encapsulated NSAIDs are reviewed and discussed.

2. Inflammation

The word "Inflammation" is derived from the latin word *inflammare*, which means to set on fire. On site heat, redness, swelling and pain are the four basic signs of inflammation (Scott et al., 2004). Obviously, inflammation is an answer of immunity system to infection and injury. Arthritis, cancer, stroke, neurodegenerative and cardiovascular disorders are diseases in which, inflammation is implicated (Ricciotti and FitzGerald, 2011) (Fig. 1).

3. NSAIDs mechanism of action and side effects development

NSAIDs belong to a group of extensively prescribed drugs, which are also available as over-the-counter medication (Bate-man, 2012). Each day about 30 million people take NSAIDs around the world. Acute and chronic inflammation in particular pain associated with inflammatory condition is effectively treated by NSAIDs (Gerstein et al., 2014; Burian and Geisslinger, 2005). In 1971, the biochemical mechanism of action of NSAIDs was reported to be due to inhibition of cyclooxygenase. In Fig. 1, the different types of cyclooxygenase enzymes (COX) and their roles in both physiologic and pathologic cases are figured out. COX-1 is constitutive while COX-2 is inducible by inflammation and found

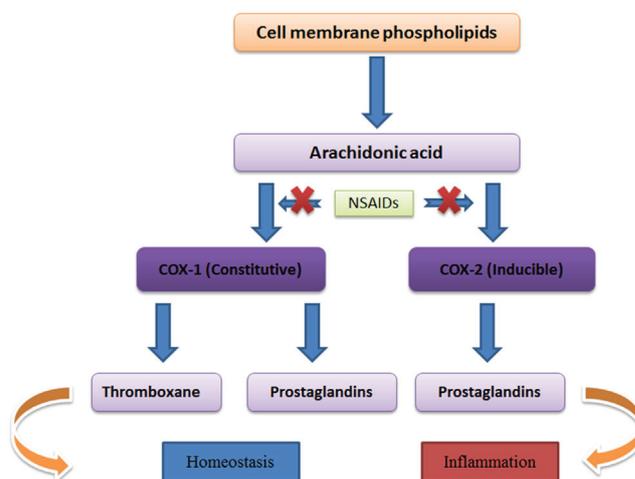


Fig. 1. Biochemical pathways in the inflammation process and NSAIDs target points.

abundantly at the site of inflammation, thereby playing a critical role in inflammatory events. During inflammation, COX-2 is responsible for production of prostaglandins (PGs), mediators of inflammation. COX-1 synthesizes PGs, which regulate physiologic processes of the gut including mucosal protection, gastrointestinal secretion and motility (Dey et al., 2006). COX-1 plays an important role in normal physiologic function of gastrointestinal (GI) system, platelets, kidneys and vascular endothelium. The therapeutic efficacy of NSAIDs is related to their action on COX-2 while their side effects are due to the inhibition on COX-1 (de Villiers et al., 2011). Selective NSAIDs have a higher affinity for COX-2 than COX-1 (e.g. Celecoxib, Nimesulide and Nabumetone) (McGeer and McGeer, 2007; Kim et al., 2010). However, it has been reported that selective COX-2 inhibitors increase the risk of heart disease particularly myocardial infarction (Chen and Ashcroft, 2007). NSAIDs are prescribed in different chronic diseases (rheumatoid arthritis, osteoarthritis and ankylosing spondylitis) and often at high dose triggering considerable number of side effects and poor patient compliance (Ejaz et al., 2004). Moreover, these drugs need a strict monitoring of patients with high risk of heart and kidney diseases. In term of pharmacokinetics, the majority of NSAIDs do not have considerable first-pass metabolism and enter the blood circulation almost completely through oral administration (Gerstein et al., 2014). In Table 1, risks that could complicate gastrointestinal side effects in patients treated by NSAIDs are shown.

4. NSAIDs classification

NSAIDs classification is based on their chemical structure. They include 8 groups such as, oxicams (meloxicam, piroxicam, tenoxicam), phenylpropionic (arylpropionic) acid derivatives (e.g. fenbufen, ibuprofen, naproxen, tiaprofenic acid, mefenamic acid, indoprofen, ketoprofen, phenoprofen, zomepirac, dexibuprofen, dexketoprofen, dexibuprofen, oxaprozin, tiaprofenic acid, suprofen), phenylacetic derivatives (diclofenac, aceclofenac, fenclofenac, alclofenac, bufexamac, nabumeton, felbinac), sulfonanilides (nimesulide), indoleacetic acid derivatives (indometacin, acemetasin, tolmetin, ketorolac, sulindac), pyrazolone derivatives (aminopyrine, metamizol, phenylbutazone, oxyphenbutazone, propyphenbutazone, amidopyrine), para- aminophenol derivatives (acetaminophen) and salicylates (acetylsalicylic acid,

Table 2

NSAIDs classification according to plasma half-life (Ziltener et al., 2010).

Short half- life	<6 h	Long half- life	>6 h
Aspirin	25–33	Diflunisal	8–12
Diclofenac	1–2	Naproxen	12–15
Ibuprofen	1–2.5	Salsalate	3.5–16
Ketoprofen	1.5–4	Sulindac	16–18
Fenoprofen	2–3	Piroxicam	24–38
Mefanamic acid	2–4	Nabumetone	24
Meclofenamate	3–4	Oxaprozin	25
Indomethacin	4–5	Phenylbutazone	77
Flurbiprofen	4–6		
Ketorolac	4–6		
Etodolac	6–7		

diflunisal, sodium salicylate, salicylic acid, salicylamide) (Bateman, 2012; Okyar et al., 2012).

Moreover, NSAIDs could be also classified based on their plasma half-life that is presented in Table 2.

5. NSAIDs administration routes

Mostly, NSAIDs are taken *via* oral route. However, *via* this route of administration, they provide not only efficient anti-inflammatory, analgesic and antipyretic properties but also gastrointestinal side effects. Furthermore, NSAIDs can be administered topically or through intramuscular injection as well. NSAIDs dermal delivery is able to overcome drawbacks associated with oral administration by reduced systemic exposure of the active substance (Beetge et al., 2000; Ziltener et al., 2010). Table 3 shows the different delivery routes of NSAIDs.

6. NSAIDs physicochemical properties

In general, NSAIDs are weak acids with pKa values ranging between 3 and 5 but despite this general rule, as shown in Table 4, there are large variations in regard to their physicochemical properties (e.g., ionization constants (pK), solubility, partition coefficients). These differences may cause variation in their bioavailability and distribution in the body (Rainsford, 1999; Peter et al., 1996), and consequently variation in both efficacy and tolerability. The physicochemical and pharmacokinetic properties of mostly used NSAIDs are comparatively described in Table 4.

Table 1

Gastrointestinal complications risk factors for NSAIDs takers, classified according to their risk (from Sostres et al., 2010 with modifications) (Sostres et al., 2010).

<div style="text-align: center;"> <div style="background-color: #0070C0; color: white; padding: 5px; margin-bottom: 10px;">Highest risk</div> <div style="font-size: 2em; color: #0070C0; margin: 0 auto;">↓</div> <div style="background-color: #0070C0; color: white; padding: 5px; margin-top: 10px;">Lowest risk</div> </div>	Complicated ulcers previous history
	Anticoagulants complementary use
	Numerous NSAID use, compromising low-dose aspirin
	Uncomplicated ulcers previous history
	NSAID high dose (or piroxicam or ketorolac use)
	Age > 60 years
	Sever disease
	<i>Helicobacter pylori</i> infection
	Complementary use of corticosteroids

Table 3
NSAIDs administration routes (from Kim et al., 2010 with modifications) (Kim et al., 2010).

Name	Administration route
Acetylsalicylic acid (aspirin)	Oral, rectal
Choline Magnesium Trisalicylate	Oral
Salsalte	Oral
Diflunisal	Oral
Diclofenac	Oral, ocular, dermal, parenteral (intravenous and intramuscular)
Etodolac	Oral
Indomethacin	Oral, rectal, intravenous
Ketorolac	Oral, topical, ocular, dermal, parenteral (intravenous and intramuscular)
Nabumetone	Oral
Sulindac	Oral
Tolmetin	Oral
Bromfenac	Dermal
Nepafenac	Ocular
Flurbiprofen	Oral, ocular
Ketoprofen	Oral
Ibuprofen	Oral, intravenous
Naproxen	Oral
Fenoprofen	Oral
Oxaprozin	Oral
Piroxicam	Oral
Meloxicam	Oral
Meclofenamate	Oral
Celecoxib	Oral

Table 4
NSAIDs physicochemical and pharmacokinetic properties (from Beetge et al., 2000 with modifications).

Properties	Ibuprofen	Indomethacin	Naproxen	Ketoprofen	Piroxicam	Diclofenac
Physicochemical						
Chemical formula	C ₁₃ H ₁₈ O ₂	C ₁₉ H ₁₆ ClNO ₄	C ₁₄ H ₁₄ O ₃	C ₁₆ H ₁₄ O ₃	C ₁₅ H ₁₃ N ₃ O ₄ S	C ₁₄ H ₁₁ Cl ₂ NO ₂
Molecular mass	206.30	357.80	230.30	254.29	331.40	296.14
Solubility in water	practically insoluble	practically insoluble	practically insoluble	practically insoluble	practically insoluble	sparingly soluble
<i>Sweetman (2009)</i>						
LogP	3.60	3.8	3.22	0.97	1.8	4.51
pKa	5.3	4.5	4.2	4.45	5.3	4.15
Melting point	76.5 °C	160 °C	156 °C	94.5 °C	199 °C	156–158 °C
Pharmacokinetic						
AUC (mg/ml per h)	88.09	243.22	258.65	269.45	527.00	82.6–103.4
Tmax (h)	2	24	2	24	30	0.5–1
t _{1/2} (h)	2.2	6.10	17.10	1.80	40.80	1.92
Cmax (mg/ml)	4.43	11.95	10.25	7.1	16.23	–

7. The promise of NSAIDs encapsulation

Poor aqueous solubility, low bioavailability, poor stability, bitter taste and unpleasant odor of some active agents are among the most encountered problems. Generally, encapsulation enables overcoming such obstacles. Encapsulation is an ingenious delivery opportunity for various drugs. For instance, it decreases systemic toxicity, protects unstable molecules from degradation in the gastrointestinal tract, provides controlled release properties and masks the disagreeable taste of drug. Controlled release, targeted delivery enhances therapeutic efficacy compared to conventional medicine. The particle size and surface charge are the factors that can influence release kinetics of nanomedicines (Arida and Al-Tabakha, 2007). Encapsulation of NSAIDs is an interesting approach for their safe usage. Improvement of therapeutic efficacy and reduction in the severity of gastrointestinal side effects through modification of drug release can be considered in NSAIDs development. From the other hand, encapsulation would decrease the mucosal contact of NSAIDs, which are mostly weak acids consequently reduce the toxicity (Reis et al., 2013). To promote the patient compliance, these formulations are designed by a change in their duration of action and plasma peak concentrations (Phillips et al., 2010; Iqbal et al., 2015; Rwei et al., 2015). In addition to the

drug and materials physicochemical properties, the formulation of nanomedicine is associated to the selection of appropriate polymeric and lipidic systems giving highest encapsulation efficiency, enhanced bioavailability and retention time.

8. Preformed polymer based carriers

In the last decades, colloidal carriers have gained remarkable attention due to active ingredient protection from inactivation or degradation and drugs toxicity diminution (Tammam et al., 2012; Barratt, 2003; Khachane et al., 2011; Mazzaferro et al., 2012).

Encapsulation could improve the efficacy of active molecules through modification of their physicochemical properties (Gagliardi et al., 2012; Heneweer et al., 2012; Herrero et al., 2012; Mora-Huertas et al., 2010). In comparison to drug conventional delivery systems, particulate carriers may provide an increase in drugs absorption and drug targeting (Mohanraj and Chen, 2006). Furthermore, unpleasant taste and odour of several drugs could be masked by encapsulation, and a prolonged release of the active molecule could be obtained as well (Levchenko et al., 2012; Poletto et al., 2012; Wang et al., 2012; Cenni et al., 2008; Sahoo et al., 2007; Miladi et al., 2013; e Silva et al., 2012). To prepare particulate carriers, various polymers are used. Despite

biodegradable and biocompatible common properties of these polymers, their physicochemical characteristics may be different. The applications of the carriers in biomedical field are growing (Ahmad et al., 2013; Soares, 2013; Miladi et al., 2014). The physicochemical properties of the nanoparticles (e.g. hydrophobicity, zeta potential), drug release characteristics (e.g. delayed, prolonged, triggered) and biological behavior (e.g. bioadhesion, improved cellular uptake) are all polymer dependent. Drugs may become non-toxic and stable in blood *via* encapsulation in biodegradable polymers (Kumari et al., 2010; Galindo-Rodriguez et al., 2005; des Rieux et al., 2006). In general, final application and toxicity of the polymer are the main concerns in the selection of the polymer. Generally, particles with modulated surface coated with substances such as polyethylene glycol (PEG) will not be uptaken by macrophages (Zafar et al., 2014). Drug release from the polymer based carriers in a time or condition-dependent way could take place either by (surface or bulk) erosion driven, or by diffusion driven release mechanisms (Gu et al., 2007).

8.1. Encapsulation methods

The techniques used for the formulation of particulate carrier systems are classified into two categories: techniques based on the use of preformed polymers and methods depended on polymerization of monomers. Techniques based on the use of preformed polymers comprise emulsion coacervation, nanoprecipitation, salting out, dialysis, spray drying, emulsion solvent diffusion and solvent evaporation. Methods relying on the use of monomer polymerization include mainly emulsion polymerization and interfacial polymerization. Each technique has to be developed individually for every class of compounds, which is costly and time-consuming. The nanoprecipitation, emulsion-diffusion, double emulsification, emulsion-coacervation, polymer coating and layer-by-layer are six classic methods, which are usually employed for encapsulation of nanoparticles. Among these techniques, Table 5 describes the most widely used techniques in NASIDs encapsulation. As listed in Table 5, each of these methods has its own advantages and limitations (Mora-Huertas et al., 2010; Lattuada and Hatton, 2011).

As reported in Table 5, nanoprecipitation technique is the most employed method for the encapsulation of drugs.

8.1.1. Nanoprecipitation

Fessi et al. developed nanoprecipitation method in 1986 for the first time, which is also named solvent displacement, or interfacial deposition method. As demonstrated in Fig. 2 this technique includes two phases, the solvent phase consists of one or mixture of solvents (acetone, ethanol, hexane, methylene chloride or dioxane). Lipophilic active ingredients or lipophilic surface-active agents are dissolved in solvent phase. The non-solvent phase could be a mixture of non-solvents and may contain surfactants. The non-solvent phase is mainly water.

In general, biodegradable polyesters, especially Poly(ϵ - caprolactone) (PCL), poly(lactide) (PLA) and poly (lactide-co-glicolide) (PLGA) are extensively employed polymers in this technique. Eudragit[®] (polymethacrylate-based copolymers) is another polymer that can be used (Mora-Huertas et al., 2010). Synthetic polymers are more preferred because of their higher purity and better reproducibility as compared to natural polymers (Khoee and Yaghoobian, 2009). It is worth mentioning that diverse types of triglycerides are also often employed because of their wide range of solubility for active substances. According Limayem Blouza et al., an enhancement of Spironolactone solubility as high as 30 mg/ml would be obtained by using the Labrafac[®] Hydro (Caprylic/Capric Triglyceride PEG-4 esters) as oil (Limayem Blouza et al., 2006). In addition, skin penetration enhancement properties, were also described for these ingredients. In fact, it was shown an enhancement of Levodopa (antiparkinson drug) transdermal delivery after using these ester types' vehicles (Lee et al., 2013). While other less frequently used oils, such as oleic acid, ethyloleate, argan oil, sunflower seed oil, soybean oil and so on, they can nevertheless give good outcomes. In w/o surfactants, sorbitan esters and phospholipids are preferred. Concerning the solvent for polymer, acetone is selected in all cases. Furthermore, solvents such as ethanol are used for dissolution of active substance or the oils. Water or buffer solutions can be used as the non-solvent with the poloxamer 188 or polysorbate 80 as stabilizing agent (Mora-Huertas et al., 2010). Particles formation is a dynamic process, which comprises three stages, namely nucleation, molecular growth, and aggregation (Lince et al., 2008). As described in Fig. 2 in this technique, after slowly addition of organic phase with moderate stirring to the aqueous phase, the nanocapsules as a particulate suspension are formed.

Table 5
Advantages and drawbacks of polymer-based encapsulation methods.

Methods	Approximately usage percentage (%)	Advantages	Drawbacks	References
Nanoprecipitation	50	Simple, fast, economic, achievement of reproducible submicronial particle size with narrow distribution without external energy source	Not efficient to water-soluble drugs, organic solvents use	Chorny et al. (2002), Legrand et al. (2007), Mora-Huertas et al. (2010)
Emulsion-diffusion	25	The employed solvent is suitable in the pharmaceutical field, Suitable encapsulation technique for both lipophilic and hydrophilic molecules	Three liquid phases are needed, organic solvents use	Mora-Huertas et al. (2010)
Double emulsification	13	The method is relatively simple, appropriate in controlling process parameters. The process can efficiently encapsulate highly water soluble compounds as proteins and peptides	Thermodynamically double emulsions are unstable systems and they have a strong tendency for coalescence, flocculation and creaming. The majority of double emulsions contain relatively large droplets, which cannot withstand storage regimes and have a strong trend to release the entrapped matter in an uncontrolled mode, organic solvents use	Giri et al., (2013), Garti (1997), Mora-Huertas et al. (2010)
Layer-by-layer	3	The simplicity of the process and equipment, Appropriate to coat most surfaces, Availability of natural and synthetic colloids, The flexible application to objects with irregular shapes and sizes,	Poor encapsulation efficiency, sudden and fast release of encapsulated drug	Giri et al. (2013), Garti (1997), Mora-Huertas et al., (2010)

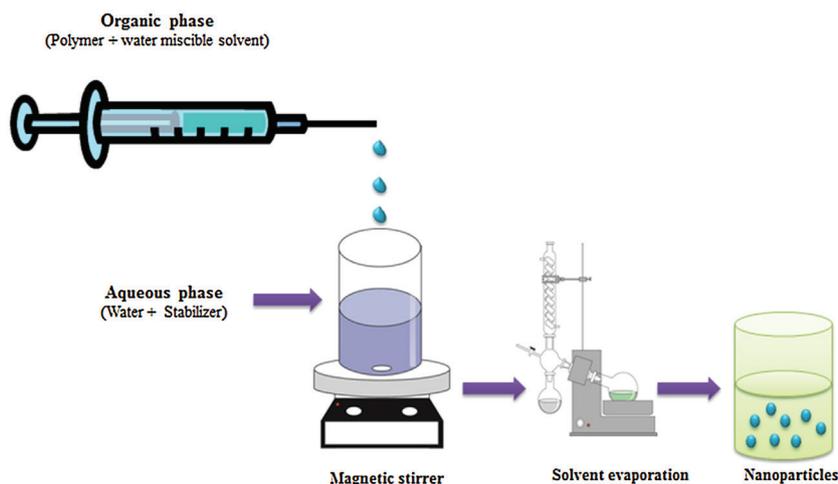


Fig. 2. Schematic representation of nanoprecipitation method.

The organic phase injection rate, aqueous phase agitation rate, the method of organic phase addition and the organic/aqueous phase ratio are the important variables of encapsulation procedure, determining the characteristics of the final product. Similarly, the nature and concentration of the components have an effect on nanocapsule features (Chorny et al., 2002; Legrand et al., 2007; Lince et al., 2008; Plasari et al., 1997).

8.1.2. Emulsion diffusion

This technique was developed by Quintanar-Guerrero and Fessi (Miladi et al., 2014) allowing the encapsulation of both lipophilic and hydrophilic active ingredients. Fig. 3 describes the procedure to gain the three phases of organic, aqueous and dilution. The organic phase contains the active substance, polymer, oil and an organic solvent. In order to encapsulate lipophilic active substance, the organic phase should not be completely miscible with water.

The aqueous phase consists of the aqueous dispersion of a stabilizer, which is prepared using solvent-saturated water whereas the dilution phase is mostly water. Insoluble components are dissolved in the organic-solvent. Biodegradable polyesters (particularly PCL and PLA) and Polymethylmethacrylate (such as Eudragit®) are the most frequently employed polymers in this method. Hydroxybutyrate-co-hydroxyvalerate (PHBV) is used to

a lesser extent. The size of the obtained particles could be affected by operating conditions like external to internal phase ratio, stirring rate at the emulsification step, temperature and volume of water for the dilution, the stabilizing agent and polymer amounts. The most used solvents include ethyl acetate, propylene carbonate, benzyl alcohol and dichloromethane (Mora-Huertas et al., 2010). Regarding external phase, water together with poly(vinyl alcohol) (PVA) are frequently used. Stabilizing agents such as poloxamer and specifically ionic emulsifiers have been used too. The diluent is mostly water. However, in order to attain better nanodispersion stability, stabilizer could be added to the diluent as well. Likewise, the particles size was explained to amplify through an augmentation of primary drug quantity (Youm et al., 2012; Mora-Huertas et al., 2010).

Nanocapsule size is correlated to applied shear rate in the emulsification step, the chemical nature of the organic phase, polymer concentration, the oil-to-polymer ratio and drop size of the primary emulsion (Guinebreteire, 2001).

8.1.3. Simple emulsion evaporation

In the development of polymer-based carriers, emulsion evaporation method is widely used. This encapsulation technique was first developed by Vanderhoff in 1979 (Miladi et al., 2014).

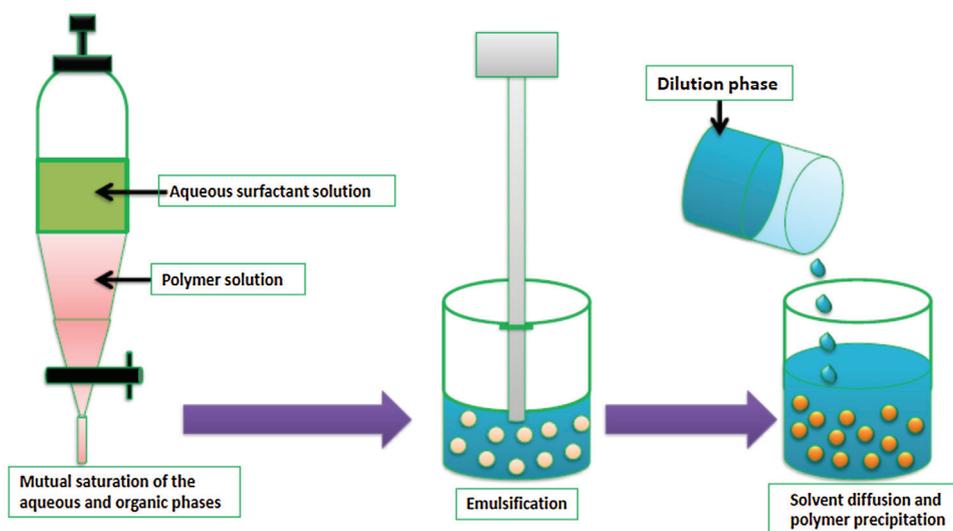


Fig. 3. Schematic representation of emulsion diffusion method.

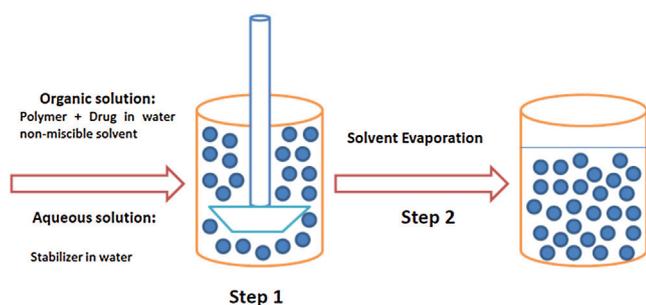


Fig. 4. Schematic representation of simple emulsion evaporation method.

Nanoparticles preparation *via* this technique comprises preparation of a simple emulsion followed by organic solvent removal through evaporation. Subsequently, polymer precipitation allows nanoparticles formation (Fig. 4). This technique allows the use of both oil in water (o/w) emulsions or water in oil (w/o) emulsions. Selection of emulsion type depends on physicochemical properties of formulation ingredients such as polymer, active ingredient, surfactants, *etc.* For hydrophobic polymer and drug, o/w emulsion is used. Polymer and drug could be dissolved in a volatile organic phase that is not miscible with water, such as chloroform, ethyl acetate or dichloromethane. The prepared organic phase, in which polymer and drug are dissolved, would be consecutively dispersed in an aqueous phase *via* high-speed homogenization or sonication. Furthermore, to assure stability of the dispersed system aqueous phase could also contain a surfactant. When a w/o emulsion is obtained, organic solvent evaporation allows polymer precipitation and subsequent nanoparticle formation. W/O type, emulsion could be employed for hydrophilic drug and polymer. Nature of surfactant and its concentration, stirring rate, ratio of organic to aqueous phase, polymer concentration and evaporation rate are the parameters, which should be managed to obtain particles with suitable properties (Miladi et al., 2014). Increasing the surfactant

amount, aqueous phase volume and stirring rate can decrease the particle size (Valot et al., 2009; Khaled et al., 2010; Su et al., 2009). In contrary, raising the volume of organic solvent and polymer concentration might increase the particle size (Budhian et al., 2007; Yadav and Sawant, 2010; Avachat et al., 2011; Doan et al., 2011; D'Aurizio et al., 2011).

8.1.4. Double emulsion evaporation

Double emulsion, or emulsions-of-emulsions, is a complex heterodisperse system. The hydrophilic molecules are more convenient to be encapsulated by this technique. There are two types of double emulsions: water-oil-water (W/O/W) and oil-water-oil emulsion (O/W/O) (Mora-Huertas et al., 2010; Charcosset and Fessi, 2005). This method consists of a two-step emulsification process *via* the usage of two surfactants: a hydrophobic one to stabilize the interface of the w/o internal emulsion and a hydrophilic surfactant to stabilize the external interface of the oil droplets for second emulsification step.

As pointed out in Fig. 5, this technique is commonly comprised of the dispersion of an aqueous phase in a non-miscible organic phase to form the first emulsion (W/O). This dispersion is carried out under high shear homogenization or low power sonication for a short time. This step is pursued through the dispersion of the achieved emulsion in a second aqueous phase containing a hydrophilic emulsifier. Again, homogenization could be carried under high shear homogenization or with a sonication probe. When sonication is used, it must be performed at low power and briefly to not break the first emulsion. After formation of the multiple emulsion, evaporation of the volatile organic solvent under low pressure (by a rotary evaporator) or at ambient temperature allows the obtaining of the particulate carriers. There are other categories of multiple emulsions, such as w/o/o or o/w/w (Giri et al., 2013). To gain complete solvent diffusion, water is generally added to the double emulsion. Surfactants play a double task in emulsions: being the steric stabilizing agent on the external interface and as a film that is a barrier to drug release at the

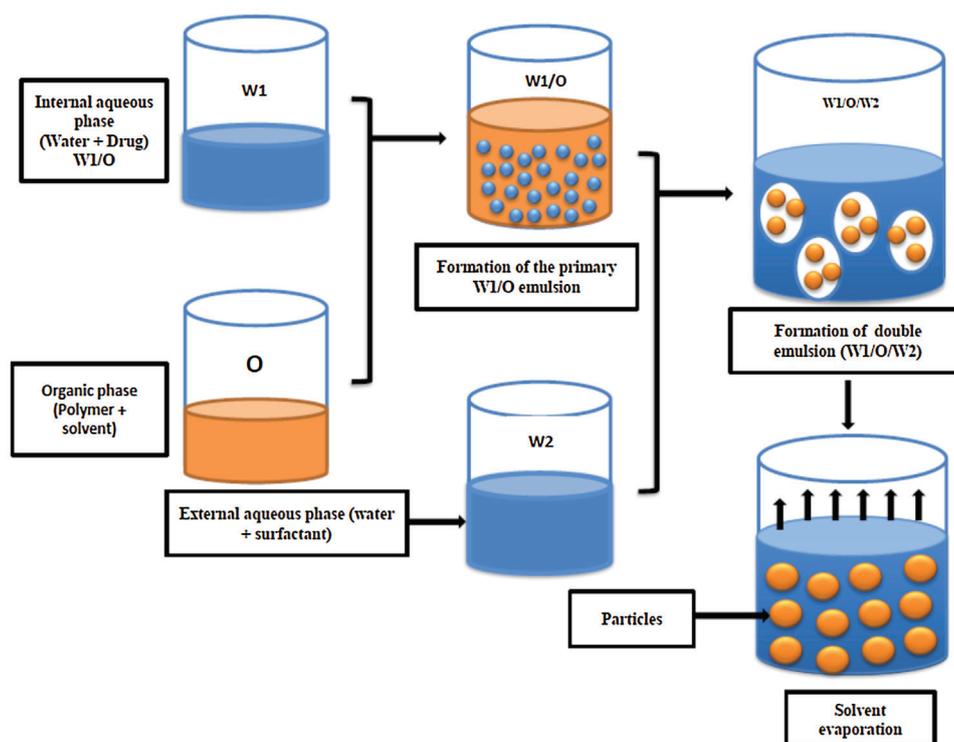


Fig. 5. Schematic representation of double emulsion evaporation.

internal interface (Khoee and Yaghoobian, 2009). Through change of type and concentration of both the w/o emulsion and stabilizer the average particle size can be influenced. The organic phase can consist of ethyl acetate, methylene chloride, dichloromethane, with PCL, PLA and PLGA as biodegradable polyesters in this phase. Concerning o/w surfactants, sorbitan esters are favored. PVA and polysorbates are frequently used stabilizing agents in the external aqueous phase. As mentioned previously, to ensure complete solvent diffusion, an optional dilution step, before extraction under vacuum nanocapsule may be helpful (Limayem Blouza et al., 2006).

8.1.5. Layer-by-layer method

Indeed, this technique through employing the biopolymers, proteins, peptides, polysaccharides and DNA, permitted the assembly of polyelectrolyte films (Powell et al., 2011). Polyelectrolytes are classified according to their origin. To name a few synthetic polyelectrolytes, poly(styrene sulfonate) (PSS), poly(dimethyldiallylammonium chloride) (PDDA), polyethylenimine (PEI), poly(*N*-isopropyl acrylamide (PNIPAM), poly(acrylic acid) (PAA), poly(methacrylic acid) (PMA), poly(vinyl sulfate) (PVS) and poly(allylamine) (PAH). Natural polyelectrolytes like deoxyribonucleic acid (DNA), proteins and polysaccharides of which alginate, chondroitin sulfate, heparin, chitosan, cellulose sulfate, dextran sulfate and carboxymethyl cellulose are most common (de Villiers et al., 2011).

Moreover, polyelectrolytes with opposite charge could be adsorbed on the surface of colloidal particles. The empty nanocapsules are then loaded with the desired active substance (Mora-Huertas et al., 2010). As shown in Fig. 6, nanocapsules are formed because of the irreversible electrostatic attraction, which leads to the adsorption of supersaturating bulk polyelectrolytes.

8.2. Characteristics of nanoparticles according to their preparation method

Chemical nature of the active ingredient and especially its polarity governs particles encapsulation efficiency. Additionally, encapsulation efficiency of hydrophilic molecules can reach the values of up to 10%, in contrast to lipophilic molecules where the encapsulation efficiency can be as high as 70% (Stella et al., 2007; Mora-Huertas et al., 2010). Generally, the mean sizes of nanoparticles loading molecules that are prepared through pre-formed polymers are 250–500 nm. Exceptions arise in techniques where the solid active substance is encapsulated directly (e.g. s/o/w emulsification and layer-by-layer methods). Nevertheless, in these cases it is still possible to achieve low mean particle sizes by employing ultrasound in the initial stages of the preparation procedure. The release of active ingredient from nanocapsules designed by nanoprecipitation, emulsion-diffusion, emulsion-coacervation and polymer-coating methods occurs in two phases; a fast initial release stage pursued by slower second release stage. The initial phase (burst release) is linked to the release of active, which is on particle surface. The second phase is more prolonged,

which correspond to the release of, encapsulate drug. However, if the drug were adsorbed on particle surface only one phase would be observed. Usually, with the application of nanoprecipitation, the pH values of the nanocapsule dispersion will be between 3.0 and 7.5. There is no information in literatures regarding the rate of drug release when other encapsulation methods are applied. One should be aware that pH determines the zeta-potential of the colloidal dispersions, which itself can affect their stability (Mora-Huertas et al., 2010).

8.3. Criteria for the selection of encapsulation method

Physicochemical characteristics of the drug are the most important criteria for the selection of the specific method for encapsulation. In addition, this includes, stability during drug delivery system production, encapsulation efficiency, mixing, method feasibility, generation of contaminants and the need for successive purification steps, time consumption, the water volume required, solvent nature, application of nanocapsule, like the preferred route and drug release profile. Each method has its advantages and restrictions so there is no ideal method of encapsulation. Concerning the water consumption, emulsion diffusion technique is definitely unfavorable (Charcosset and Fessi, 2005; Limayem Blouza et al., 2006). Nanoprecipitation as shown in Fig. 2, is the most used method and this method is appreciated for its low cost, simplicity of procedure, reproducible carrier size and high encapsulation efficiency (Mora-Huertas et al., 2010). The choice of encapsulation method depends on the desired application, the nature of the active ingredient and that of the polymer and their physicochemical properties. The nature of carriers plays a crucial role in selection of appropriate method for drug encapsulation (Beija et al., 2012). To provide a formulation bearing proper properties for the *in vitro* and *in vivo* applications, right selection of the encapsulation method is vital. Furthermore, in the selection of materials for drug encapsulation it is crucial to take into account the nature and interaction between these materials.

8.4. Recently encapsulated NSAIDs

Table 6 provides encapsulated NSAIDs with employed encapsulation techniques. Aim of encapsulation, obtained particles size, zeta potential, polydispersity index and entrapment efficiency are shown here as well. Table 6 figures out the encapsulated NSAIDs, the employed encapsulation technique, polymer, NSAIDs molecule, particle size, zeta potential, encapsulation objective, polydispersity index and entrapment efficiency.

Table 6 pinpoints that mostly, utilized method, NSAIDs molecules, polymer and delivery approach of NSAIDs are respectively nanoprecipitation, ketoprofen, aceclofenac, poly(ϵ -caprolactone), chitosan, Eudragit[®] and sustained release drug delivery system design.

9. Lipid based carriers

Lipid based carriers include liposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). Liposomes were the first examples of developed lipid based carriers. They are vesicles that contain lipid bilayers. Surfactants that could be used for production of lipid based carriers include biological membrane lipids such as lysophospholipids, bile salts (e.g., sodium taurocholate), and biocompatible nonionic molecules (e.g., ethylene oxide/propylene oxide copolymers, sorbitan esters, fatty acid ethylates). Although, such carriers have numerous advantages in comparison to other vehicles in general, they show some shortcomings such as: low drug loading capacity and drug expulsion

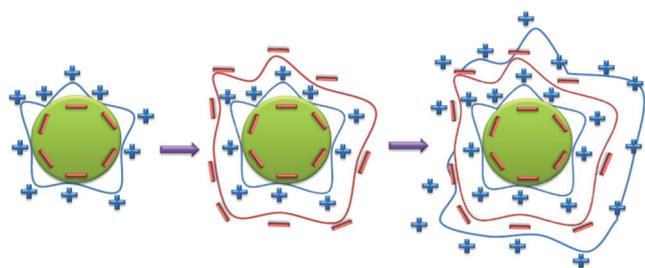


Fig. 6. Schematic representation of layer-by-layer method.

Table 6
Encapsulated NSAIDs via polymer-based encapsulation techniques.

Method	Polymer	NSAIDs	Particle size (nm)	Zeta potential (mV)	Goal of encapsulation	Poly dispersity Index	Entrapment efficiency (%)	Reference
Modified Nanoprecipitation	Different grades	Eudragit [®] Naproxen	100–500	77–67	Development of argan oil based nanocapsules as vehicle of naproxen	–	90	Rosset et al. (2012)
Nanoprecipitation	Eudragit [®] RL 100	Aceclofenac	134.97	30.5	Prolongation of corneal contact time with drug	0.186	95.73	Katara and Majumdar (2013)
Nanoprecipitation	Poly (d,l-lactide-co-glycolide), (PLGA)	Etodolac	184	–7.6	Etodolac Loaded PLGA Nanoparticles	0.03	17	Çirpanlı et al. (2009)
Nanoprecipitation	Polycaprolactone (PCL)	Indomethacin	290–350	–40 up to –50	Development of polymer based nanocapsules containing Argan oil and indomethacin	–	65–75	Badri et al., (2015)
Nanoprecipitation	PCL	Diclofenac	150	–50	Attaining of colloidal system with good stability and good encapsulated drug release through the change of nature of the oil	–	–	Mora-Huertas et al. (2012)
Nanoprecipitation	PCL	Nimesulide	344.6	–	Increasing of anti-inflammatory activity of topical nimesulide	0.251	–	Lenz et al. (2012)
Nanoprecipitation	Dextran	Ibuprofen,	223.1	–50	Drug release adjustment	0.152	55.76	Hornig et al., (2009)
Nanoprecipitation	Dextran	Naproxen	282	–	Drug release adjustment	0.136	51.7	Hornig et al. (2009)
Nanoprecipitation	Magnetic biodegradable poly(D,L- lactide) polymer (PLA)	Indomethacin	250	–	Indomethacin target drug delivery in magnetic biodegradable polymer nanoparticles	–	80	Závišová et al. (2007)
Nanoprecipitation	Eudragit [®] L100 and Eudragit [®] L100- PLGA	Diclofenac sodium	196.35	1.13	Sustaining the diclofenac sodium release profile	–	46.55	Cetin et al. (2010)
Nanoprecipitation	Eudragit [®] S100	Ibuprofen	345	–26.9	Preparation of Ibuprofen loaded Eudragit [®] S100 small, stable and high efficiency nanoparticles	–	96.47	Vineela and Krishna Sailaja, (2014)
Nanoprecipitation	PCL	Ibuprofen and indomethacin	345.25 and 285	–17.55 and –25.67	Comparative study of Ibuprofen and Indomethacin loaded nanoparticles	0.252 and 0.097	36.25 and 61.25	Suksiriworapong et al. (2010)
Emulsification solvent diffusion	Chitosan et PLGA	Celecoxib	113.3 and 154.67	36.92 and –36.5	Developing and optimizing and sustained release, mucoadhesive and biodegradable nanoparticles formulations of celecoxib for topical ocular delivery.	0.971 and 0.724	89.88–75.38	Ibrahim et al. (2013)
Emulsion-diffusion	PCL	Diclofenac	200	–	Attaining of colloidal system with good stability and good encapsulated drug release through the change of nature of the oil	–	–	Mora-Huertas et al. (2012)
Layer- by-layer	Poly (dim imethyl-dally ammonium chloride)	Ketoprofen	41–111	–28 to –15	Obtaining of prolonged release delivery system	–	–	Arida and Al-Tabakha (2007)
Layer- by-layer	Sodium Alginate, Chitosan	Ketoprofen	2.202 μm	7	Ketoprofen transdermal drug delivery	0.151	–	Gupta et al. (2009)
Double- emulsion solvent evaporation	Chitosan	Aceclofenac	39 and 55	–	Achievement of controlled drug release of Aceclofenac microspheres and minimizing the local side-effects in the digestive tube	–	53–72	Nagda et al. (2010)
Double-emulsion solvent evaporation	Polymethylmethacrylate (PMMA)	Diclofenac sodium (DFS)	215 μm	–	Drug release control	–	98.71	Pal et al. (2011)
Double-emulsion solvent evaporation	Chitosan	Ketoprofen	1.79–58.96	–	Study of microsphere characteristics under the influence of different process condition and preparation	–	>67	Pavanetto et al. (1996)
Emulsion-solvent evaporation	PLGA	Nimesulide	0.7 μm	–15 ± 3	Contributory treatment of prostate cancer	–	>83	Huerta et al. (2015)
Emulsion-solvent evaporation	Ethyl cellulose	Aceclofenac	912 μm	–	Preparation of microcapsules to provide sustained release in localized areas	–	32.85	Yadav and Sawant (2010)
Emulsion-solvent evaporation	Eudragit [®] RS100	Naproxen	378–644	–	To improve the physicochemical characteristics of the drug	0.12–0.29	–	Adibkia et al. (2011)

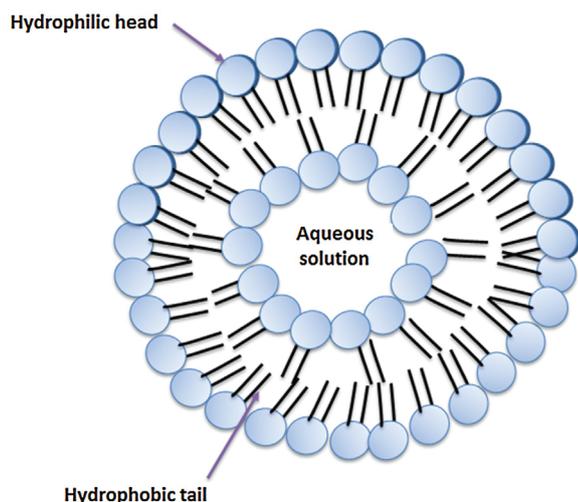


Fig. 7. Liposome structure.

during storage due to transition of highly ordered lipid particles (Araújo et al., 2009; Pardeike et al., 2009; Zhai and Zhai, 2014).

9.1. Liposomes

Liposomes are vesicles made of lipid bilayers, which surround an aqueous core (Fig. 7). Main constituents of liposomes are cholesterol and phospholipids. As demonstrated in Fig. 8, liposomes could be classified in unilamellar, oligolamellar, multilamellar and multivesicular liposomes (Zhai and Zhai, 2014).

Many techniques are used for preparation of liposomes. The most used method is film hydration that was developed in 1965. The first step in preparation of liposomes consists of drying a lipid solution, which allows the formation of a thin film on the bottom of a round-bottom container (See Fig. 9). Subsequent hydration of this film with an aqueous buffer along with vortexing allows liposomes formation. Sonication or extrusion could be applied to reduce particle size and to obtain unilamellar vesicles. Other methods are called reverse phase evaporation techniques and they are based on mixing the organic phase containing the lipids solution with the aqueous phase. Slow evaporation of the organic solvent leads to the formation of liposomes. Other methods are based on rapid injection of lipids dissolved in organic solvent into

an excess of aqueous solution (Batzri and Korn, 1973). Ethanol injection method allows the obtaining of small liposomes with narrow size distribution (below 100 nm). It is based on injecting an ethanol-containing lipid solution in water, without the need of any size reduction method (Laouini et al., 2012; Nogueira et al., 2015). Table 7 shows properties and applications of liposomes containing NSAIDs. It can be seen that thin film hydration is by far the most used preparation technique. Most encountered applications are bioavailability enhancement and topical delivery. In fact, liposomes are well recognized to enhance the interactions with biological membranes.

9.2. Lipid based particles

Lipid based particles could either be SLN or NLC.

9.2.1. Solid lipid nanoparticles

SLN are O/W emulsions in which the internal oil droplets are replaced by one or more solid lipid particles (Fig. 10). SLN have been developed at the beginning of 1990s. SLN average particle size is ranged between 40 and 1000 nm. SLN composition includes 0.1% (w/w) to 30% (w/w) solid lipid dispersed in an aqueous medium. Furthermore, when needed 0.5% (w/w) to 5% (w/w) surfactant could be employed for stabilization.

9.2.2. Nanostructured lipid carriers

NLC differ from SLN in the way that solid lipids are replaced by solid and liquid lipids blend (Fig. 11). Such carriers have been developed to overcome the SLN drawbacks (drug expulsion during storage, loading capacity limitation and gelation risk) (Pardeike et al., 2010). In fact, in case of highly pure lipids, the particles could form relatively perfect lipid crystals and then tend to recrystallize. Thus, SLNs have major drawbacks of low drug loading and expulsion due to advancing lipid crystallization or transformation during stored procedures (Chen et al., 2013; Schwarz et al., 2013; Barua and Mitragotri, 2014). For a number of active compounds, NLC showed higher loading capacity as compared to SLN. Like SLN, NLC are well-tolerated carriers for dermal application (Pardeike et al., 2009; Weber et al., 2014). NLCs are mixtures of solid and liquid lipids. It is reported that the liquid lipid can embed into solid lipid matrix or localize at the interface of solid matrix and the surfactant layer (Štecová et al., 2007). In general, these spatially different lipids lead to imperfectible crystal structure, which provide more space for accommodating the encapsulated drugs.

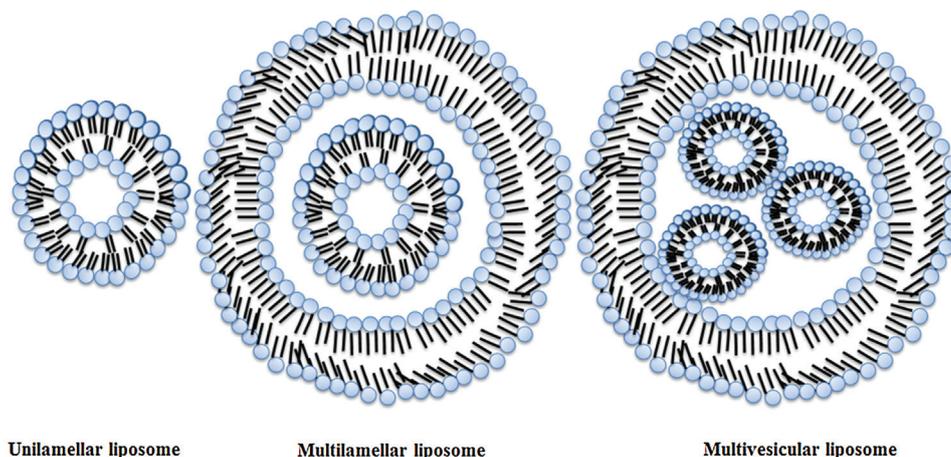


Fig. 8. Liposomes types.

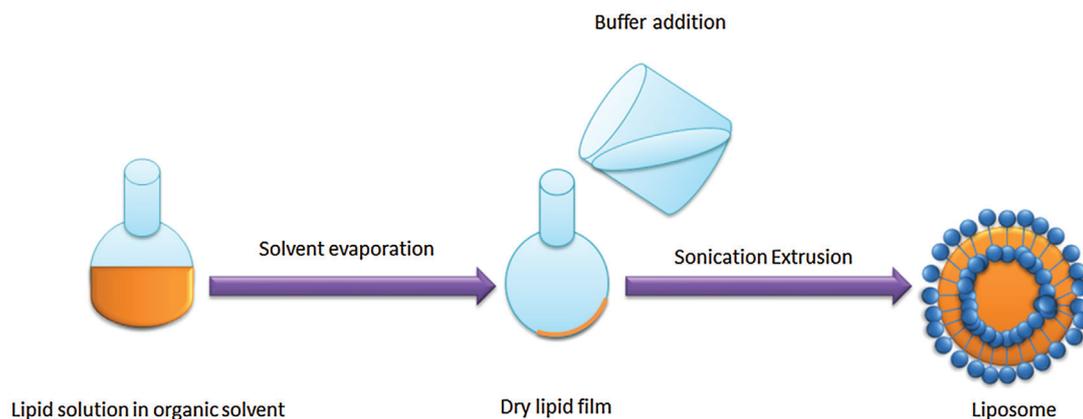


Fig. 9. Schematic representation of lipid film hydration method.

Table 7
NSAIDs encapsulated in liposomes.

Method	Active ingredient	Particle size (nm)	Encapsulation efficiency (EE%)	Polydispersity index (PDI)	Zeta potential (mV)	Goal of encapsulation	References
Thin film hydration	Piroxicam	1660–66.76	12.5–36.6	0.28–1.00	–1.31 to –2.06	Functionalization of gauzes with liposomes entrapping an anti-inflammatory drug	Ferreira et al. (2013)
Thin lipid film hydration	Bromfenac (BRF)	171.74	>90	0.22	–23.29	Appropriate liposomal formulations for ophthalmic delivery	Tsukamoto et al. (2013)
Thin lipid film hydration	Celecoxib (CLX)	5450–6230	96.32	–	–	Design of celecoxib liposomal delivery system	Deniz et al. (2010)
Lipid hydration-extrusion	Indomethacin	150–200	93	0.069	–	Prevent the transfer of indomethacin across the placenta to the fetus	Refuerzo et al. (2015)
Thin film hydration	Diclofenac sodium	135–186	29–34	–	–46 to –34	Enhancement of bioavailability	Jukanti et al. (2011)

Hence, compared to SLNs, NLCs were characterized by higher drug incorporation rate and better stabilization after preparation (Zhai and Zhai, 2014).

9.2.3. Lipid based nanoparticles preparation

There are many systems for production of lipid based nanoparticles. Commonly used methods for preparation of SLNs are high pressure homogenization at elevated or low temperatures (including hot homogenization and cold homogenization), solvent emulsification, evaporation or diffusion, supercritical fluid

methods, ultrasonication or high speed homogenization and spray drying (Kohli et al., 2014; Drummond et al., 2008). High-pressure homogenization technology has emerged as a potent technique for production of lipid nanoparticles. Two processes of the homogenization were developed, namely hot and cold processes. In both processes, active substance is first dissolved or dispersed in a melted lipid. High-pressure (100–2000 bar) moves the fluid in the narrow gap in homogenizer. Average particle size is in sub-micron region. This method has many advantages including large-scale production, absence of organic solvent, enhanced product stability and enhanced drug loading, but specifically high pressure and temperature conditions are actual challenges (ALHaj et al., 2008; Naseri et al., 2015). In this technique, SLN are prepared at temperatures higher than lipid melting point. Drug loaded lipid

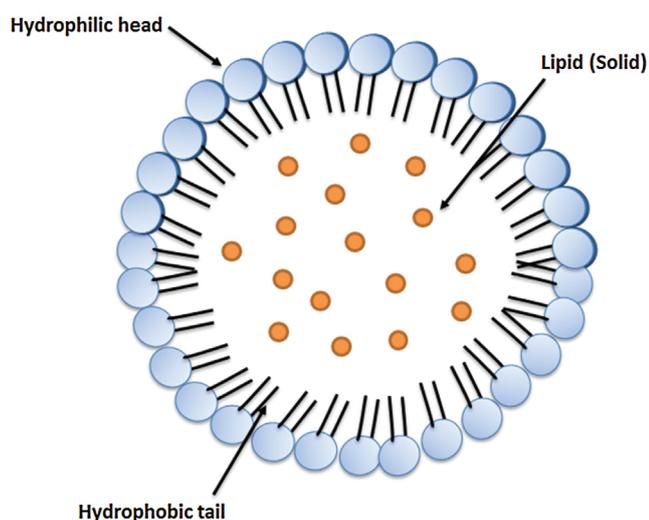


Fig. 10. Schematic representation of solid lipid nanoparticle (SLN).

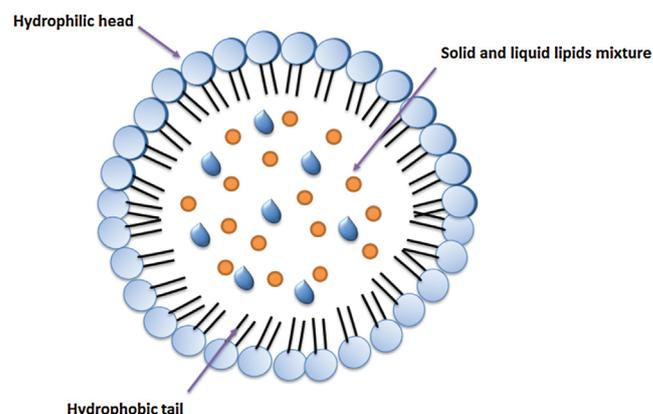


Fig. 11. Schematic representation of nanostructured lipid carrier (NLC).

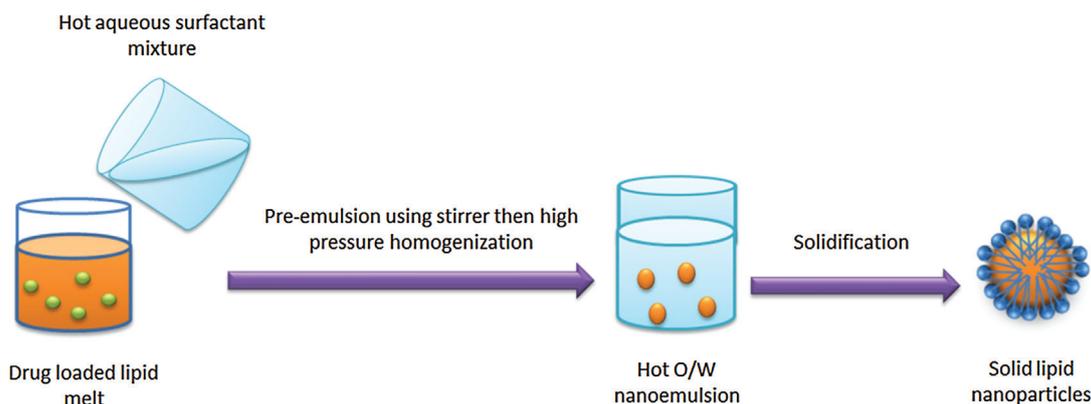


Fig. 12. Schematic representation of hot homogenization.

melt is dispersed in hot aqueous surfactants phase (isothermal) by mixing device (Ultra-Turrax) and leads to the formation of pre-emulsions. Generally, at higher temperatures, the viscosity of the inner phases is reduced and consecutively, particles size is decreased (e Silva et al., 2012). Nevertheless, drug and carrier degradation speed could be increased. This technique is illustrated in Fig. 12. Hot homogenization has three basic problems. The first one is temperature-dependent degradation of the drug, the second one is the drug penetration into the aqueous phase during homogenization and third one is complexity of crystallization step of nanoemulsion leading to several modifications and/or super-cooled melts (Parhi and Suresh, 2012; Mehnert and Mäder, 2001).

In cold homogenization method, the drug is dissolved in the lipid melt and then rapidly cooled by liquid nitrogen or dry ice. Milling leads to formation of nanoparticles in the range of 50–100 nm. Such particles are dispersed in a cold surfactant solution, which forms a pre-suspension. High-pressure homogenization is done at ambient temperature that leads to breaking of the nanoparticles to SLNs. Cold homogenization technique has been expanded to resolve the problems of hot homogenization technique such as, thermolabile drug degradation (Parhi and Suresh, 2012), Schematic diagram of this method is depicted in Fig. 13 (Naseri et al., 2015).

Other methods for lipid nanoparticles preparation are ultrasonication and high-shear homogenization. These techniques are simple (Manjunath et al., 2005). The lipid phase is dispersed in a phase containing a large amount of surfactant. The high surfactant amount is however a disadvantage. These methods produce particles with large particle size distribution (Tables 8–10).

10. *In vivo* applications of encapsulated NSAIDs

Table 11 shows the different carriers loaded with NSAIDs that were tested *in vivo*. Both nanoparticles and liposomes have been tested. Studies were performed either in rats or in rabbits. The most targeted ways of administration routes in *in vivo* investigations, were ocular and intravenous routes. Furthermore, skin,

oral and nasal delivery routes of administration were employed. Several outcomes have been confirmed such as bioavailability enhancement, improvement of drug accumulation in targeted site and prolonged release. Tolerance has been also evaluated for all studies and it was concluded that these carriers turned out to be safe. Most common actives that were studied *in vivo* include flurbiprofen, diclofenac, indomethacin and oxicams. It has been also shown that encapsulated NSAIDs were more efficient than conventional dosage forms. The most common studied formulations are liposomes, polymeric nanoparticles and NLC. With these encouraging results, it would be interesting to pay more attention to test encapsulated NSAIDs in human.

11. Opportunities and challenges

NSAIDs are one of the most prescribed drugs around the world, for the treatment of pain, fever and inflammation. NSAIDs side effects are still less risky in comparison to the side effects of corticosteroids, which can be used for treatments where NSAIDs become ineffective. Even so, to overcome these obstacles; carriers such as polymer based particles, liposomes, SLN and NLC could play crucial role. The function of the aforementioned carriers has been validated by several clinical researches in *in vivo* setting (Table 11). The pharmaceutical development of drug-loaded nanoparticles is based on three features: usage of less toxic reagents, economic scale-up by simplification of the procedure and optimization to progress yield and entrapment efficiency. There are different options for nanocapsule synthesis by using polymers; the selection of a specific method is generally determined by the drug physicochemical properties, especially aqueous solubility of the drug. Currently safe, simple and reproducible techniques exist to prepare drug-loaded nanoparticles. The chosen method for drug vehicle preparation could have an effect on produced nanocapsules. It must be taken into account that under the operating condition the selected method should not disturb the stability of the active ingredient. All NSAIDs are chemically organic acids but they have different physicochemical properties so single

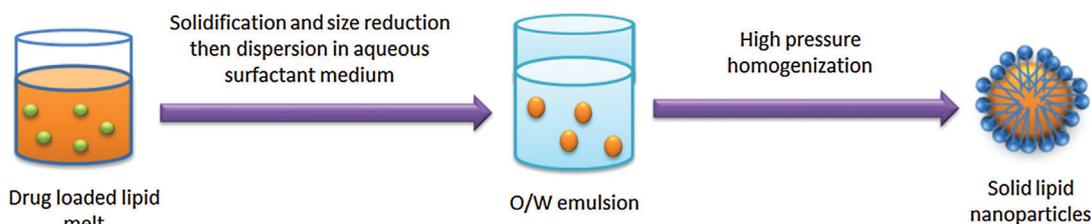


Fig. 13. Schematic representation of cold homogenization.

Table 8

List of excipients used in SLN preparation (from Yadav et al., 2013 with modifications) (Yadav et al., 2013).

Lipids	Surfactants
Fatty Acids	Alcohols
para-acyl-calix-arenes	Ethanol
Hydrogenated coco-glycerides	Butanol
Cyclic complexes	Butyric acid
Acidan N12	Diocetyl sodium sulfosuccinate
Behenic acid	Monooctylphosphoric acid sodium
Decanoic acid	Bile salts
Palmitic acid	Sodium cholate
Stearic acid	Sodium glycocholate
Acyl glycerols	Sodium taurocholate
Glyceryl monostearate	Sodium taurodeoxycholate
Glyceryl distearate	Phospholipids
Glyceryl monooleate	Soy lecithin
Glyceryl behenate	Egg lecithin
Glyceryl palmitostearate	Phosphatidylcholine
Hard fat types	Ethylene oxide/propylene oxide copolymers
Triglycerides	Poloxamer 188
Tricaprin	Poloxamer 182
Trilaurin	Poloxamer 407
Trimyristin	Poloxamine 908
Tripalmitin	Sorbitan ethylene oxide/propylene oxide copolymers
Tristearin	Polysorbate 20
	Polysorbate 60
	Polysorbate 80
	Alkylaryl polyether alcohol polymers
	Tyloxapol

encapsulation method may not be ideal for all. Nowadays, for achievement of an efficient entrapment of the drug, it is possible to select the best method of preparation and the best polymer. The selected method must give minimal loss of the drug or its pharmacological activity. However, certain problems need to be solved. In fact, residual solvent analysis must be more extensively investigated and the post preparative steps, such as purification and preservation, particularly important for nanocapsules, are to be optimized. Currently, most utilized methods for NSAIDs encapsulation are nanoprecipitation, double-emulsion solvent evaporation, and emulsion-solvent evaporation. Mainly used polymers are poly(ϵ - caprolactone), chitosan, Eudragit[®] and sustained release drug delivery system design. Mostly, prepared

vehicles were nanocarriers. Main applications compromise promoted stability, sustained release and bioavailability enhancement. According to our state of the art, ketoprofen has been among the first encapsulated NSAIDs. More recently, molecules like indomethacin, nimesulide, aceclofenac and celecoxib are encapsulated. According to our best of knowledge, lipid based carriers nanostructured lipid carriers (NLC) could be the best carrier for NSAIDs delivery in order to attain different objectives such as, gastrointestinal side effects decrease, solubility and bioavailability enhancement, stability increase, unpleasant taste cover, systemic toxicity reduction and half-life control. Most encapsulated NSAIDs are ketoprofen, aceclofenac and indomethacin. However, *in vivo* efficiency has been mostly performed on molecules like

Table 9

Encapsulated NSAIDs in SLN.

Method	Active ingredient	Particle size (nm)	Encapsulation efficiency (EE %)	Polydispersity index (PDI)	Zeta potential (mV)	Goal of encapsulation	References
Hot homogenization	Indomethacin	140	72	0.16	–21	Development and characterization of Indomethacin-loaded solid lipid nanoparticles for ocular drug delivery	Hippalgaonkar et al. (2013)
Hot homogenization	Diclofenac sodium	115.8	90	0.29	–34.7	Diclofenac sodium for ophthalmic delivery	Attama et al. (2008)
Hot homogenization	Flurbiprofen	190	90	–	–	Flurbiprofen development of novel solid lipid nanoparticle	Din et al. (2015)
Microemulsion	Ketoprofen	75 ± 4	97	0.2	–15 to –17	Ketoprofen –loaded SLNs preparation by a mixture of beeswax and carnauba	Kheradmandnia et al. (2010)
Modified high shear homogenization and ultrasonication	Meloxicam	325–1080	61.94 to 85.33	–	–17.6 to –38.6	Meloxicam solid lipid nanoparticles (MLX SLNs) development for topical delivery	Khalil et al. (2014)
Hot high pressure homogenization	Lornoxicam	136.6	90.07	0.22	–	Solid lipid nanoparticles as delivery system for lornoxicam	Kumar et al. (2014)
Microemulsion template	Meloxicam	239	52	0.267	–24	Quick onset and prolonged action	Khurana et al. (2015)
Ultrasonication	Indomethacin	102–215	83	–	–	–	Castelli et al. (2005)
Hot homogenization followed by sonication	Flurbiprofen	250–350	>90	0.3–0.5	–21–42	Enhance oral bioavailability	Bhaskar et al. (2009)

Table 10
Encapsulated NSAIDs in NLC.

Method	Active ingredient	Particle size (nm)	Encapsulation efficiency (EE%)	Polydispersity index (PDI)	Zeta potential (mV)	Goal of encapsulation	References
High pressure homogenization	Celecoxib	217 ± 20	90	–	–	Celecoxib encapsulated in lipidic nanocarriers, anticancer activity evaluation	Patel et al. (2013)
Microemulsion template technique	Celecoxib	160	35	0.624	–	Rapid onset and prolonged release	Joshi and Patravale (2008)
Ultrasound	Flurbiprofen	288–459	92	0.245–0.639	–29.9 to –26.2	–	Gonzalez-Mira et al. (2011)
Ultrasound	Ketoprofen	335	–	–	–	Prolonged topical drug delivery	Puglia et al. (2008)
High Pressure homogenization	Naproxen	380	–	–	–	–	–
Ultrasound	Ketoprofen	215	–	–	–	–	–
Ultrasound	Naproxen	404	–	–	–	–	–
Ultrasound	Indomethacin	80–270	76	–	–	–	Castelli et al. (2005)
Hot high-pressure homogenization	Ibuprofen	130–160	–	0.065–0.249	–15 to –7	Development of Ibuprofen loaded NLC	Sütő et al. (2016)
Hot melt homogenization	Celecoxib	217	95	0.2	–25	Enhanced lung deposition time	Patlolla et al. (2010)
Modified hot sonication method	Aceclofenac	134–542	77–99	–	–	Better skin permeability	Phatak and Chaudhari (2013)
Hot homogenization followed by sonication	Flurbiprofen	250–320	>90%	0.3–0.5	–16.6–40.9	Enhance oral bioavailability	Bhaskar et al. (2009)

Table 11
Carriers already tested *in vivo*.

Encapsulated NSAIDs	Carrier	Animal	Administration route	Study outcome	Reference
Ibuprofen sodium	PEGylated gelatin nanoparticles	Sprague Dawley rats	Intravenous delivery	Improved plasma half-life of ibuprofen sodium	Narayanan et al. (2013)
Flurbiprofen	Nanostructured lipid carriers based gel	Male Wistar rats	Skin delivery	–	Han et al. (2012)
Flurbiprofen	Nanostructured lipid carriers	Male albino rabbits	Ocular delivery	Ocular tolerance	Gonzalez-Mira et al. (2011)
Carprofen	PLGA nanoparticles	Male albino rabbits	Ocular delivery	Significant reduction of conjunctival inflammation and iris hyperemia compared to free drug	Parra et al. (2015)
Diclofenac	Liposomes	Albino rabbits	Ocular delivery	1.8 fold increase in diclofenac accumulation in the retina-choroid compared to aqueous solution	Fujisawa et al. (2012)
Diclofenac	Liposomes	Male Wistar rats	Intravenous delivery	Higher accumulation and prolonged retention at inflammation site	Jukanti et al. (2011)
Indomethacin	Liposomes	Male Wistar rats	Intravenous delivery	Enhanced bioavailability and local drug amount at inflammation site.	Srinath et al. (2000)
Meloxicam	Polymeric nanoparticles	Male Sprague Dawley rats	Nasal and oral delivery	Bioavailability enhancement	Kürti et al. (2013)
Piroxicam	Polymeric nanoparticles	Rabbits	Ocular delivery	More significant inhibition of inflammation than drug microsuspension	Adibkia et al. (2007)
Indomethacin	Polymeric nanoparticles	Albino rabbits	Ocular delivery	Increased active delivery to external and internal ocular tissues	Badawi et al. (2008)

flurbiprofen and diclofenac that have been especially intended for ocular delivery. Persisting safety and tolerability, scale-up and quality of providing health services are the crucial issues to overcome as prospective for a better tomorrow. However, in spite of this interesting success on animals, encapsulated NSAIDs are not yet commercialized. Till now researches have been carried out only on animals in order to prove NSAIDs efficacy and safety. Therefore, it is necessary to perform more studies in humans to attain successful clinical application using carriers in the near future.

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II.2. Plant extracts: from encapsulation to application

Summary

This work explains the research performed in the elaboration and use of polymeric nanoparticles for the encapsulation and application of plant extracts. The review describes in detail, the different encapsulation methods, key physicochemical characteristics of the nanoparticles, and toxicity tests. In addition, results acquired from *in vivo* or *in vitro* studies are highlighted as well here.

Plants are a natural source of different products with various biological activities offering treatment for several diseases since long time. Plant extracts are a complex mixture of compounds that have antioxidant, antibiotic, antiviral, anticancer, antiparasitic, antifungal, hypoglycemic, anti-hypertensive and insecticide properties. Approximately three quarters of the world's population trust on the use of plants extracts as remedies for different afflictions. The organic solvents (e.g. methanol, ethanol, hexane, dichloromethane, ethyl acetate, etc.) are involved in the extraction of these extracts that not only complicates the formulations but also makes it difficult to directly use the plant extracts for humans. In addition to this, plant extracts protection, conservation, and targeted delivery (into organism) is another barrier to overcome their potential use as a treatment for diseases. In order to tackle these challenges, recent study has been dedicated to the development of new methods for plant extracts formulation and their safe delivery to provide a high efficacy. Plant extracts complex composition; toxicity risks and instability are the major obstacles towards their clinical applications. However, encapsulation can be fruitfully employed to reduce plant extracts toxicity, to make available targeted drug delivery and to overcome stability associated problems. In fact, polymeric nanoparticle can be defined as a particle of polymer of any shape with the size range of 1 to 100 nm while polymer based microparticle is a particle of polymer of any shape with size range of about 0.1–100 μm . Natural polymers such as chitosan, albumin, gelatin, or synthetic polymers such as methacrylates can be used for the encapsulation of drugs. Generally these polymers are biodegradable and biocompatible; Polymers selection is commonly based on the criteria of final application and toxicity of polymers. The physicochemical properties, drug release profile, and biological characteristics of nanoparticles are possible to be altered via using polymers. Encapsulation drugs within biodegradable polymers make them nontoxic, stable in blood, and noninflammatory. Nanoparticles thanks to their size and distinctive physicochemical properties, can design formulations with several advantages. There are two classes of methods including preformed polymers dispersion (solvent evaporation, nanoprecipitation, solvent diffusion, and dialysis) and monomers polymerizations (miniemulsion, microemulsion, interfacial polymerization, and radical polymerization) are used for the nanoparticles preparation. Best of our knowledge all encapsulation techniques would not use for the natural plant extracts encapsulation. Therefore, in this review the preformed polymers-based methods, which are mostly used for the encapsulation of natural plant extracts, are described. A progressively growing inclination for the usage of polymer based nanoparticles encapsulating plant extracts will be perceived in future medicinal therapies. Recently, researchers are more and more focused on the elaboration of polymer based

nanoparticles containing plant extracts to associate plant extracts broad biological activities with the nanoparticles advantages. These formulations are designed for the potential application in the fields of medicine, food, and cosmetics. The last research on formulations loaded with natural extracts, commonly reports regarding standardizing methods for obtaining carriers, physicochemical characterization of the formulation, encapsulation of the drug molecule; extract release profiles from the nanoparticles, biological evaluations both free extract and encapsulated extract and stability studies of the formulation.



Plant extracts: from encapsulation to application

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REVIEW

Plant extracts: from encapsulation to application

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ABSTRACT

Introduction: Plants are a natural source of various products with diverse biological activities offering treatment for several diseases. Plant extract is a complex mixture of compounds, which can have antioxidant, antibiotic, antiviral, anticancer, antiparasitic, antifungal, hypoglycemic, anti-hypertensive and insecticide properties. The extraction of these extracts requires the use of organic solvents, which not only complicates the formulations but also makes it difficult to directly use the extracts for humans. To overcome these problems, recent research has been focused on developing new ways to formulate the plant extracts and delivering them safely with enhanced therapeutic efficacy.

Areas covered: This review focuses on the research done in the development and use of polymeric nanoparticles for the encapsulation and administration of plant extracts. It describes in detail, the different encapsulation techniques, main physicochemical characteristics of the nanoparticles, toxicity tests and results obtained from *in vivo* or *in vitro* assays.

Expert opinion: Major obstacles associated with the use of plant extracts for clinical applications include their complex composition, toxicity risks and extract instability. It is observed that encapsulation can be successfully used to decrease plant extracts toxicity, to provide targeted drug delivery and to solve stability related problems.

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1. Introduction

Since ancient times, it is known that plants are a natural source of various products with diverse biological activities. These products have been used for the treatment of different diseases.[1] Actually, about three quarters of the world's population rely on the use of particular plant extracts as a remedy for various afflictions.[2] Within the natural products, we can find essential oils, plant extracts, tea, salves, etc.

Natural extracts are complex mixtures of chemicals with biological properties derived mainly from the leaves, the stems, the fruits, or roots of medicinal plants. Among the biological activities presented by plant extracts, the most prominent ones include the antioxidant, antibiotic, anticancer, antifungal, antiparasitic, hypoglycemic, and antihypertensive properties.[3–9]

Even though, the plant extracts are suitable for treatment for various diseases, studies show that their therapeutic use is still limited because of their complex composition and toxicity when they are applied in organisms with more complex metabolic systems. Furthermore, for obtaining these extracts, generally organic solvents (e.g. methanol, ethanol, hexane, dichloromethane, ethyl acetate, etc.) are used. Hence, the final vehicle in which the extracts are found prevents their direct application in organisms. In addition to this, the protection, conservation, and targeted delivery (into organism) of

plant extracts are another challenge to overcome their potential use as a treatment for diseases.[10]

At present, research is more focused on the composition of plant extracts, whereas, solutions that enable the efficient, safe, and direct application of these natural products need to be more focused on. One of the newest and most current ways for the application of the natural extracts, which also reduces the limitations outlined, is the use of polymeric nanoparticles (nanoparticles). Polymeric nanoparticle is a particle of polymer of any shape and an equivalent diameter from 1 to 100 nm and polymeric microparticle is a particle of polymer of any shape with an equivalent diameter from approximately 0.1–100 µm. Polymers can be of natural source, e.g. chitosan, albumin, gelatin, etc. or synthetic, e.g. methacrylates. Due to their size and unique physicochemical characteristics of nanoparticles, they generate formulations with several advantages, such as: (i) encapsulation of compounds of different chemical nature in the same formulation (mixture of compounds), (ii) targeting of specific organs (low toxicity), (iii) easy removal of organic solvent during the development of the nanoparticles (effective purification procedures), (iv) protection and conservation of the encapsulated active (enzymes damage, environment, etc.), and (v) controlled release of incorporated actives. [11–13]

In order to combine the diversity of biological activities of plant extracts and the advantages offered by the

Article highlights

- About three quarters of the world's population rely on the use of plants extracts as remedies for various afflictions.
- The use of plants extracts, as treatment is still limited due to their complex composition and toxicity when they are used directly in larger organisms.
- The polymeric nanoparticles are colloidal systems, which could act as carriers of natural extracts.
- The polymeric nanoparticles encapsulating plants extracts find applications in the fields of foods industries, medicine, cosmetics and health sector.
- Different formulations based on polymeric nanoparticles have demonstrated enhanced biological activity (e.g. anticancer, antibacterial, antidiabetic, antihypertensive, cosmetology, etc.) of plant extracts.
- A gradually increasing tendency for the usage of polymeric nanoparticles encapsulating plant extracts will be observed in future medicinal therapies.

This box summarizes key points contained in the article.

nanoparticles, recently, researchers are increasingly focused on designing formulations consisting of nanoparticles constituted from different polymers and containing encapsulated plant extracts. These formulations are developed for potential application in the field of medicine, food, and cosmetics.

The latest research on formulations containing natural extracts encapsulated in nanoparticles, generally reports about standardizing techniques for obtaining carriers, physicochemical characterization of the formulation (size, morphology, zeta potential, etc.), encapsulation of the active (percentages of encapsulation), the release profiles of the extract from the nanoparticles, biological evaluations both free extract and embedded extract (*in vitro* and *in vivo* models) and stability studies of the formulation. The aim of this review is to present the most recent data on polymeric nanoparticles encapsulating plant extracts, highlight their characterization, and report the results obtained from studies related to biological activities *in vitro* and *in vivo* models.

2. Elaboration of carriers using preformed polymers

The use of colloidal carriers has gained tremendous interest during the past decades, which paved the way for further advancements in biomedical and biotechnology field. These particulate carriers find applications in both *in vivo* and *in vitro* studies. Colloidal carriers have distinct advantages that make them a preferable choice over a simple solution of active molecules. These carriers protect active pharmaceutical ingredients from degradation or inactivation (by light or enzymatic attack), reduce toxicity of drugs.[14–17] Therapeutic efficacy of active is enhanced, since after the encapsulation the drug's biodistribution depends no longer on its own physicochemical properties but on carrier's ones.[18–21] Encapsulation of the actives in carriers also masks the unpleasant taste and odor associated with some drugs. When compared to drug solutions, carriers may give better membrane absorption and drug targeting to the tissues where pharmacotherapeutic action occurs. Reproducible and prolonged release of the active is thus provided.[22–28] The biomedical applications of the

carriers are constantly increasing.[29–31] The different polymers employed to construct these carriers may differ in physicochemical properties but mainly are biodegradable and biocompatible. Drug's encapsulation in biodegradable polymers makes them nontoxic, stable in blood, and noninflammatory. By using polymers, the physicochemical properties (e.g. hydrophobicity and zeta potential), drug release characteristics (e.g. delayed, prolonged, and triggered), and biological behavior (e.g. bioadhesion and improved cellular uptake) of the nanoparticles can be modified.[32–34] The uptake of the particles by the macrophages may also be prevented by modulating the particle's surface with coating of substances, such as polyethylene glycol (PEG). The selection of the polymer is generally made considering its final application as well as its toxicity.[35] Various techniques are available to formulate the colloidal carriers. These techniques differ by their principles or the nature of the active, which is to be encapsulated. Correct selection of the technique is very important to obtain a formulation bearing suitable properties for the *in vitro* and *in vivo* applications. Broadly speaking, there are two techniques namely: dispersion of preformed polymers and polymerization of monomers. Under preformed polymers techniques, such as solvent evaporation, nanoprecipitation, solvent diffusion, and dialysis are included. On the other hand, polymerization of monomers includes processes, such as miniemulsion, microemulsion, interfacial polymerization, and radical polymerization. After careful search through literature, it is observed that not all encapsulation techniques have been employed for the encapsulation of natural plant extracts. So here, we will briefly describe the basic principles involved in the preformed polymers-based techniques that are very frequently used for formation of particles encapsulating natural plant extracts.

2.1. Emulsion solvent evaporation

The technique emulsion solvent evaporation was introduced by Vanderhoff and colleagues.[36] This method consists of two major steps: formation of single or double emulsion and organic solvent evaporation. Evaporation results in polymer precipitation and subsequent particles' formation (Figure 1). [35] Microencapsulation by solvent evaporation is mostly used in pharmaceutical industries to get controlled release formulations. Different methods are available to use microencapsulation by solvent evaporation technique. The selection of a method that will give adequate drug encapsulation usually depends on the hydrophilic or hydrophobic character of the active molecules.[37] In single emulsification (e.g. o/w), polymer (selected according to desired properties) is dissolved in volatile, water immiscible solvent, such as dichloromethane, ethyl acetate, or chloroform. This organic phase is then emulsified in an aqueous phase already containing a dissolved surfactant. High speed homogenization or sonication is the key to good dispersion of the oil phase in aqueous phase.[35] However, this technique fails when it comes to encapsulation of highly hydrophilic agents. This is because the active agent may diffuse into the continuous phase during the formulation or it may not get dissolved in the organic solvent. Multiple emulsions are more suitable in such cases. In this case, primary

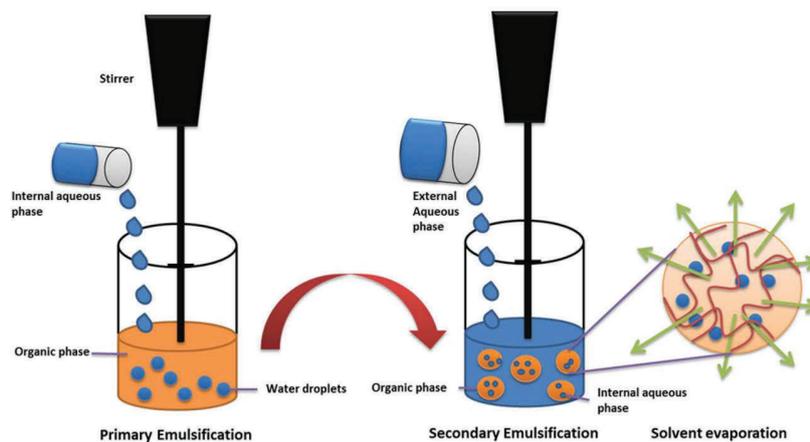


Figure 1. Emulsion solvent evaporation technique (w/o/w type).

emulsion formed via single emulsification (generally w/o) is again dispersed in second aqueous phase.[38] Most hydrophilic drugs have been encapsulated via (w/o/w) method.[39–41] In both single and double emulsification, the organic solvent is evaporated either by stirring at room temperature or under low pressure and high temperature conditions. The formed particles can be obtained by ultracentrifugation or filtration and finally can be washed or lyophilized.[28] Stabilizers play dual role in emulsions. They form films and provide barrier to drug release at internal interface and act as steric stabilizer on the external aqueous phase.[21,35,42]

2.2. Nanoprecipitation

Nanoprecipitation, also known as solvent displacement or interfacial deposition, is considered quite simple and reproducible method, which allows the production of polymer-based sub-micrometer particles (Figure 2). It is also thought to be one of the first developed techniques employed for the encapsulation of the active molecules. This method was introduced by Fessi

et al. [43] Since its development, this technique is mostly limited to encapsulation of mainly hydrophobic actives (because of the miscibility of the solvent with the aqueous phase) in either nanocapsules or nanospheres.[44–50] However, hydrophilic actives have also been encapsulated via nanoprecipitation.[51–55] Many different polymers have been used for this technique, especially biodegradable polyesters, such as polylactide (PLA), polylactide-co-glycolide (PLGA), and poly- ϵ -caprolactone (PCL).[56] Basically, this technique requires two miscible phases: an organic solvent in which polymer is dissolved and an aqueous phase (non-solvent of the polymer). Commonly used organic solvents are ethanol and acetone. Such solvents are miscible in water and can be removed by evaporation. The addition of one phase to the other under moderate magnetic stirring causes the interfacial deposition of the polymer after displacement of the organic solvent from the organic solution. This leads to the formation of nanoparticles. The formation of particles involves three basic steps: particle nucleation, molecular growth, and aggregation. The rate of every step is quite important for the particles size distribution.

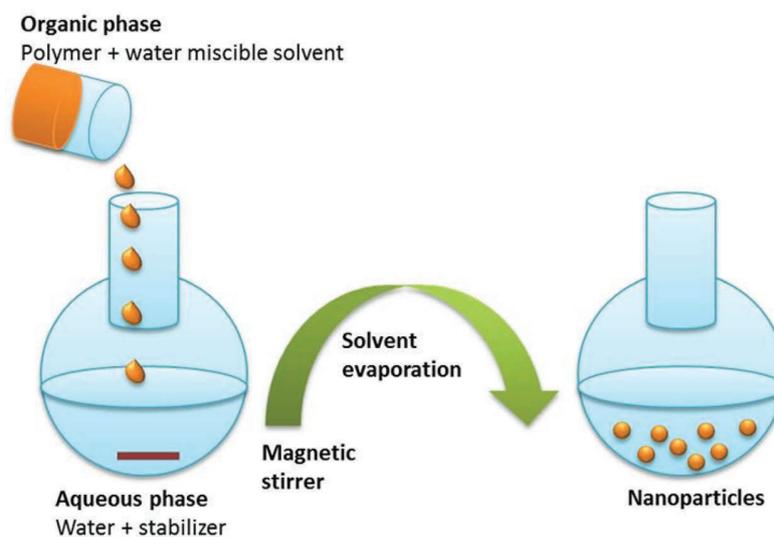


Figure 2. Nanoprecipitation technique.

Different formulation parameters that should be controlled include organic-non-organic phase ratio, polymer concentration, stabilizer concentration, and the amount of active molecules. Each of these parameters may influence the characteristics of the obtained nanoparticles (size, uniformity, and charge). This technique has many advantages over other encapsulation methods, such as simplicity, ease of scalability, good reproducibility, submicrometer particles with narrow size distribution, avoidance of usage of large amounts of toxic solvents, and no high energy input required.[57] Membrane contactor and microfluidic technologies have been successfully used to improve reproducibility and increase the convenience of application of nanoprecipitation in industries.[58]

2.3. Emulsion solvent diffusion

This method was introduced by Leroux et al. in 1995.[59] This technique basically requires the presence of three liquid phases: an aqueous phase (stabilizer dissolved in water), an organic phase (polymer in organic solvent and a hydrophobic active), and a dilution phase. The organic solvent must be partially soluble in water. The aqueous and organic phases are mutually saturated and emulsified using high-speed homogenization. Finally, addition of large volume of water allows the

diffusion of organic solvent from dispersed phase to external aqueous phase. This causes the precipitation of the polymer and, hence, the formation of particles (Figure 3).[35,60] The solvent is removed depending on its boiling point either by evaporation or filtration. Usually encapsulation efficiency is approximately 70%. This method is mainly used for the entrapment of hydrophobic actives. However, hydrophilic molecules may also be encapsulated by a modified solvent diffusion method using an aqueous inner phase.[61] The different operating conditions affecting the obtained particles include external/internal phase ratio, emulsification stirring rate, volume, and temperature of water for dilution, amount of polymer and the concentration of the stabilizer.[21,62]

2.4. Ionic gelation (IG)

IG is considered as a mild process since the use of toxic organic solvents and surfactants is avoided. It mainly involves the usage of hydrophilic natural polymers for preparation of particulate carriers. These polymers include chitosan, gelatin, agarose, and alginate. The basic principle of this method is the electrostatic interaction between the oppositely charged polymer and a polyelectrolyte (Figure 4). A solution of the charged polymer when added drop-wise under constant stirring to an

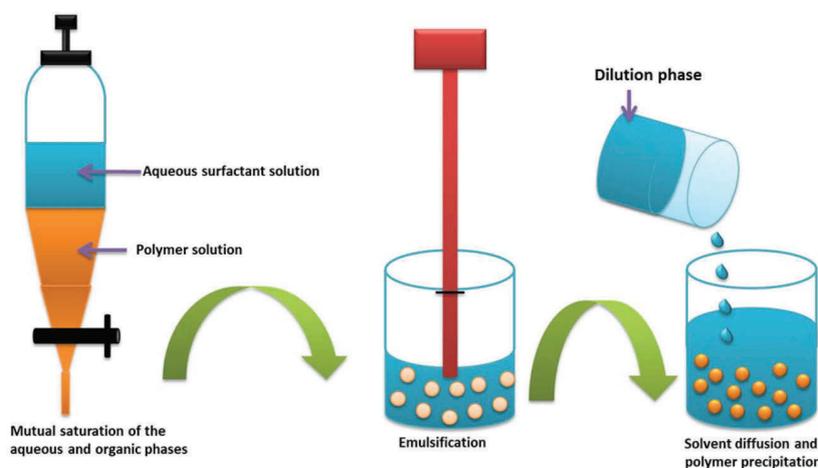


Figure 3. Emulsion solvent diffusion technique (adopted from Miladi et al. [28])

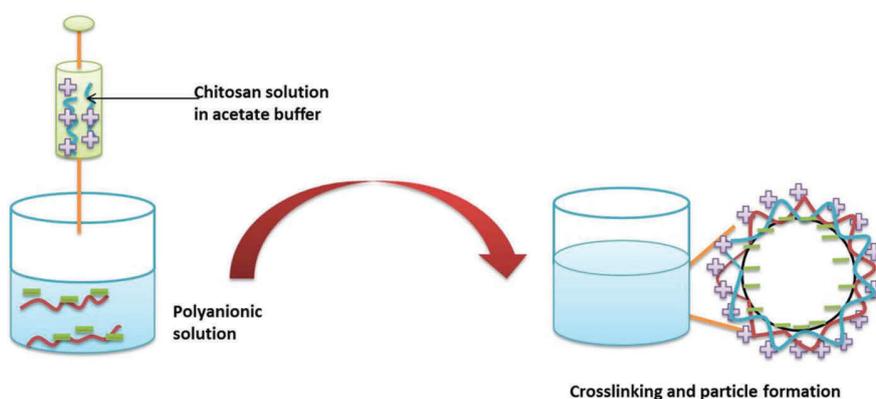


Figure 4. Ionic gelation technique (Adopted from Miladi et al. [28])

oppositely charged polyelectrolyte causes the cross-linking of the two entities and, hence, the particulate formation.[63,64] Gelatin-based particles are obtained after hardening of the droplets of emulsified gelatin solution. The gelatin emulsion droplets are cooled below the gelation point in an ice bath to obtain the particles. Alginate particles are obtained by drop-by-drop extrusion of the sodium alginate solution into the calcium chloride solution. Sodium alginate is a hydrophilic polymer, which gels in the presence of multivalent cations such as calcium. This technique was widely used to prepare chitosan particles. Electrostatic interaction occurs between positively charged chitosan (in an acidic medium) and negatively charged tripolyphosphate or arabic gum.[65–67]

3. Applications of encapsulated plants extract

3.1. Cosmetic applications

The use of plant extracts for skin application is limited because some of their active compounds exhibit high volatility, low solubility in aqueous systems and/or a short residence time on the skin. The use of controlled release systems, such as nanoparticles enables an increase in the amount of active incorporated in aqueous systems and increased bioactivity, protection and stability of the actives. Due to their size, the nanoparticles can penetrate intracellular or intercellular spaces and through the hair follicles, which makes them a suitable pharmaceutical carrier of the extracts for the skin application.

In recent years, several works support the feasibility of using nanoparticles for the release of plant extracts in the skin. Tachaprutinun et al.[68] developed formulations containing both free *Garcinia mangostana* Linn extract and encapsulated *Garcinia mangostana* Linn extract in ethylcellulose or methylcellulose. They evaluated the intracellular and follicular penetration of the nanoparticles by tape stripping technique and fluorescence microscopy, respectively. The nanoparticles loaded with extract showed an average size of 625 ± 20 nm, a zeta potential of 3.6 ± 0.2 mV. Both free extract and encapsulated extract were incorporated into creams or aqueous systems, and it was determined that both formulations in cream (free extract and encapsulated) penetrated deeper into the hair follicles as compared to those applied in aqueous systems. Furthermore, *G. mangostana* Linn extract incorporated into nanoparticles showed higher and more homogeneous distribution in the layers of the stratum corneum compared to the free extract. Hence, the results showed that the viability and dermal penetration of plant extract was increased when it was encapsulated in nanoparticles.[68]

The lavender extract is one of the most commonly used extracts in cosmetics. However, its application is limited by its physicochemical instability. Pereira et al. encapsulated methanolic extracts of *Lavandula stoechas* and *L. pedunculata* in nanoparticles of PLGA for increasing the chemical stability of the extract. Before the encapsulation process, it was determined that the methanolic extracts of both plants contained a high amount of flavonoids and, therefore, they possessed high antioxidant properties and low toxicity to human keratinocytes (up to $15 \mu\text{g/mL}$ during 24 h), which favored their dermal application. The physicochemical characterization of

the loaded nanoparticles showed a particle size of 300 nm for formulations prepared with both extracts. The obtained zeta potential was -15.74 ± 9.93 and -19.35 ± 8.42 mV for *L. stoechas* and *L. pedunculata*, respectively. The scanning electron microscopy showed nanoparticles with spherical form and smooth surface. The percentage of encapsulation was 95.8% for *L. stoechas* and 96.7% for *L. pedunculata*. The loaded nanoparticles were not tested for skin penetration; therefore, it was difficult to determine if the nanoparticles increased the deposition of the lavender extracts in skin. However, this work provides fundamentals for the elaboration of nano-objects containing lavender extracts for application in cosmetics or dermatological treatments.[69]

3.2. Food additives applications

In recent years, increasing interest in eating healthy and less processed foods, has led to the reduction of synthetic compounds and they are being replaced by natural sources such as plant extracts products. However, the replacement of these compounds is not easy due to the poor chemical stability shown by plant extracts during the processing steps in food industry. Thus, nanoparticles composed of different polymers have been developed for this purpose.

In 2011, Anbinder et al. developed a formulation from alginate, chitosan, and aqueous extract of *Ilex paraguariensis* (yerba mate) in order to use it as a delivery system of antioxidants and add them to functional foods. The extract was incorporated in three different systems (composed of calcium alginate, chitosan, and a mixture of both polymers) and their physicochemical characteristics were compared by scanning electron microscopy, thermal analysis, and infrared spectroscopy. The system based on the mixture of both polymers showed an interaction between calcium alginate matrix and the external layer of chitosan. The chitosan nanoparticles showed enhanced release of polyphenols in the simulated intestinal fluid. This suggested a greater interaction between chitosan and the extract. The thermal analysis indicated that the systems do not decompose at high temperatures, making it feasible for the systems to be used in the production of fortified foods.[70] Subsequently, in 2013, the same research team, studied the release of polyphenols from loaded yerba mate nanoparticles (hydrated or dried) elaborated with mixture of calcium alginate/chitosan. The release of polyphenols from the hydrated nanoparticles was immediate in acidic pH due to the erosion of the system, while dried nanoparticles enabled release of the compounds after a period of swelling (hydration of the system). The results supported the implementation of these nanoparticles in instant soups, thereby increasing the amount of natural antioxidants available from foods.[71]

It is well known that plant extracts are a source of antimicrobial agents, which can be used as preservatives in different types of food. However, sometimes the addition of large amounts of extract to foods may cause odor and taste disturbances. Moreover, many of these extracts are hydrophobic, limiting their potential application. To resolve this problem, biodegradable nanoparticles have been developed. Hill et al. encapsulated root extract of cinnamon using biodegradable

PLGA in two different ratios (65:35 and 50:50) to promote the antimicrobial activity of the extract. The size of nanoparticles was 145–167 nm with a percentage encapsulation of 39% and 48% for PLGA 65:35 and PLGA 50:50, respectively. Release tests showed an initial burst effect due to the extract present in the surface of the nanoparticles, followed by a controlled release of the extract from the polymeric matrix. The two types of nanoparticles inhibited the growth of *Salmonella Typhimurium* and *Listeria monocytogenes* after 24 and 72 h in a concentration range of 224–529 mg/mL. In conclusion, the PLGA-based nanoparticles of cinnamon improved the release and antimicrobial activity of hydrophobic compounds in aqueous media, commonly found in foods.[72]

Pereira and collaborators used nanoparticles of PLGA (ratio 65:35 and 50:50) for encapsulation of an extract rich in carotenoids obtained from the fruit of guabiroba (*Campomanesia xanthocarpa* O. Berg) to improve the antioxidant and antimicrobial activity. The size of nanoparticles obtained was in a range of 145–162 nm. All nanoparticles showed a spherical shape and smooth surfaces. Encapsulation efficiency of the extract was 83.7% and 98.5% for nanoparticles of PLGA 50:50 and PLGA 65:35, respectively. Furthermore, release profile of the extract (based on the measurement of carotenoids release) from the nanoparticles showed an initial burst effect followed by a decrease in cumulative release over time for both formulations. The initial burst effect was more pronounced in nanoparticles of PLGA 50:50 (92% in an hour) compared with 37% obtained from the nanoparticles of PLGA 65:35. The antimicrobial activity was enhanced when the extract was encapsulated in PLGA 50:50 nanoparticles. Assays for determination of antioxidant activity showed the highest activity for the PLGA 50:50 based nanoparticles. Moreover, a lower concentration of encapsulated guabiroba extract was required to reduce reactive oxygen species (oxidation) in human colon adenocarcinoma HT-29. Overall, the results showed that PLGA nanoparticles could be used to incorporate extracts rich in carotenoids or other functional lipids as delivery systems with increased biological activity.[73]

In the food area, nanoparticles may also provide controlled release formulations of nutraceutical compounds. The root and rhizome of *Pichorhiza kurroa* has demonstrated significant hepatoprotective activity that improves liver function. However, principal molecules in the extract (picoside I and II) show poor gastrointestinal absorption and bioavailability (low aqueous solubility). Recently, Jia et al. developed biodegradable nanoparticles constituted using PLA and pluronic F-68. The observed size was about 175 and 155 nm and encapsulation efficiency 60% and 67% for picoside I and II, respectively. The value of zeta potential for both formulations was -27.87 and -31.5 for blank and loaded nanoparticles, respectively. This confirmed good stability of nanoparticles for long periods of storage. Meanwhile, in the release studies, an initial burst effect followed by a sustained release up to 210 h was observed. The formulation could be used as a nutraceutical with hepatoprotective effect. However, it is important to undertake studies to demonstrate the biological activity of the extract after the encapsulation process.[74]

3.3. Phytotherapy applications

Despite the great potential of plant extracts as source of treatments for various diseases, there are few investigations that support their use in formulations, because, in general, the extracts show lower *in vitro* biological activity when are compared with pure chemical compounds. It has been demonstrated that a strong synergy exists between the compounds present in the extracts, which, in many occasions is lost if these compounds are isolated. Moreover, the poor water solubility, physicochemical instability, and complexity of such extracts further limit their use. The application of nanoscale release systems for extracts provides an increase in solubility of the compounds, improved bioavailability, dose reduction, steady plasma levels and enhanced stability.

3.3.1. Antidiabetic activity

In this context, Samadder et al. developed formulation, which encapsulated the ethanolic extract of the seeds of *Syzygium jambolanum*. The aim was to improve treatment of hyperglycemic stress induced by arsenic poisoning. The average size of these nanoparticles was 122 nm, with spherical shape and smooth surface. Constant release of *Syzygium jambolanum* from its nano-encapsulated form was observed from 0 to 30 min in L6 cells. The studies showed that the application of extract encapsulated in nano-objects allowed increased consumption compared to the application of free extract. This may be because the loaded nanoparticles enable better functioning of the cellular glucose transporter GLUT4. In addition, the loaded nanoparticles showed a potential protection/recovery of cells damaged by arsenic. In *in vivo* (mice) models, it is determined that the free extract and encapsulated extract decreased the glucose and glycated hemoglobin levels induced by arsenic. Finally, by fluorescence techniques it was observed that the nanoparticles could effectively cross the blood brain barrier, whereby the formulation could provide targeted treatment in this important area. Thus, formulation showed a significant potential for use in the treatment of hyperglycemia induced by arsenic poisoning.[75]

3.3.2. Anti-inflammatory activity

Because of the wide range of biological activities offered by the extracts of the fruit of *Embllica officinalis*, Renuka et al. encapsulated ethanolic and aqueous extracts of this plant in polyvinylpyrrolidone (PVP)-based nanoparticles and evaluated their antioxidant and anti-inflammatory activity. The encapsulation efficiency of the nanoparticles with extracts was found to be between 58% and 70%. The particle size was determined to be in the range of 550–825 nm. The obtained zeta potential was in a range of -7.49 to -13 mV, indicating a high stability for the systems. The release profile of the nanoparticles was better in 50 mg of ethanolic or aqueous extract. The formulation prepared with 50 mg of ethanolic extract showed the highest antioxidant activity, which could be attributed to the greater amount of flavonoids present in this kind of extract. In the anti-inflammatory activity test, the formulations prepared with the aqueous extracts showed greater activity than those containing ethanolic extracts. Generally, the results showed

that the encapsulation of aqueous and ethanolic extracts of *E. officinal* in nano-objects increased their antioxidant and anti-inflammatory activities.[76]

In 2014, with the aim of increasing anti-inflammatory activity of *Scutellaria baicalensis*, Choi et al. developed lectin nanoparticles loaded with aqueous or ethanolic extract of the plant. Nanoparticles loaded with ethanolic extract showed an average size of 94 nm and a percentage encapsulation of 62%. The antioxidant activity of nanoparticles was established by a 60% inhibition in the activity of DPPH radical. In addition, a concentration of 0.5 mg/mL of loaded nanoparticles inhibited the production of nitric oxide and produced only a concentration of 743.7 pg/mL PGE2 in macrophages RW264.7. Furthermore, this study was the first to show that the penetration of nanoparticles in human fibroblasts using scanning confocal microscopy.[77]

3.3.3. Anticholesterolemia activity

Considering the medicinal properties of *Clerodendrum infortunatum*, Suman et al. encapsulated root ether extract in nanoparticles of PLGA for treating hypercholesterolemia. The formulation showed particle size of about 608 nm with a zeta potential, drug efficiency and encapsulation efficiency of -30 mV, 32.8%, and 98.40%, respectively. Based on these characteristics, the nanoparticles-based preparation could be used for the treatment of hypercholesterolemia by passive targeting to the liver. However, there is no solid evidence about it.[78]

3.3.4. Antiulcerolitic activity

The crude extract (CE), the fraction obtained with ethyl acetate (EAF), and the residual aqueous fraction obtained from leaf *Passiflora serratodigitata* L. show a potential antiulcer activity due to the presence of a high content of flavonoids. To increase the aqueous solubility and provide increased stability of these substances, Strasser et al. developed poly(ϵ -caprolactone) (PCL)-based nanoparticles encapsulating *P. serratodigitata* extract, which could be used for gastroprotective properties. PCL-CE and PCL-EAF nanoparticles presented a size of 379 and 383 nm and a zeta potential of -20.2 and -27.3 mV, respectively. The encapsulation efficiency (based on the total amount of flavonoids) was 90.6% and 79.9% (w/v), respectively. *In vivo* studies showed that the nanoparticles loaded with CE provided four times more gastric protection than the free CE extract. The nanoparticles-EAF required 10 times less extract for achieving the same gastroprotective activity as that of EAF free extract. Probably, the nanoparticles-EAF was more potent due to the high purity of the extract. These results showed that both crude extracts of *P. serratodigitata* L., and their nanoencapsulated form had a high antiulcer activity and would be potential candidates for treatment of gastric ulcers.[79]

3.3.5. Anticancer activity

A large number of plants show a significant cytotoxic activity against various cancer cells. However, due to their toxicity they could be potentially fatal if administered for long periods. Nowadays, the use of nanoparticles for the delivery of plant extracts against cancer is one of the major focused fields. The studies related to nanoparticles for this purpose includes the

physicochemical characterization, *in vitro* or *in vivo* toxicology analysis, cell internalization studies and the influence on apoptosis and genetic damage.

In this field, Bhattacharyya et al. developed PLGA-based nanoparticles of ethanolic extract (tincture) of *Gelsemium sempervirens* and studied the effect of the formulation on their cellular internalization and *in vitro* bioactivity. Loaded nanoparticles had an average size of 122 nm. Their morphology showed spherical particles with smooth surface. The zeta potential was -14.8 mV, which suggested that the formulation was physically stable. The extract loaded nanoparticles showed to be more potent and active compared to non-encapsulated extract. In comparison with the free extract, the nanoparticles were better internalized. For the cellular effect of formulation, the results showed that loaded nanoparticles caused increased expression of p53 and caspase-3 (two key proteins markers in apoptosis) and downregulation of survivin, cyclin-D1, and PCNA; thus providing evidence about the working mechanism of these carriers. Test results of Annexin V-FITC and TUNEL FACS techniques confirmed that encapsulated extract induced greater degree of apoptosis with little necrotic potential as compared to free extract. *In vivo* model studies are needed to use these formulations in cancer treatment.[80]

The ethanolic extract of the root of *Polygala senega* cause death and apoptosis in lung cancer cell line A549 but show no toxicity in normal lung cells. Paul et al. encapsulated methanolic extract of this plant in PLGA-based nanoparticles in order to increase bioavailability and cellular internalization of the active in the A549 cell line for use in cancer therapy. Neither free extract nor the loaded nanoparticles showed cytotoxic effects on normal lung cells. However, after 24 h, a dose of 200 μ g/mL of free extract or encapsulated extract inhibited 68.65% and 77.46% of the growth of cancer cells A549, respectively. Fluorescence techniques showed that loaded nanoparticles were internalized faster than free extract (30 and 45 min, respectively). Both formulations saturated cancer cells after 240 min. Furthermore, both free extract and encapsulated extract induced apoptosis of A549 cells, which was associated with decreased expression of survivin, PCNA, and increased expression of caspase-3 and p53 in cell line A549. These results showed the great therapeutic potential of ethanolic extract loaded in PLGA nanoparticles.[81]

Another plant extract with potential anticancer activity in cell line A549 was obtained from root of *Phytolacca decandra*. Das et al. developed nanoparticles loaded with ethanolic extract of this plant and studied their therapeutic effectiveness *in vitro* and *in vivo* models. The nanoparticles showed a size around 110 nm, a zeta potential of -17.5 mV, and a smooth surface. The Fourier transform infrared spectroscopy, high performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance analysis showed the incorporation of a tri-terpenoid (derivative of betulinic acid) in biodegradable nanoparticles. The encapsulation efficiency of the loaded nanoparticles was 82.15%, from which, only 56% had a constant release for 24 h. The loading of *P. decandra* in PLGA nanoparticles provided more effective release of the extract with higher antitumor efficacy. Extract loaded nanoparticles

also showed a significant modulation of apoptosis and increased bioactivity of the active probably due to their small size, high cellular/tissue internalization and improved bioavailability.[82]

Helicobacter pylori bacterium is one of the principal causes for the development of gastric cancer and over the past years it has shown resistance to antibiotics. The extract of *Garcinia mangostana* L. shows anticancer and anti-*H. pylori* activity. However, its therapeutic application in the stomach mucosa requires a delivery system that can withstand the acidic condition for a sufficient period of time. Pan-In et al. formulated nanoparticles loaded with *G. mangostana* using ethyl cellulose (EC) and methylcellulose (MC) as polymer to improve the residence time and increase resistance to damage by the acidic conditions of the stomach. EC:MC nanoparticles showed an encapsulation efficiency of 98.9% and loading capacity of 49.7%, with an average particle size of 500 nm and a zeta potential of 1 mV. Loaded nanoparticles presented a sustained release of the extract at pH 2 and pH 7.4. In addition, encapsulation of extract in the nanoparticles did not reduce their anti-*H. pylori* activity. *In vitro* antiadhesion activity of the extract of *G. mangostana* was enhanced with their nanoencapsulation, preventing the infection of *H. pylori*. Moreover, in oral administration *in vivo* studies, the encapsulated extract showed a greater ability to combat *H. pylori* in mice stomach. The results showed that the extract of *G. cambogia* encapsulated in nanoparticles had high potential as anti-*H. pylori*. [83] In 2014, the same research team developed a similar formulation and tested the internalization and cytotoxic activity of nanoparticles in the HeLa cell line. The nanoparticles loaded with *G. mangostana* of EC:MC with a size of 250 nm showed twice as much cytotoxic activity than the nanoparticles developed with only EC. Based on the *in vitro* tests, it was established that the loaded nanoparticles were internalized by endocytosis mechanism mediated by clathrin and mobilized internally through endolysosomal pathway.[84]

Due to chemical complexity posed by plant extracts, current studies on nano-object-based formulations not only focus on cytotoxic-bioactive behavior of nanoparticles both *in vitro*

and *in vivo* models, but also focus on establishing experimental designs that will produce more efficient formulations or functionalization of the nanoparticles to achieve an increased anticancer properties of the extracts. In this context, Ribeiro et al. elaborated biodegradable PLGA-based nanoparticles loaded with hydroalcoholic extract of *Uncaria tomentosa*, which present a significant antitumor activity. Besides, obtaining a formulation with potential therapeutic application in cancer, the most novel aspect of this work was the development of a fractional factorial design study based on behavior variables during the formulation to facilitate selection of optimal conditions and develop the best nano-objects with the plant extract of *U. tomentosa*. [85] Another new study was developed by Narayanan et al. They obtained PLGA-based nanoparticles loaded with grape seed extract with a size of approximately 100 nm and encapsulation efficiency of 60%. Through a process of chemical conjugation, folic acid was added to the surface of the nanoparticles, generating a specifically functionalized preparation for the folate receptors, which are overexpressed in cancer cells. Fluorescence and flow cytometry made evident a high and specific internalization of the functionalized nanoparticles. Moreover, it was possible to relate the functionalization of the nanoparticles with an increase death of HeLa cells. The IC₅₀ of the functionalized nanoparticles-based preparation was about three times less than free extract and showed a higher rate of apoptosis. This *in vitro* study establishes bases for the use of functionalized preparation to increase bioavailability and anticancer activity of plant extracts.[86] Table 1 summarizes the most commonly used techniques for the encapsulation of plant extracts.

4. Conclusion

Currently, the great potential of the plant extracts, as the source of various chemical compounds with important biological activities is very well known. Plant extracts might be used in different areas, such as food, therapy, and cosmetics. However, many of these plant extracts have shown poor

Table 1. Encapsulation methods and applications of plant extracts.

Extract name	Encapsulation method	Application	Encapsulation efficiency (EE, %)	Reference
<i>Garcinia mangostana</i>	Nanoprecipitation	Anticancer	87	[84]
<i>Picrorhiza kurroa</i>	Nanoprecipitation	Hepatoprotective	63.6	[74]
<i>Polygala senega</i>	Nanoprecipitation	Anticancer	80	[81]
<i>Passiflora serrato-digitata</i>	Nanoprecipitation	Antiulceritic	90.6	[79]
<i>Syzygium jambolanum</i>	Nanoprecipitation	Antidiabetic	Not mentioned	[75]
<i>Gelsemium sempervirens</i>	Nanoprecipitation	Anticancer	81.6	[80]
<i>Garcinia mangostana</i>	Nanoprecipitation	Skin penetration enhancement	98.94	[68]
<i>Vitis vinifera</i>	Nanoprecipitation	Nutraceuticals bioavailability enhancement and effective targeting of cancer	Not mentioned	[86]
<i>Emblica officinalis</i>	Solvent evaporation	Anti-oxidant and anti-inflammatory	58–70	[76]
<i>Phytolacca decandra</i>	Nanoprecipitation	Better chemo-preventive action against lung cancer	82.15	[82]
<i>Uncaria tomentosa</i>	Emulsion solvent evaporation	Anticancer	64.6	[85]
<i>Cinnamomum</i> spp.	Emulsion-solvent evaporation	Antimicrobial	47.6	[72]
<i>Lavandula</i> ssp.	Modified-spontaneous Emulsification solvent diffusion method (mSEDM)	Anti-aging and antioxidant agents	>96%	[69]

solubility in aqueous systems, short residence time onto the site of application, high chemical instability, and chemical complexity, which affects the biological activity of their different compounds. These challenges limit their direct and efficient application. Controlled release systems such as polymeric nanoparticles offer a solution to these problems. Right selection of the encapsulation method is the key element to obtain a formulation (nanoparticles) bearing suitable characteristics for the *in vitro* and *in vivo* applications. Generally, it is observed that nanoprecipitation technique provides most suitable nanoparticles in terms of size and encapsulation efficiency. Among the advantages provided by nanoparticles as carriers of plant extracts are: (i) protection against enzymatic degradation, (ii) protection in the application environment, (iii) design of sustained release systems of bioactive compounds, (iv) enhancement of solubility, (v) bioavailability improvement, (vi) affected areas targeting, (vii) boosting of cell or tissues internalization, (viii) reduction of toxicity, and (ix) masking of unpleasant odor and taste. Literature also shows that mostly dermal and oral routes are employed for the delivery of formulations based on encapsulated plant extracts.

5. Expert opinion

In recent years, one of the biggest challenges in the area of pharmaceutical technology is the development and efficient implementation of new therapies. The products obtained from natural sources (e.g. extracts, essential oils, infusions, etc.) represent a novel and efficient alternative to conventional treatment. Many of these natural products, particularly plant extracts, have important biological activities. However, both the formulation and the implementation thereof represent an even greater challenge due to the complex chemical nature of these extracts. Other obstacle associated with using conventional plant extracts-based formulations for humans is the risk of toxicity. Instability of such plant extracts is another concern to be dealt with.

To resolve these problems, recently, it has been proposed to use nanoparticles as carriers of plant extracts. Today, there are a considerable number of investigations are performed in which the nanoparticles are evaluated not only for the administration of the extracts, but also to increase their biological properties. Due to encapsulation of the plant extracts in the nanoparticles (embedded, adsorbed, or bonded), these vehicles represent an excellent approach for controlled release of actives, thereby decreasing the number of doses and toxic effects that occur with conventional delivery.

Among the most important features of nanoparticles in the area of pharmaceutical technology, are: (1) nanometric size, which allows greater interaction of actives with specific cells or tissues, (2) nanoparticles formulations are generally more stable as compare to simple plant extracts solutions, (3) natural extracts are protected by the polymeric wall from external factors (e.g. light, temperature, humidity) and within biological systems (e.g. enzymes, pH,) and (4) rough surface of nanoparticles provides better interactions with biological systems (e.g. cells, tissues, and organs). All these characteristics

can increase the therapeutic efficiency of nanoencapsulated plant extracts.

In the investigations discussed above, formulations containing nanoparticles and encapsulated plant extracts, mainly, maintain and increase the biological activity of the extracts incorporated. Important activities, such as, anti-cancer, anti-diabetic, antiulcerolitic, antioxidant, and antimicrobial are tested in these formulations. The results obtained from the formulations with encapsulated plant extracts show the great potential of nanoparticles for use as carriers of this type of actives.

However, it is very important to carry out careful studies regarding the toxicological aspects involved in the administration of the nanoparticles loaded with plant extracts in biological systems. There is still no specific evidence to support the use of nanoparticles without any risk. In addition, toxic effects, accumulation in biological systems and removal mechanisms must be established. Moreover, the methodologies that are conventionally used for evaluation *in vivo* and *in vitro* of plant products must be adapted to provide reliable results. Another important aspect to consider is to scale-up these processes in industries.

Because of all favorable physical and biological characteristics presented by the nanoparticles, it is clear that their usage is one of the newest routes for the delivery of actives with different chemical properties. In coming years, nanotechnology employment in plant extracts delivery for safe usage in the areas, such as health, medicine, food, cosmetics, and environment, among others could be an interesting field for research.

Declaration of interests

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II.3.Nanoprecipitation process: From encapsulation to drug delivery

Summary

In this part of the literature review the encapsulation of drugs by nanoprecipitation encapsulation method; encapsulation based marketed products, precipitation mechanism throughout nanoprecipitation technique described in details. Furthermore, in vitro release profile, of encapsulated drugs and their applications in medicine, food and agriculture are explained in this review. A part of this review is dedicated to the clinical trial of drug loaded nanoparticles prepared by nanoprecipitation, industrial scale-up, and advantages and disadvantages of nanoprecipitation description. Encapsulation of drug molecules is a promising technique for the drug delivery. Nanospheres, nanocapsules, microspheres, microcapsules and liposomes are the main pharmaceutical forms that can be designed by the encapsulation methods. Since nanoprecipitation looks to be the most simple and reproducible encapsulation method among all encapsulation techniques, thus it is the most frequently used technique. In fact, 40 % of the available drug molecules on the market are poorly soluble and 90 % of drug in active molecules in development pipeline are categorized as poorly soluble. The biocompatible, safe, easily administered, comfortable and inert system of drug delivery can be designed by drug encapsulation within biodegradable polymers. The commercialization of nanotherapeutics encounter major barriers and challenges as: (a) quality control deficit; (b) separation from unwanted nanostructures; (c) scalability correlated issues; (d) production scale enhancement; (e) batch to batch reproducibility in terms of size distribution of particles, charge, porosity, and mass; (f) high manufacturing price; (g) information shortage concerning nanosystems and living cell interaction; (h) nanotherapeutics therapeutic capacity optimization; (i) investment doubt by pharmaceutical industries on nanotherapeutics. (j) nanomaterials negative features regular target by media, without a clear scientific proof. Nanoprecipitation encapsulation method is mainly used for the encapsulation of hydrophobic drug molecules. In nanoprecipitation method it is needed to prepare the solvent and nonsolvent phases followed by the addition of organic phase to aqueous phase under moderate magnetic stirring. Consecutively, organic solvent evaporation at ambient temperature or with a rotavapor allows the obtaining of nanoparticles (NPs) suspension in water. Mostly employed solvents in nanoprecipitation technique are ethanol, acetone, hexane, methylene chloride or dioxane. Typically, water is non-solvent (or aqueous phase). Principle of nanoparticles preparation by nanoprecipitation method is the reduction of the solvent quality in which the central composition of nanoparticles is dissolved. This solvent quality variation can be achieved by modifying the pH, salt concentration, solubility conditions, or the addition of a non-solvent phase. Non-solvent based precipitation process comprising four steps of supersaturation generation, nucleation, growth, and coagulation. Supersaturation preform when the solution encloses more dissolved solute than that given by the equilibrium saturation value. Indeed, the addition of non-solvent decreases solvent potency to dissolve the solute, which put the system in a

supersaturation state. The *in vitro* release of drug from nanoparticles prepared by nanoprecipitation method commonly involves of two phases: a first phase of “burst release” and second phase (prolonged release). The first phase is because of the drug substance release that is adsorbed on nanoparticles surface or that is dispersed near to the surface. The second phase is thanks to release of drug that is located in the central compartment. To approve clinically a formulation, its transition from laboratory to industrial grade is important. Nevertheless, this transition has to be controlled via a scale-up strategy to create industrial scale parameters that lead to the mass production of laboratory-like formulations. Nanoparticles formulations scaling-up are frequently successful and have advantages over laboratory scale production. In comparison to pilot-scale smaller polymers amounts are needed to produce nanoparticles in pilot-scale. Furthermore, pilot-scale processes are further reproducible than laboratory-scale ones. In addition, polymer precipitation throughout nanoparticles production is more efficient in pilot-scale as formulation parameters are well controlled. Numerous research works were performed to use nanoprecipitation in a conventional way whereas other researches concentrated on the enhancement of nanoprecipitation scalability, reproducibility and safety by scale-up. To this end, Tee mixer and flash nanoprecipitation are among the techniques that were introduced. Throughout several studies advantages of submicron carriers prepared by nanoprecipitation in the biomedical and agricultural fields has confirmed. Nanoprecipitation was extensively employed for the preparation of nanoparticles. Even though several advances have been noted, extra *in vivo* human studies are required.



Review

Nanoprecipitation process: From encapsulation to drug delivery



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ABSTRACT

Drugs encapsulation is a suitable strategy in order to cope with the limitations of conventional dosage forms such as unsuitable bioavailability, stability, taste, and odor. Nanoprecipitation technique has been used in the pharmaceutical and agricultural research as clean alternative for other drug carrier formulations. This technique is based on precipitation mechanism. Polymer precipitation occurs after the addition of a non-solvent to a polymer solution in four steps mechanism: supersaturation, nucleation, growth by condensation, and growth by coagulation that leads to the formation of polymer nanoparticles or aggregates. The scale-up of laboratory-based nanoprecipitation method shows a good reproducibility. In addition, flash nanoprecipitation is a good strategy for industrial scale production of nanoparticles. Nanoprecipitation is usually used for encapsulation of hydrophobic or hydrophilic compounds. Nanoprecipitation was also shown to be a good alternative for the encapsulation of natural compounds. As a whole, process and formulation related parameters in nanoprecipitation technique have critical effect on nanoparticles characteristics. Biodegradable or non-biodegradable polymers have been used for the preparation of nanoparticles intended to *in vivo* studies. Literature studies have demonstrated the biodistribution of the active loaded nanoparticles in different organs after administration *via* various routes. In general, *in vitro* drug release from nanoparticles prepared by nanoprecipitation includes two phases: a first phase of “burst release” which is followed by a second phase of prolonged release. Moreover, many encapsulated active molecules have been commercialized in the pharmaceutical market.

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1. Introduction

Conventional drug delivery systems like tablets, capsules, solutions *etc.* are still the most used ways for medicines administration. Such formulations present many advantages such as, full control of preparation processes, common availability of manufacturing facilities and efficacy. Furthermore, major advances have been made to enhance drugs solubility and sustained release. Various excipients could be added to improve drugs properties and biodistribution. These advances enabled widening of used arsenal against diseases. However, in most cases, *in vivo* activity of conventional dosage forms remains limited to drug physicochemical properties. Consequently, stability, taste and absorption concerns are still observed. In addition, targeting specific tissue or cells could not be reached. For these reasons, encapsulation appeared as an interesting approach for drug delivery. In fact, major advances have been made, since the last decades, toward preparation of drug delivery systems that are based on entrapment of actives in various structures. Several techniques have also been used for encapsulation such as, emulsion solvent evaporation, nanoprecipitation, emulsion solvent diffusion, ethanol injection, ionic gelation *etc.* Major pharmaceutical forms prepared *via* these techniques are nanospheres, nanocapsules, microspheres, microcapsules and liposomes. Among these techniques, nanoprecipitation seems to be the most simple and reproducible. This made it one of the most commonly used approaches for the nanoparticles preparation. Several polymers are used to encapsulate drugs with nanoprecipitation. Among them, we could cite biodegradable polyesters such as, polylactide (PLA), polylactide-co-glycolide (PLGA) and poly- ϵ -caprolactone (PCL). Obtained particles could be either nanocapsules or nanospheres. Nanocapsules are vesicles with core-shell structure in which the drug is confined within a cavity surrounded by a polymeric membrane. Nanospheres are, however, particles in which the drug is either dissolved or dispersed within the polymer matrix (Mora-Huertas et al., 2010; Letchford and Burt, 2007). Nanoprecipitation is based on the interfacial deposition of a polymer following the displacement of a

semi-polar solvent miscible with water from a lipophilic solution (Fessi et al., 1989). It is an easy and reproducible technique that has been widely used in the preparation of nanoparticles. In this review, the state of the art of this technique is performed. Definition of the method is provided. Nanoprecipitation mechanism and applications are also discussed.

2. Encapsulation of active pharmaceutical ingredients

Encapsulation has been broadly explored in the fields of pharmaceuticals, agriculture, food, cosmetics, and textile industries over the past decade (Ghosh, 2006). Modern technologies recently paved the way to the evolution of indigenous pharmaceuticals. Therefore, advanced drug deliveries are successively taking the place of conventional dosage forms that were less flexible and less sophisticated. The fact of overcoming these conventional dosage forms constraints attracted a special attention. Bioavailability, stability, taste, and odor could be among the aforementioned barriers. In this regard, encapsulation takes a crucial part in order to overwhelm these challenges (Iqbal et al., 2015). According to the biopharmaceutical classification system (BCS), 40% of the currently commercialized drug molecules on the market are poorly soluble while 90% of drug molecules in drug development pipeline are also categorized as poorly soluble (Loftsson and Brewster, 2010). In addition, drugs encapsulation could play an important role in prevention of active ingredients from degradation and obtaining of controlled or targeted drug release systems. In fact, following active molecule encapsulation, biodistribution would no longer be related to drug itself but to carriers physicochemical properties (Armendáriz-Barragán et al., 2016). Drug encapsulation as the best approach through the employing of biodegradable polymers may provide the biocompatible, easily administered, safe, comfortable and inert drug delivery system. Indeed, delivery system possessing such properties could be defined as an ideal drug delivery system (Kalani and Yunus, 2011). The adequate encapsulation method should be selected based on the hydrophobic or hydrophilic properties of

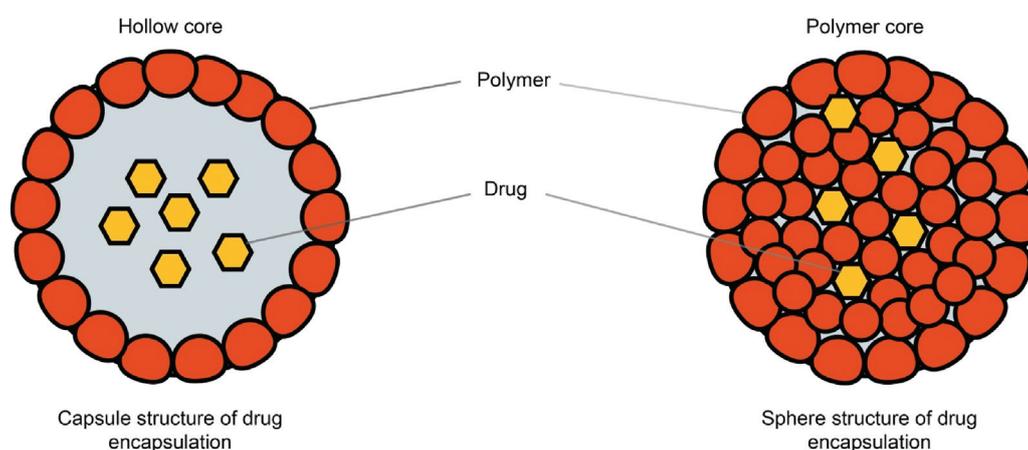


Fig. 1. Schematic representation of encapsulation forms.

drugs (Jelvehgari and Montazam, 2012). Fig. 1 shows examples of structures that could be obtained by encapsulation.

To resume, drugs encapsulation might be used for several reasons such as:

- Drug release prolongation,
- Design of targeted drug delivery,
- Mask unfavorable organoleptic properties (taste, odor, color),
- Protection of sensitive drugs from digestive tube contents degradation effect,
- Insurance of drug molecules stability toward environmental destructive factors such as, oxygen, temperature, moisture and light,
- Reducing the vaporization of volatile materials,
- Prevention of drugs incompatibility,
- Toxicity moderation,
- Hygroscopic characteristic decline of substances,
- Design of new dosage forms, (Singh et al., 2010)

3. Encapsulation based marketed products

The main aim of nanotherapeutics research and development in the pharmaceutical industry is to provide new approaches for the treatment of diseases. Some encapsulation based drug delivery systems are already marketed (see Table 1). Nevertheless, nanotherapeutics commercialization faces major challenges and hurdles such as: (a) deficit of quality control; (b) separation from unwanted nanostructures (e.g., products and starting materials); (c) scalability related issues; (d) improvement of production scale; (e) batch to batch reproducibility in terms of particles distribution of size, charge, porosity, and mass; (f) high manufacturing price; (g) information shortage concerning nanosystems and living cell interaction (e.g., biocompatibility and toxicity); (h) therapeutic capacity optimization of nanotherapeutics; (i) investment doubt by pharmaceutical industries on nanotherapeutics. (j) regular targeting of negative features of nanomaterials by media, in absence of clear scientific proof (Hafner et al., 2014).

4. Nanoprecipitation

Nanoprecipitation was patented by Fessi et al. in 1989 (Fessi et al., 1989). After its development, it was mostly employed for

encapsulation of hydrophobic drug molecules (nanocapsule or nanosphere forms). To this end, several polymers, notably, biodegradable polyesters like polylactide (PLA), polylactide-co-glycolide (PLGA) and poly- ϵ -caprolactone (PCL), have been used (see Table 2). As reported by Fessi et al. (1989), in this method solvent and nonsolvent phases preparation is required which is followed by the addition of one phase to another under moderate magnetic stirring (See Fig. 2). Organic solvent evaporation at ambient temperature or with a rotavapor allows the obtaining of nanoparticles (NPs) suspension in water. Ultracentrifugation and freeze drying are two methods that could be employed in next step for aqueous phase removal. Basically, the solvent phase comprises a film-forming material, one or more drug molecules, a lipophilic surfactant, and one or more organic solvents. Solvent and nonsolvent phases are usually named as organic and aqueous phases, respectively. Film-forming materials could be natural, synthetic or semi-synthetic polymers. To provide nanocapsules instead of nanospheres, mineral oil or vegetable oil would be added. The NPs aggregation could be avoided by adding surfactants into the formulation (Miladi et al., 2016). Surfactants can affect NPs characteristics as well. For instance, D - α -tocopheryl polyethylene glycol 1000 succinate (TPGS) is broadly used in nanoprecipitation technique. It is recognized as an excellent emulsifier due to its bulky structure and large surface area (Zhu et al., 2016). This water-soluble derivative of a natural vitamin is also suggested as copolymer to form amphiphilic block biodegradable copolymers. Its potential to form polymeric NPs by self-ensemble effects is due to the hydrophobic-lipophilic interactions (Zeng et al., 2013). TPGS has been successfully co-ensembled to PLA (Wang et al., 2015; Zhu et al., 2016), PLGA (Tao et al., 2016, 2015) and PCL (Cao et al., 2015) for cancer treatment applications. In nanoprecipitation technique, parameters modification causes crucial change in physicochemical characteristics of NPs such as, size, drug encapsulation efficiency and so on. Process and formulation related parameters impacts are figured out in Table 3.

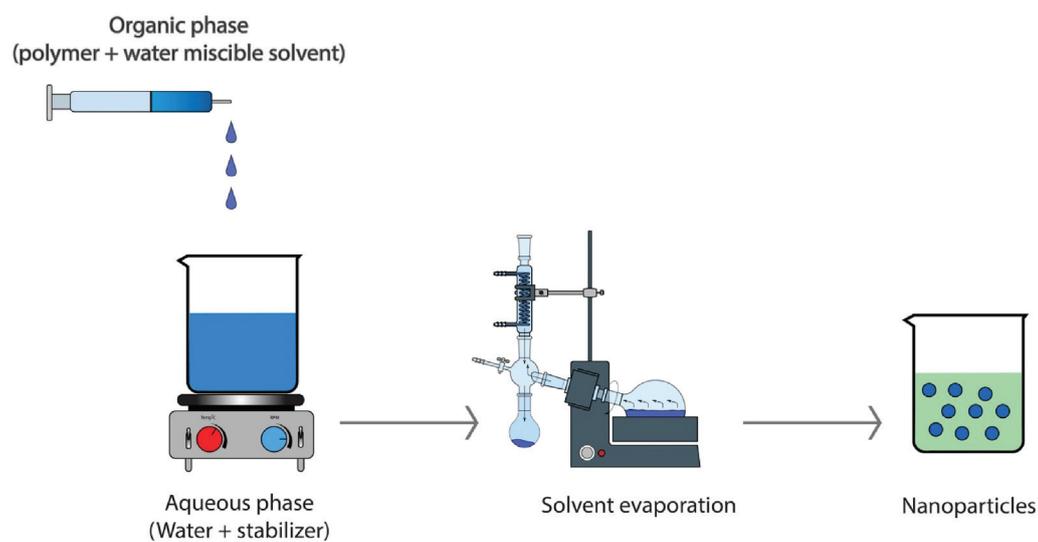
The most used solvents in nanoprecipitation method are ethanol, acetone, hexane, methylene chloride or dioxane. Mostly, non-solvent (or aqueous phase) is water. However, hydrophilic excipients could be also added to the nonsolvent phases. Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) or dynamic light scattering (DLS) could be used in order to characterize produced particles in terms of size and surface morphology (Mora-Huertas et al., 2010; Miladi et al.,

Table 1
Examples of marketed drug delivery carriers with their composition and date of approval in EU and US (Bomgaars et al., 2004; Chang and Yeh, 2012; Mitchell, 2005; Schmidt et al., 2011; "Vincristine Liposomal—INEX," 2004; Wacker, 2013).

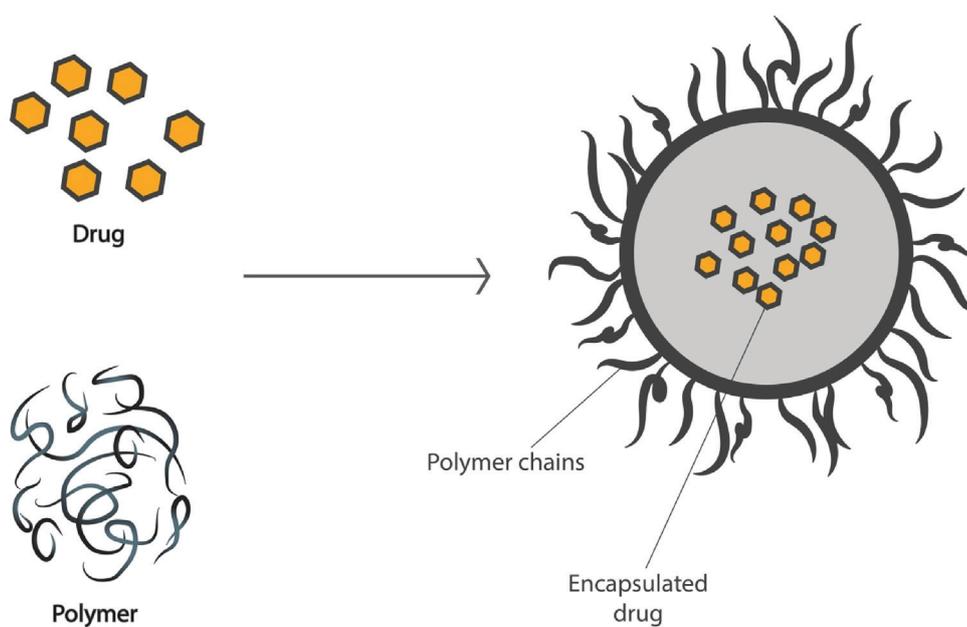
Encapsulated drug	Trade name	Drug carrier material	Carrier system	Administration route	Application	Approval
Leuprolide	Lupron Depot [®]	Poly lactic acid	Microparticles	Intramuscular	Analog of gonadotropin-releasing hormone	1989, USA
Amphotericin B	Ambisome [®]	Phospholipids	Liposomes	Intravenous	Visceral leishmaniasis treatment	1990, Europe 1997, USA
Doxorubicin	Doxil [®]	Phospholipids	Liposomes	Intravenous	Anticancer therapy	1995, USA 1996, Europe
Daunorubicin	DaunoXome [®]	Phospholipids	Liposomes	Intramuscular	Anticancer therapy	1996, Europe 1996, USA
Cytarabine	Depocyt [®]	Phospholipids	Liposomes	Intrathecal	Lymphomatous meningitis treatment	1999, USA
Doxorubicin	Myocet [®]	Phospholipids	Liposomes	Intravenous	Anticancer therapy	2000, Europe
Verteporfin	Visudyne [®]	Phospholipids	Liposomes	Intravenous	Photodynamic treatment of age-related macular degeneration	2000, USA
Morphine	DepoDUR [®]	Phospholipids	Liposomes	Epidural	Analgesia	2004, USA
Vincristine	Onco TCS [®]	Phospholipids	Liposomes	Intravenous	Anticancer therapy	2004, USA
Doxorubicin	Transdrug [®]	Poly-iso-hexyl-cyanoacrylate	Nanoparticles	Hepatic intra-arterial	Hepatocellular carcinoma treatment	2005, USA
Paclitaxel	Abraxane [®]	Human serum albumin	Nanoparticles	Intravenous	Anticancer therapy	2005, USA 2008, Europe

Table 2
Mostly used polymers in nanoparticles preparation by nanoprecipitation method.

Type	Group	Polymer Name (Common abbreviation)	References
Biodegradable	Polysaccharide	Starch	Qin et al. (2016)
		Chitosan	Luque-Alcaraz et al. (2016)
	Protein	Gelatin	Han et al. (2013)
		Bovine serum albumin (BSA)	Ge et al. (2012)
	Polyester	Poly(lactic acid) (PLA)	Bazylińska et al. (2014)
		Poly(ϵ -caprolactone) (PCL)	Mazzarino et al. (2012)
Polyether	Poly(lactic-co-glycolic acid) (PLGA)	Siqueira-Moura et al. (2013)	
	Poly(ethylene glycol) (PEG)	Şimşek et al. (2013)	
Non-Biodegradable	Polymethacrylate Acrylate	Eudragit®	Averina and Allémann (2013), Katara and Majumdar (2013), and Kumar et al. (2016)



(a)



(b)

Fig. 2. (a). Nanoprecipitation schematic representation, (b). Illustration of drug encapsulation into a preformed polymer.

Table 3
Formulation and process dependent parameters effect on the characteristics of the nanoparticles (Miladi et al., 2016).

Affected Variable	Outcome	Parameter	Modification	Explanation	Reference
Size	Increases	Stirring rate	Increases	Faster diffusion rate will facilitate solvent diffusion	Asadi et al. (2011)
		Organic phase flow rate	Increases	High nucleation rates promotion that will reduce strongly the mean particle size.	Lince et al. (2008)
	Increases, then decreases Decreases	Organic/aqueous phase ratio	Volume of the aqueous phase increases	Increased diffusion of the water-soluble solvent in the aqueous phase. At a certain point, this diffusion of solvent to the aqueous phase becomes so rapid that the polymer immediately precipitates before agglomerating into particles.	Budhian et al. (2007)
		Surfactant concentration	Increases	Prevents coalescence with each other. Increased viscosity of the aqueous phase reduces the net shear stress available for droplet breakdown	Budhian et al. (2007), Contado et al. (2013), and Zeng et al. (2013)
		Polymer concentration	Increases	Favors particle growth with respect to particle nucleation. Higher organic solution viscosity.	Badri et al. (2017), Dong and Feng (2004), and Lince et al. (2008)
	Polymer molecular weight	Increases	Higher organic solution viscosity	Limayem Blouza et al. (2006) and Martin-Banderas et al. (2012)	
Drug Encapsulation Efficiency	Increases Decreases	Polymer concentration	Increases	It is related to size, larger size higher drug entrapment	Chorny et al. (2002) and Dong and Feng (2004)
		Organic/aqueous phase ratio	Volume of the aqueous phases increases	Amount of drug that can dissolve in the aqueous phase increases, which increases the drug loss into the aqueous phase.	Budhian et al. (2007) and Limayem Blouza et al. (2006)
	Drug concentration	Increases	The polymer itself may have a limited capacity to encapsulate the specific amount of drug. Beyond its maximum capacity, more drug might be wasted during the fabrication process.	Dong and Feng (2004)	

2016). In nanoprecipitation, many parameters modification cause crucial changes in the physical characteristics of NPs in terms of size, drug encapsulation efficiency and so on. Fig. 3 shows SEM images of NPs prepared *via* nanoprecipitation.

Another crucial measured parameter is the surface charge of NPs called zeta potential. It measures the magnitude of the electrostatic interactions. This parameter is too crucial for the stability of NPs and their behavior in a biological environment. The positive or negative zeta potential values could be determined by identifying towards which electrode particles are moving during electrophoresis. In fact, an electric field is applied and the

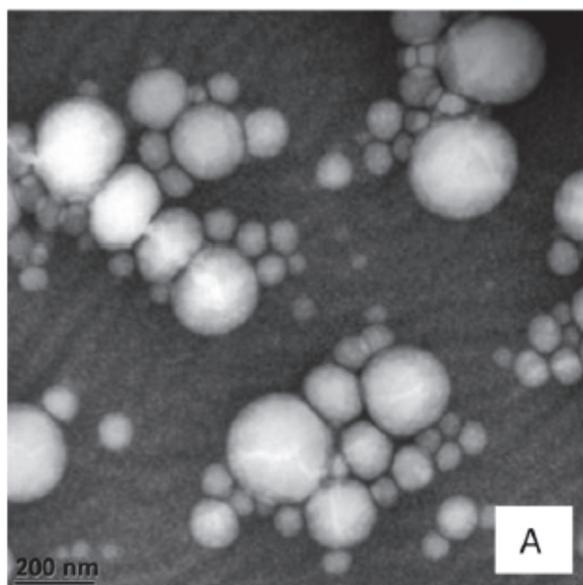


Fig. 3. TEM micrograph of typical PCL spheres prepared by solvent displacement process. Reproduced with permission from Mora-Huertas et al. (2011).

electrophoretic mobility of the particles is measured by electrophoretic light scattering (Bhattacharjee, 2016). Loading and encapsulation efficiency are variables that are related to the quantification of the incorporated active ingredients within NPs. These parameters could be established by analytical methods such as UV–vis spectrophotometry, High Performance Liquid Chromatography or Gas Chromatography (for volatile actives *i.e.* essential oils). In addition, thermodynamic characterization of NPs could provide information about their chemical properties and it is carried out by the following methods:

- i Thermal Gravimetric Analysis (TGA): determines endothermic and exothermic weight loss upon heating or cooling of NPs. In fact, TGA uses heat to force reactions and physical changes in materials. Thermogravimetric curves characterize specific compounds due to the unique sequence from physicochemical reactions occurring over the specific temperature ranges.
- ii Differential Thermal Analysis (DTA): based on the principle that the substance upon heating undergoes reactions and phase changes that involve absorption or emission of heat. Identification of a substance is accomplished by comparing DTA curves obtained from the unknown substance with the DTA curves that are provided by known elements.
- iii Differential Scanning Calorimetry (DSC): based on heat release from a chemical process, either a chemical reaction or a conformational alteration. The heat of reaction or $\Delta_r H$ is defined as the change in enthalpy associated with a chemical reaction (Singh, 2016).

Fourier Transform Infrared Spectroscopy or FT-IR is also a useful tool for the identification of drugs. It permits continuous monitoring of the spectral baseline and simultaneous analysis of different components of the same sample (Bansal et al., 2013). Molecular structure and composition of nanoparticle-forming polymers before and after nanoprecipitation could be analyzed *via* this technique (Qin et al., 2016; Wang and Tan, 2016).

5. Precipitation mechanism

Nanoprecipitation is based on the reduction of the quality of the solvent in which the main composition of NPs is dissolved. Such variation in solvent quality can be achieved by altering the pH, salt concentration, solubility conditions, or the addition of a non-solvent phase (Miladi et al., 2014). The non-solvent based precipitation process includes four steps: generation of supersaturation, nucleation, growth, and coagulation (see Fig. 4) (Joye and McClements, 2013). Supersaturation occurs when the solution contains more dissolved solute than that given by the equilibrium saturation value. In fact, the addition of non-solvent decreases solvent potency to dissolve the solute, which put the system in a supersaturation state. The supersaturation ratio (S_r) is expressed as follows:

$$S_r = \frac{C_s}{C_\infty}$$

Where C_s is the ratio of the particle solubility at the interface, and C_∞ is the bulk solubility. The supersaturation rate can affect final NPs properties where a higher supersaturation leads to a decrease in particle size.

After supersaturation, nucleation step starts in order to gain thermodynamic stability. It is induced when the supersaturation of the system reaches the boundaries of a critical level that is solvent/non-solvent specific. In other words, the energy barrier (ΔG) has to be overcome to form nuclei.

$$\Delta G = \frac{16\pi\sigma^3 v^3}{3K^2 T^2 (\ln S_r)^2}$$

Where c is a constant, σ is the interfacial tension at the solid-liquid interface, v is the molar volume of solute, K is the Boltzmann constant, and T is the temperature (D'addio and Prud'homme, 2011).

The local fluctuations in the concentration caused by supersaturation lead to the formation of primary nuclei, which, by its turn,

increases in size by the association of solute molecules until it reaches a critical size that is stable against dissolution (see Fig. 2). The nucleation step will carry on until the growth of earlier nuclei depletes the solution supersaturation. The nucleation rate (Nr) could be expressed by the following mathematical equation (D'addio and Prud'homme, 2011).

$$Nr = c \cdot \exp\left[\frac{16\pi\sigma^3 v^2}{3K^2 T^3 (\ln S_r)^2}\right]$$

Nucleation stops when the solute concentration is reduced below the critical supersaturation concentration, and nuclei grow by either condensation or coagulation. Condensation is the addition of single molecules to the particle surface. It takes place in two steps: a diffusional step in which the solute is transported from the bulk fluid through the solution boundary layer adjacent to the nuclei surface, and a deposition step in which the adsorbed solute molecules are integrated into the nuclei matrix. When the non-adsorbed solute concentration is reduced below the equilibrium saturation concentration, condensation stops. The rate of condensation is decreased by coagulation (D'addio and Prud'homme, 2011).

On the other hand, coagulation is the adhesion of particles to each other's. It occurs when the attractive interactions (Van Der Waals, hydrophobic interactions, etc.) are stronger than the repulsive interactions (steric or electrostatic repulsion) (see Fig. 4). The factor that rules the coagulation step is the collision frequency, which depends on particle concentration, size, and motion. The number of collisions that leads to coagulation is called collision efficiency and depends on the attractive and repulsive ratio of interaction between particles. To protect particles from coagulation, stabilizing agents can be added during the preparation. Such agents could adsorb to NPs surface and introduce a repulsive interaction (Joye and McClements, 2013).

In addition to the precipitation with a non-solvent, pH-controlled precipitation is also an important approach to be discussed. In this method, the polymer switches from dissolved to

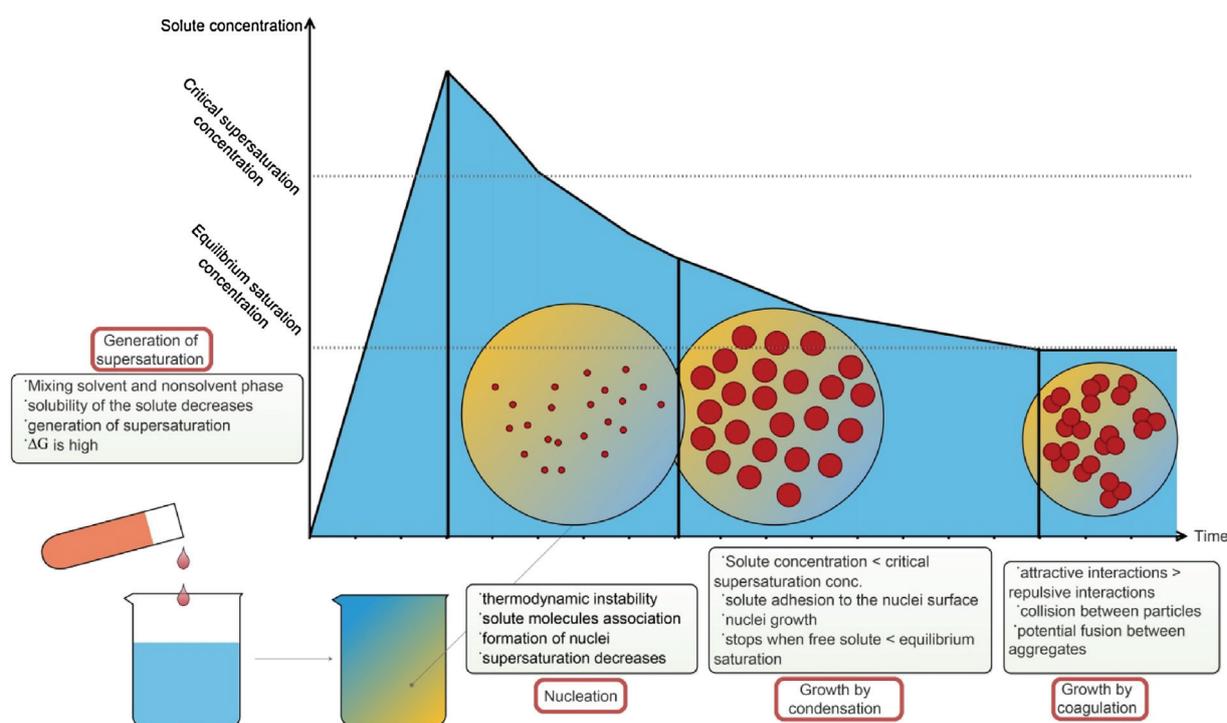


Fig. 4. Schematic illustration of non-solvent precipitation process.

non-dissolved phase by a simple pH variation in the medium. This will lead to the precipitation and formation of NPs (Pereira et al., 2006). Das et al. prepared Eudragit® RL100 based NPs using this method. Polymer was dissolved in organic phase of acetone and methanol, and the pH was adjusted to 4. The solution was later added to water where the precipitation occurs. Results showed that NPs were successfully prepared *via* pH-nanoprecipitation. In addition, due to unique particle size and positive zeta potential, particles have a good ocular retention property and a storage stability for 2 months (Das et al., 2010).

In another study, dexamethasone-loaded Eudragit® L100 based NPs were prepared by nanoprecipitation. Polymer was dissolved in organic phase of acetone and ethanol. In this case, aqueous phase pH was adjusted to 4. Obtained NPs showed no cytotoxic or oxidative stress on normal human keratinocytes. These results suggest that these particles are good candidates for the delivery of poorly soluble drugs to the skin (Sahle et al., 2017).

6. *In vitro* release profile

In vitro drug release from NPs prepared by nanoprecipitation generally consists of two phases: a first phase of “burst release” which is followed by a second phase of prolonged release. The first phase is due to the release of drug substance, which is adsorbed on NPs surface or which is dispersed near to the surface. The second phase is due to release of drug which is located in the core compartment (Wang and Tan, 2016). Many mathematical models have been also used to explain drug release mechanism. Most commonly used mathematical modeling that fitted drug delivery are the Higuchi model and Korsmeyer-Peppas model (Chourasiya et al., 2016; Das et al., 2010). The Higuchi model expresses cumulative percentage of released drug *versus* square root of time and it is presented by the following equation:

$$Q = k\sqrt{t}$$

Where Q is the absolute cumulative amount of released drug at time t and k is the constant reflecting the design variables of the system.

Higuchi model describes drug release as a diffusion process based on Fick’s law. Therefore, when release kinetics fit this model, active release from particles would be mainly controlled by diffusion through polymer matrix (Sinha et al., 2004). However, Korsmeyer-Peppas model is presented by the following equation:

$$Q = kt^n$$

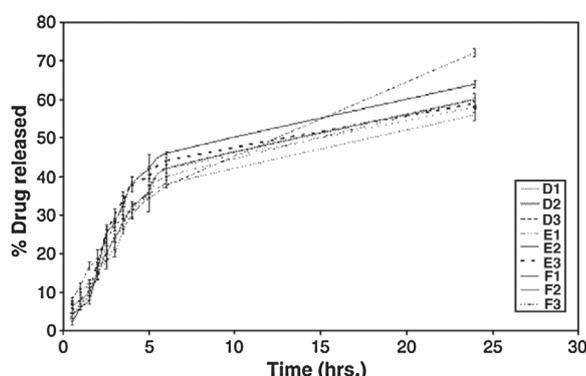


Fig. 5. *In vitro* drug-release profile of different nanoparticles formulations (D1, D2, D3, E1, E3, F1, F2, F3) through artificial membrane. Reproduced with permission from Das et al. (2010).

Where Q is the cumulative amount of released drug at time t and n is the release exponent which is indicative of drug release mechanism. In Korsmeyer-Peppas model, n values of 0.43 indicate that the drug release is controlled by Fickian diffusion. Conversely, n values between 0.43 and 0.85 imply a non-Fickian diffusion process. The latter could be described as a combination of drug diffusion and polymer chain relaxation as long as the solvent diffuse into the polymeric matrix. However, if $n \geq 0.85$, this indicates that drug release is only governed by polymer relaxation (Puga et al., 2012). Fig. 5 shows an *in vitro* drug release profile of a drug encapsulated by nanoprecipitation technique.

Chourasiya et al. studied atenolol, which is used for cardiovascular disorders (Chourasiya et al., 2016). Atenolol loaded PLGA NPs, which are intended for the oral route, were prepared by nanoprecipitation. Different kinetics models were used to analyze *in vitro* drug release profile. In the case of *in vitro* drug release study of atenolol loaded PLGA NPs, dialysis bag diffusion method was used. Dialysis bag was immersed in a receptor compartment containing phosphate buffer (pH 7.4) stirred at 100 rpm and kept at a temperature of 37 ± 1 °C. To know the mechanism and kinetics of NPs drug release, *in-vitro* drug release data were fitted to various kinetic models such as first order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas. The optimized formulation showed biphasic release profile comprising an initial burst release followed by sustained release. The preliminary fast release was due to drug molecules which are adsorbed on NPs surface. After a while, the release rate decreased which reflected the release of drug entrapped in the polymer. On the basis of best fit with the highest correlation (r^2) value, it is concluded that the formulation follows the Korsmeyer-Peppas model. Correlation value $r^2 = 0.99133$ and a release exponent value n of 0.650 were obtained. The magnitude of the release exponent n indicates that the release mechanism is an anomalous transport or non Fickian diffusion, which is related to a combination of both diffusion of the drug and dissolution of the polymer (Chourasiya et al., 2016).

Cosco et al. used the same technique for the encapsulation of 9-cis-retinoic acid (9-cis-RA) in poly (ethylene glycol)-coated PLGA. Such NPs were indicated for the treatment of undifferentiated tumor. Drug release of 9-cis-RA from PEG-PLGA NPs was evaluated by using the dynamic Franz-type diffusion cells separated by a cellulose acetate membrane. The receptor fluid was made up of a water/ethanol mixture (70:30 v:v). The PEG-PLGA NPs showed a prolonged release of the drug. An initial phase with a rapid drug release and a second phase with a more gradual release were observed (40 and 90% of released drug after 10 and 48 h, respectively).

Lie et al., investigated the encapsulation of green tea catechin derivative, lycopene, in PLGA NPs coated with chitosan. Lycopene-loaded NPs were prepared by nanoprecipitation followed by coating within chitosan to form a shell. Chitosan was coated onto the surface of lycopene NPs because chitosan exhibits pH-dependent behavior which allows to overcome the harsh environment. To understand the pH-dependent behavior effect on the release kinetics of lycopene, time-dependent release of lycopene in simulated gastric fluid and simulated intestinal fluid was studied. A known amount of lyophilized NPs was dispersed in simulated gastric juice or simulated intestinal fluid. In simulated gastric fluid, NPs released 5% of the total lycopene compared to 12% of total lycopene released in simulated intestinal fluid in 24 h. 7% of total lycopene was released in the burst-release phase from NPs in simulated gastric fluid compared to 16% of total lycopene released in simulated intestinal fluid in 24 h, both formulations showed pH-dependent release. Authors proposed that chitosan release is based on lysozyme degradation and swelling (Li et al., 2017).

7. Applications

7.1. Medicine

7.1.1. Synthetic compounds encapsulated in polymeric particles

Due to its easy manipulation, nanoprecipitation has become an important strategy in pharmaceutical development. Thus, particles for several drugs prepared by nanoprecipitation are in preclinical development (Chen et al., 2004). Paclitaxel, an anti-tumor, was loaded within poly (lactic-co-glycolic acid) (PLGA) NPs prepared by nanoprecipitation (Fonseca et al., 2002). It was shown that the 200 nm sized NPs have high EE towards paclitaxel (almost 100%). Moreover, by comparing paclitaxel loaded PLGA NPs to the already existing formulations such as, Taxol[®], it was shown that PLGA NPs strongly enhanced the antitumor activity of paclitaxel. Such formulations did not have the same composition compared to marketed products. This could be important in cancer therapy by paclitaxel since commercially available formulations are accompanied with severe hypersensitivity reactions which are caused by the used excipients (Fonseca et al., 2002). Nanoprecipitation is stated as an efficient and usual method for hydrophobic drugs encapsulation (Miladi et al., 2015). However, hydrophilic drugs were considered incompatible with this method due to rapid migration and drug loss in the aqueous phase (Govender et al., 1999). Thus, the encapsulation of water-soluble drugs into NPs using nanoprecipitation method was investigated. Procaine hydrochloride loaded PLGA NPs were prepared by nanoprecipitation (Govender et al., 1999). The obtained NPs were spherical with a size of 210 nm and a low drug entrapment. However, the study showed that drug entrapment could be increased by changing variables in the method such as, by increasing the aqueous phase pH and replacing procaine hydrochloride with procaine dihydrate (Govender et al., 1999). In another study, sodium cromoglycate loaded PLA NPs were prepared by nanoprecipitation. Different technique related parameters were modified in order to increase entrapment efficiency of the hydrophilic drug into NPs. The pH also affected EE. By lowering the pH, drug entrapment increased from 10% to approximately 70% (Peltonen et al., 2004). These results show the ability of nanoprecipitation method to encapsulate hydrophilic drugs through optimization of the method parameters. Peltonen et al., studied several parameters to increase the loading of the hydrophilic sodium cromoglycate in PLA NPs (See Table 5). Specifically, salt addition (sodium chloride) to the inner or/and outer phase affected the osmotic gradient between phases (Peltonen et al., 2004). However, the best EE (70%) was achieved by adding HCl. In fact, aqueous phase pH affects the ionization of the drug substance and, hence, its solubility (Peltonen et al., 2004). Yordanov et al., prepared poly(butyl cyanoacrylate) (PBCA) nanospheres loaded with epirubicin hydrochloride (EPI·HCl) (Yordanov et al., 2012). Effect of aqueous phase pH and EPI·HCl concentration on drug loading efficiency was evaluated (Table 5). In this study, larger amount of EPI was loaded in PBCA at higher pH (7.4) (Yordanov et al., 2012). In another study, Miladi et al., encapsulated alendronate sodium in poly- ϵ -caprolactone (PCL) NPs. The effects of drug to polymer ratios, PCL molecular weights and organic to water phase ratio were determined (Table 5). EE reached 18.8% with PCL of 80,000 g/mol, 1:10 drug to polymer ratio and 1–2.5 organic: water phase (Miladi et al., 2015). Another key parameter to enhance entrapment is electrostatic charges of actives and polymers. In a comparative study, Zhou et al. encapsulated bovine serum albumin (BSA) in lactosylated PLGA and used ϵ -polylysine (ϵ -PL) used as an antiadhesive agent (Zhou et al., 2015). All protein-loaded NPs had small sizes (<100 nm) with relatively uniform size distributions. The best EE of BSA was in Lac-PLGA/ ϵ -PL. This could be explained by the fact that ϵ -PL has abundant positive charges indicating that the negatively charged proteins were easier to be

loaded into Lac-PLGA/ ϵ -PL NPs via electrical interaction. In addition, electrical attraction between ϵ -PL and BSA played an important role in the sustained release of BSA. The *in vitro* releases of BSA- and trypsin-loaded NPs were investigated in PBS solution (pH 7.4). Release of BSA was observed after 8 days, and 15.8% of BSA was released after 32 days. Moreover, BSA initial burst release was effectively avoided. Conversely, trypsin exhibited a faster release rate than trypsin-loaded Lac-PLGA NPs; more than 80% was released after 32 days (Zhou et al., 2015). Hydrophobic nature of the polymers can contribute to low entrapment efficiency (Arpicco et al., 2016). To overcome it, hydrophilic polymers can be used. Lee et al., prepared tizanidine hydrochloride, gatifloxacin and fluconazole-loaded gelatin NPs which were uncrosslinked or crosslinked with glutaraldehyde. EE was around 14% except for fluconazole which could not be loaded (Lee et al., 2012).

Modification of the method parameters can also affect the physicochemical characteristics of NPs prepared by nanoprecipitation. Polymer concentration, solvent and non-solvent nature, and solvent/non-solvent volume ratio can influence size, surface charge, size distribution. These variables were tested by Gonzalez et al. using BSA NP (Galisteo-González and Molina-Bolívar, 2014). It was shown that NPs characteristics could be modulated by altering BSA concentration, pH, salt concentration, temperature, ethanol volume, and ethanol addition rate. It was shown that these parameters have a huge effect on size and surface charge of NPs (Galisteo-González and Molina-Bolívar, 2014). In another study, PLGA NPs characteristics were also modified by changing method parameters (Bilati et al., 2005). Bian et al., encapsulated a synthetic triazole antifungal agent called itraconazole (ITZ) (Bian et al., 2013). They developed ITZ loaded poly (lactic-co-glycolic acid) (PLGA) nanospheres. The modified parameters are shown in Table 4. Optimal formulation were chosen considering particle size (178 nm), PDI (homogeneous distribution) and EE (72%) (Bian et al., 2013).

7.1.2. Natural compounds encapsulated in polymeric particles

Natural products are the source of most of the active ingredients of medicines (Harvey, 2008). Since prehistoric times, humans have used natural products, such as plants, in traditional treatments of various diseases (Bharali et al., 2011; Palombo, 2011). The focus on encapsulation of natural molecules is increased due to the interest for additionally conferring them enhanced stability and/or less volatility (Kayser et al., 2005; Asbahani et al., 2015). Nanoprecipitation was shown to be a good alternative to load these actives into NPs. Cucurbitacin I has a potent anticancer effect (Yuan et al., 2014). Alshamsan et al., used this triterpene hydrocarbon isolated from plants belonging to the species Cucurbitaceae and Cruciferae (Alshamsan, 2014). They compared the efficiency to encapsulate this polar water-insoluble drug by three emulsion based NPs formulations and nanoprecipitation. The different formulations were CI-NP1 (single emulsion o/w starting with 1000 μ g of drug), CI-NP2 (double emulsion w/o/w starting with 250 μ g of drug) and CI-NP3 (double emulsion w/o/w with 500 μ g of drug) and CI-NP4 prepared by nanoprecipitation with 1000 μ g of drug. EE was around 1%, 4%, 7% and 48%, respectively. These results showed nanoprecipitation is more efficient than emulsion solvent evaporation method to encapsulate cucurbitacin I (Alshamsan, 2014). Quercetin is another active ingredient obtained from fruits and vegetables. Sahu et al. proposed encapsulated quercetin as potential anti-cancer topical agent. *Ex vivo* study demonstrated drug release and retention in the skin (Sahu, 2013). Moreover, α -tocopherol (a form of vitamin E) is a commonly found compound in plants which has antioxidant effect (Ching and Mohamed, 2001). Noronha et al., prepared PCL nanocapsules containing α -tocopherol (Noronha et al., 2013). Table 4 shows the conditions studied to optimize formulation. In general, PCL

Table 4
Hydrophobic and hydrophilic active molecules loaded in polymeric nanoparticles prepared by nanoprecipitation and studied parameters during the process.

Active molecule	Nanoparticle-forming polymer	Parameter studied	Effect reported	Potential use	Reference
Itraconazol	PLGA	Surfactant concentration Polymer:drug ratio	Poloxamer 188 concentration increased, size increased. PLGA amount increased, size and EE% increased.	Antifungal	Bian et al. (2013)
Cucurbitacin I	PLGA	N/S	N/S	Anticancer	Alshamsan (2014)
Quercetin	Ethylcellulose	Polymer amount	Ethylcellulose amount increased, drug loading decreased, EE% increased and percentage <i>in vitro</i> release after 24 h decreased	Anticancer	Sahu (2013)
α -tocopherol	PCL	Drug amount Lecithin concentration Surfactant concentration	α -tocopherol amount increased, particle size increased and EE% decreased. Does not seem to exert any influence Pluronic F68 concentration increased, size increased	Antioxidant	Noronha et al. (2013)
Brazilian red propolis extract	PCL	N/S	N/S	Leishmanicidal	do Nascimento et al. (2016)
<i>Zanthoxylum rhoifolium</i> essential oil	PCL	Essential oil amount	<i>Zanthoxylum rhoifolium</i> essential oil increased, EE% decreased	Insecticidal	Christofoli et al. (2015)
<i>Achyrocline satureioides</i> essential oil	PLC	N/S	N/S	Hepatoprotective effect, antioxidant	Ritter et al. (2017)
Sodium cromoglycate	PLA	Drug percentage (related to the amount of polymer) Solvent and co-solvent selection salt addition pH effect	Drug percentage increased, EE% decreased Combination of Dichloromethane and methanol, EE% increased Sodium chloride in the inner and outer phases, EE% increased Acid pH in the outer phase, EE% increased	Preventive reducer of bronchoconstriction	Peltonen et al. (2004)
Epirubicin hydrochloride (EPI-HCl)	PBCA	pH effect Drug concentration	Higher pH, EE% increased EPI-HCl concentration increased, EE% decreased	Anticancer	Yordanov et al. (2012)
Alendronate sodium	PCL	Drug:polymer ratio Polymer molecular weights Organic:water phase ratio	PCL amount increased particle size and EE% increased. PCL molecular weight increased, particle size and EE% increased. Water phase increased, particle size and EE% decreased	Osteoporosis treatment	Miladi et al. (2015)
Bovine serum albumin (BSA)/Trypsin	Lactosylated PLGA	Negative or positive drug	Negative BSA with ϵ -polylysine, EE% and sustained release increased	Model proteins and cell culture	Zhou et al. (2015)
Tizanidine hydrochloride (TZN), gatifloxacin(GTX) and fluconazole	Gelatin	Uncrosslinked or crosslinked with glutaraldehyde	TZN crosslinked NPs, EE% increased. GTX uncrosslinked, EE% increased. No loading was observed for fluconazole	α^2 -adrenergic agonist and myotonic muscle relaxant; antibacterial and antifungal, respectively	Lee et al. (2012)
Cocoa-derived polyphenolic extract	Gelatin	Polymer concentration Surfactant concentration	Gelatin concentration increased, size increased Tween 80 concentration increased, size decreased	Antioxidant	Quiroz-Reyes et al. (2014)
Protamine sulphate, diclofenac sodium and N6-cyclopentyladenosine (CPA)	PLGA/PLA	Drug amount PLGA substituted by PLA for CPA	Drug amount increased, drug loading increased and EE% decreased. The size of the particles did not increase. PLGA substituted by PLA, EE% increased	Anticoagulant activity inhibitor of heparin; anti-inflammatory, analgesic and antipyretic effect; selective agonist of adenosine A ₁ receptors, respectively	Dalpiaz et al. (2016)

PLGA: poly(lactic-co-glycolic acid); PCL: poly- ϵ -caprolactone; PLA: polylactic acid; PBCA: poly(butyl cyanoacrylate).

N/S: not studied.

NPs: Nanoparticles.

nanocapsules showed a negative charge and homogeneous size distribution. The optimal formulation had an EE of 99.97% (Noronha et al., 2013). Other studies focused on the encapsulation of natural compounds obtained from extracts or essential oils. In fact, do Nascimento et al., who investigated propolis which is recollected by bees of the species *Apis mellifera* from plant exudates (do Nascimento et al., 2016). Propolis has been widely used in

alternative and traditional medicine to treat several diseases. In this study, leishmanicidal activity against *Leishmania (V.) braziliensis* was proved. Red propolis raw material was collected and extracted by maceration, then the extract was loaded into NPs. The organic phase was composed by PCL and red propolis extract while the aqueous phase contained pluronic F-108 copolymer. Five formulations were prepared with values of particle size varying

between 208 and 280 nm (do Nascimento et al., 2016). Ritter et al., developed *Achyrocline satureioides* essential oil loaded nanocapsules (Ritter et al., 2017). When tested, nanocapsules showed potency to protect hepatic tissue against cytotoxic damage caused by *Trypanosoma evansi* (Ritter et al., 2017). Essential oils could also have insecticidal activity. Their encapsulation into NPs can potentially improve their activity, offer better protection against degradation and oxidation processes by light and heat. Essential oils could also be used against agricultural pests such as *Bemisia tabaci* (Christofoli et al., 2015). Due to large number of compounds that constitute an extract or an essential oil, some researchers quantified the major component and used the obtained data to determine EE or loading rate of the essential oil (do Nascimento et al., 2016). Quiroz-Reyes et al. (2014) obtained cocoa-derived polyphenolic extract in gelatin NPs. They dissolved the polyphenolic extract in a water-methanol solution, adding a specific quantity of gelatin. The mixture was then stirred and maintained at 45 °C for 30 min. The resulting solution was added dropwise to an ethanolic solution of tween 80. Finally, glutaraldehyde was added as a crosslinking agent. With 2% w/v of tween 80 and gelatin, it was possible to obtain a formulation with a loading efficiency of 77% (Quiroz-Reyes et al., 2014). Another strategy is used by Dalpiaz et al., who dissolved protamine sulphate, diclofenac sodium and N⁶-cyclopentyladenosine (CPA) and PLGA in acetone phase and added them drop-wise to the cottonseed oil (oil phase) and tween 80 (Dalpiaz et al., 2016). For CPA, authors substituted PLGA with PLA, which is characterized by a lower hydrophilicity. Using PLA instead of PLGA increased CPA loading when 5 mg of drug were used as the initial amount, doubling the EE value (from 7% to 15%) (Dalpiaz et al., 2016).

7.1.3. Protein based particles

Proteins constitute an important class of biopolymers that gained lately importance in drug delivery field. Proteins have several advantages. They are biocompatible, biodegradable, and their biodegradation products are often non-toxic. Moreover, since they are derived from animals or plant sources, they are lacking of monomers or initiators found in synthetic polymers (Pathak and Thassu, 2009). Because of their importance, protein based nanoparticle systems are already found in the market, such as albumin bound paclitaxel NPs (AbraxaneTM) (Langer et al., 2008). Protein based NPs prepared by nanoprecipitation method could be often found in literature. Lee et al. prepared gelatin based NPs by nanoprecipitation (Lee et al., 2012). Water and ethanol were used as solvent and non-solvent, respectively. It was shown that the non-crosslinked particles have an irregular shape due to particle aggregation. However, the cross linked particles have a unimodal size of 251 nm, low polydispersity index (0.096), and uniformly round shape (Lee et al., 2012). This suggests that nanoprecipitation is a suitable method for the preparation of gelatin NPs. In another study, curcumin loaded zein NPs were prepared by nanoprecipitation. Results showed that the average particle size can be controlled through the solvent system and the zein/curcumin ratio. This formulation enhanced the stability of curcumin at all physiological pH and following UV irradiation. The formulation was also found stable in the gastrointestinal tract. Furthermore, due to the fact that zein is an edible protein, the ability to use such formulation by including it in oral products was suggested (Patel et al., 2010). Whey protein were also used to prepare NPs. Ethanol was used as non-solvent and added at a speed rate of 1 ml/min in order to achieve a solvent/non-solvent volume ratio of 1:5. Obtained particles were spherical with a relatively small size (less than 100 nm). These particle properties are obtainable at pH3, a desirable pH for food applications. Moreover, it was found that particle size can be controlled by a combination of heating and homogenization (Gülseren et al., 2012). These results show that

nanoprecipitation is a successful and promising approach for the preparation of NPs using the natural, biodegradable, non-toxic proteins as starting materials.

7.2. Applications in agricultural and food industry

The success of nanoprecipitation method in the pharmaceutical field shed the light on the application of this technique in the agricultural industry. In fact, the growth of world population requires food sources increase which leads to an augmentation of fertilizers and pesticides use. However, this could result in soil depletion and environmental pollution (Bareras-Urbina et al., 2016). Thus, the need of a controlled release system is crucial to reduce environmental problems associated with the use of pesticides (Boehm et al., 2003). For this reason, nanoprecipitation has been used to prepare polymeric NPs as an insecticide formulation (Boehm et al., 2003). Eudragit[®] S100 based NPs showed small size and high EE, but they did not provide a controlled release of the active ingredient. However, they enhanced the penetration of the active in the plant due to their small size (Boehm et al., 2003). Nanoprecipitation is also important in the food industry since particles could be prepared by natural food compound such as, starch or proteins (Castro-Enríquez et al., 2012). Moreover, α -tocopherol loaded poly ϵ -caprolactone NPs were prepared. These particles showed high values of recovery and EE. They could potentially be used as food antioxidants and preservatives in food packaging (Noronha et al., 2013).

8. In vivo testing

In vivo studies give a closer idea about NPs action in the human body (Popov et al., 2016). Biodegradable and biocompatible polymers as PLA, PGA, PCL, poly(γ -valerolactone) and copolymers such as, PLGA (Nicolas et al., 2013) are used for preparation of NPs by nanoprecipitation. *In vivo* studies could give relevant information about drug transportation up to the targeted organs. For example, Sharma et al. demonstrated that intranasal NPs can potentially transport the encapsulated drug *via* nose-to-brain (Sharma et al., 2015). They used diazepam (DZP), which is widely used as sedative hypnotic, antianxiety, and antiepileptic drug. The administration routes were intranasal and intravenous in Sprague-Dawley rats. DZP was labeled using technetium-99m-labeled (^{99m}Tc), loaded into PLGA NPs (DNP) and applied. Gamma scintigraphy and biodistribution study were carried out to follow DNP and DZP solution (DS) in rats. The scintigraphy images indicated the high uptake of ^{99m}Tc-DNP into the brain. Presence of high radioactivity was observed in rat brain after administration of ^{99m}Tc-DNP intranasally compared to intravenous ^{99m}Tc-DS and intranasal ^{99m}Tc-DS. Biodistribution studies showed significantly higher brain uptake of intranasal ^{99m}Tc-DNP as compared to intranasal ^{99m}Tc-DS and intravenous ^{99m}Tc-DS (Sharma et al., 2015). After intravenous administration, it has been demonstrated that these particles provided sustained drug delivery. Bian et al. (2013) showed that systemic bioavailability of itraconazole (ITZ) loaded PLGA nanospheres was more important than Sporanox[®] formulation. This is consistent with the observed sustained plasma drug level for up to 24 h after administration by PLGA-ITZ-NS formulation (Bian et al., 2013). NPs have been found in spleen, liver and lungs (Zhou et al., 2015). Intratracheal instillation in male mice has been used to evaluate pulmonary delivery by Popov et al. (2016). Fluticasone propionate (FP) was loaded in poly(lactide)-based particles of around 200 nm diameter prepared by nanoprecipitation. NPs pulmonary residence was assessed by measuring FP levels in mouse lungs over 24 h. Higher FP levels were observed with PLA-based NPs during 24 h while free FP was rapidly eliminated from lungs following instillation (Popov et al., 2016).

These studies demonstrated the presence of active loaded NPs in different organs. Jeannot et al., administrated intravenously and intrapulmonary NPs in healthy mice, H358-tumor bearing mice or A549-tumor bearing mice (Jeannot et al., 2016). Two different sizes of NPs, 30 and 300 nm (NP30 and NP300, respectively), were prepared by nanoprecipitation. NP30 and NP300 were internalized in H358 and A549 cells and cell labeling and internalization were stronger with NP30 than NP300. In both lung tumor models, intrapulmonary nebulized NPs were accumulated in lungs, but not in the tumor nodules. This means that direct administration of these NPs into the airways failed to increase their uptake by tumors. Despite a significant liver capture, intravenous injection led to a better accumulation of the NPs in the lung tumors compared with the surrounding healthy lung tissues. The pharmacokinetic constants were calculated, and the theoretical distribution and elimination half-lives for NP30 were higher than for NP300, which showed that NP30 had longer circulation times than NP300 (Jeannot et al., 2016). The *in vivo* distribution and the cellular uptake of NP depended on their size (Dufort et al., 2012).

Focusing on natural products, *in vivo* study has carried out for *Achyrocline satureioides* essential oil loaded PCL nanocapsules (AS-NC) proving their capacity to protect liver. Here, Ritter et al., infected female Wistar rats with *Trypanosoma evansi*, a widely distributed protozoan that parasites the blood of wild and domestic animals, and rarely humans (Ritter et al., 2017). Four groups administrated by oral gavage were used: uninfected/saline, uninfected/AS-NC, infected/saline and infected/AS-NC. Infected/AS-NC group showed lower parasitemia than animals of the infected/saline group. Moreover, *T. evansi* infection causes decreased cell viability on hepatic tissue after excessive production of reactive oxygen species (ROS) and nitric oxide metabolites. Treatment with AS-NC was able to protect the hepatic tissue against cytotoxic effect caused by parasite due to the capacity of to avoid exacerbated production of ROS. Thus, the protective effect of AS-NC might be related to antioxidant properties of *A. satureioides* essential oil (Ritter et al., 2017). Studies of Danhier et al., has proved that NPs can be an effective anticancer drug delivery system for cancer chemotherapy (Danhier et al., 2009). Paclitaxel (PTX) a major anticancer drug isolated from the plant *Taxus brevifolia* was loaded in PEGylated PLGA NPs. NPs inhibited tumor growth more efficiently than Taxol[®] (commercial product of PTX) (Danhier et al., 2009). Oral administration of Eudragit[®] E PO based NPs loaded with meloxicam prepared by nanoprecipitation resulted in an enhanced anti-inflammatory effect and in a decrease of the adverse effects associated with the treatment (Khachane et al., 2011). Singh and Pai prepared Eudragit[®] RL 100 based NPs for encapsulation of trans-resveratrol with mean particle size around 180 nm (Singh and Pai, 2014). These NPs showed higher plasma levels than free resveratrol. Active accumulation in the brain, heart, liver, lungs, kidneys and spleen after oral administration over a period of 24 h was also higher than pure drug and marketed formulation (Singh and Pai, 2014). Eudragit[®] RL 100 was used for encapsulation of amphotericin-B (AmB), a polyene antifungal antibiotic that has broad-spectrum activity (Das et al., 2010). NPs sizes ranged from 134 to 290 nm. The selected formulation administered *via* ocular route in male albino rabbits showed no eye irritation. *In vivo* study suggests that AmB NPs could have potent ocular antifungal effect with minimal eye-irritating effect (Das et al., 2010). Fig. 6 depicts most common administration routes and targeted organs for NPs prepared by nanoprecipitation.

9. Clinical trials of drug loaded nanoparticles prepared by nanoprecipitation

In general, FDA drug approval process can be separated into preclinical, clinical, and post-marketing phases. The gathered data

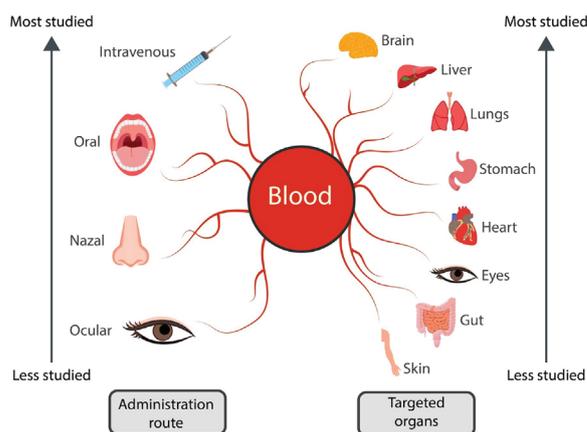


Fig. 6. Most common administration routes and targeted organs for nanoparticles prepared by nanoprecipitation.

during the preclinical phase is used to support an Investigational New Drug (IND). If during the clinical phase, the drug is considered safe and efficacious, the manufacturer files a New Drug Application (NDA) (see Fig. 7) (Eifler and Thaxton, 2011). Despite the potential advantages of these new drug delivery systems, few NPs formulations are approved for clinical use and face challenges and hurdles at different stages of development (Desai, 2012).

The following features are studied during formulation development process and have to be well known before clinical trials:

- Adequate particle size
- Stability of the nanosystem
- Drug release from the complex matrix
- Targeting
- Efficacy of biological activity
- Toxicology in cell lines and animals
- Pharmacology
- Scale up (manufacturing process)
- Production of sterile forms at laboratory and scale up

Mostly clinical studies are focused on cancer therapy (see Table 5).

The *in vivo* findings, showed that loaded NPs prepared by nanoprecipitation gave satisfactory results. Due to the versatility of nanoprecipitation technique, a broad range of polymeric materials which have already been approved by FDA could be used. Dong and Feng elaborated paclitaxel-loaded NPs of poly(D,L-lactide)/methoxy poly(ethylene glycol)-poly(lactide) (PLA/MPEG-PLA) blends of various blend ratio from 100/0 to 0/100 by the nanoprecipitation method to control the release of paclitaxel. NPs with hydrodynamic diameter of 230 to 74 nm, encapsulation efficiency of 69–55% and zeta potential of 19.6–0.3 mV were obtained. DSC analysis suggested the miscibility of PLA and MPEG-PLA. The pure PLA NPs (100/0) exhibited the slowest drug release rate with 37.3% of encapsulated drug released from the NPs for 14 days. MPEG-PLA NPs (0/100) provided the fastest drug release with 95.9% drug release in the same period (Dong and Feng, 2004). Genexol-PM[®] Cremophor EL-free based on paclitaxel loaded methoxy-PEG-poly(lactide) NPs is marketed in Europe and Korea for breast cancer and small cell lung cancer (Lohcharoenkal et al., 2014; Pillai, 2014). Based on its simplicity and versatility, nanoprecipitation is chosen as encapsulation method for the development of new nanoparticle-forming polymers with potential used in biomedical field. An example is poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA), which is in clinical phase. This polymer is water-soluble, synthetic, vinyl-based polymer with

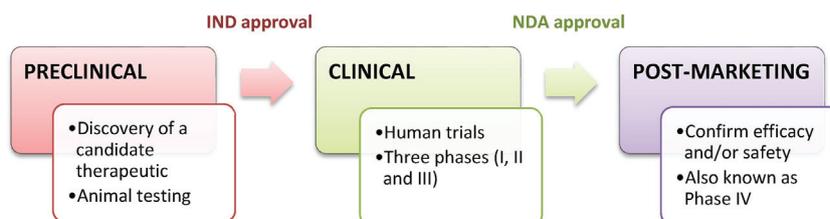


Fig. 7. Phases of drug development and approval by the US Food and Drug Administration. Reproduced with permission from Eifler and Thaxton (2011).

Table 5

Polymeric nanosystems in clinical use for anticancer therapy.

Product name	Polymer-forming nanoparticle	Active molecule	Reported Clinical Phase	Cancer type	Reference
NK105	PEG–poly(aspartic acid) block copolymer	Paclitaxel	I	Pancreatic, bile duct, gastric and colonic cancer	Hamaguchi et al. (2007)
NK911	PEG–poly(aspartic acid) block copolymer	Doxorubicin	I	Gastric Cancer Metastatic pancreatic cancer	Kato et al. (2012) Matsumura et al. (2004)
CRLX101	PEG–Cyclodextrin copolymer	Camptothecin	II	Various types (non-small cell lung)	Svenson et al. (2011)
NC-6004	PEG–poly(glutamic acid) block copolymer	Cisplatin	I	Various types (i.e. colon and lung)	Plummer et al. (2011)

singular nonimmunogenic and nontoxic characteristics (Yan et al., 2017).

In the pharmaceutical industry, the production of nanoparticles by nanoprecipitation has not been applied due to the organic solvent usage issue. Nevertheless, the production equipment for large-scale has been marketed (Tran et al., 2016). Despite the employment of organic solvent within the process, pre-clinical and clinical studies have suggested to this technique as a potential preparation technique of nanoparticles in order to be applied in organism. Thus, nanoprecipitation could be a recommended technique for the nanoparticles preparation.

10. Industrial scale-up of nanoprecipitation method

The transition from laboratory to industrial grade is crucial for any clinically approved formulation. However, this transition should be controlled by a scale-up strategy in order to create industrial scale parameters that lead to the mass production of laboratory-like formulations (Galindo-Rodríguez et al., 2005). Scaling-up of NPs formulations is often successful and have advantages over laboratory scale production. Smaller polymers amounts are needed to produce NPs in pilot-scale than in

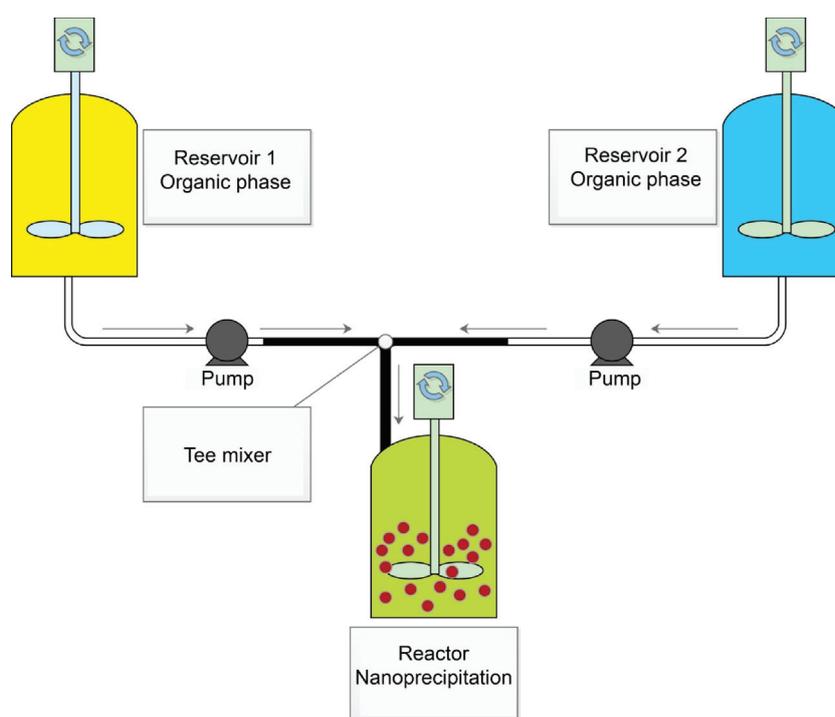


Fig. 8. Experimental set-up for pilot-scale nanoprecipitation.

laboratory-scale. In addition, pilot-scale processes are more reproducible than laboratory-scale ones. Moreover, polymer precipitation during NPs production is more efficient in pilot-scale since formulation parameters are well controlled (Marchisio et al., 2006).

The success of nanoprecipitation depends on the way the aqueous and organic phases are mixed together to lead to polymer precipitation and create NPs. It is known that the mixing time must be faster than the time required to induce nanoparticle formation (Johnson and Prud'homme, 2003). In lab scale, the mixing conditions are ideal since the amount of solutions is relatively small (in ml), and the initial conditions are maintained stable during the whole process. However, in industrial scale, the production of large amount of NPs needs large amounts of both phases. This is where the keeping of the ideal conditions during the whole process becomes difficult.

Galindo-Rodríguez et al., assessed a scaling-up procedure for ibuprofen loaded poly(vinyl alcohol) NPs prepared by nanoprecipitation (Galindo-Rodríguez et al., 2005). Particles were prepared at laboratory scale and at pilot scale by increasing the volume 20 fold from 60 ml to 1.5 l. The scale-up of nanoprecipitation was performed using the experimental set up showed in Fig. 8. This set-up consists of two reservoirs, one for the aqueous phase and another for the organic one. Each reservoir is connected to an independent pump that continuously supplies the two phases. The two phases meet at the "Tee mixer" where the precipitation occurs instantaneously. Finally, the set up includes a reactor where particles are maintained under agitation (see Fig. 7).

Laboratory and pilot-scale NPs were compared. It was found that the particles prepared by laboratory scale have a bigger size (141 nm) than the ones prepared by pilot scale (105 nm). However, the polydispersity index at pilot scale (0.130) is higher than the laboratory scale (0.082). This could be caused by the higher turbulence generated in the pilot scale which improves the diffusion of solvent and, by its turn, leads to smaller NPs. In addition, drug loading and entrapment efficiency at lab scale (4.5% and 50% respectively) were higher than pilot scale (3.2% and 39% respectively). Moreover, the pilot batches show reproducibility and each batch requires 120 min to be produced. These results show

that the pilot scale production of polymer NPs by nanoprecipitation method was successful. However, the major drawback of this method is related to the low concentration of polymer, which leads to a difficult NPs recovery in the final dispersion (Galindo-Rodríguez et al., 2005).

Another approach of precipitation optimization is flash nanoprecipitation (FNP) method. This modified version of nanoprecipitation is based on stimulating the supersaturation conditions required for the precipitation using a jet mixer to mix the two phases (See Fig. 9) (Pustulka et al., 2013). In this method, the characteristic mixing time of the two phases is in the order of milliseconds. Such rapid mixing induces a high supersaturation that initiates precipitation (Johnson and Prud'homme, 2003). Zhang et al. prepared polystyrene NPs using FNP method (Zhang et al., 2012). It was found that formulations have a comparative size distribution when the diameter of particles was less than 150 nm. In addition, NPs size was tunable by modifying the polymer concentration. An increase in polymer concentration leads to an increase in particle size (Zhang et al., 2012). FNP as an enhanced version of nanoprecipitation could lead to more accurate and reproductive results. In addition, an advantage of this method is that it could be run at laboratory scale with small amounts of solutions, and the process performance could be easily duplicated at a pilot-scale (Johnson and Prud'homme, 2003).

11. Advantages and disadvantages of nanoprecipitation

Nanoprecipitation technique is based on the interfacial deposition of polymers following the displacement of a semi-polar solvent miscible with water from a lipophilic solution. Generally, actives loaded into nanoparticles show stability, controlled release or targeting potential. Different parameters during nanoprecipitation process can be modified to obtain a formulation with the desirable characteristics in terms of size, storage stability, active encapsulation and electrostatic charges. Advantages of nanoprecipitation over other encapsulation techniques are: (1) simplicity (2) ease of scalability (3) good reproducibility (4) safety (large amounts of toxic solvents are avoided) (5) obtaining of submicron particle sizes with narrow size distribution

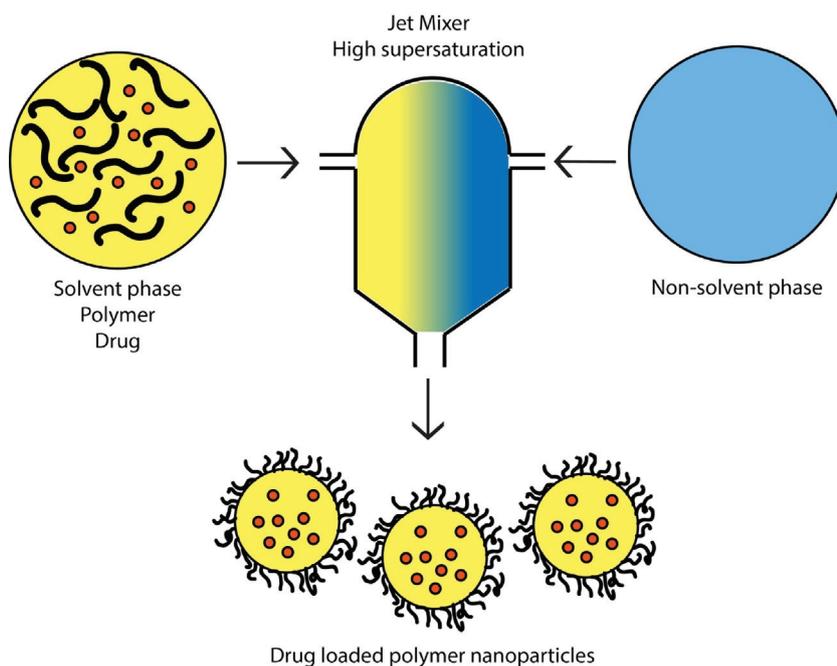


Fig. 9. Flash nanoprecipitation method.

Table 6
Advantages and drawbacks of nanoprecipitation method (Miladi et al., 2016; Paliwal et al., 2014).

No	Advantages	Drawbacks
1	Rapid	Not adequate method for water soluble molecules Particles growth controlling complication
2	Profitable	
3	Simple	
3	Production of colloidal dispersion with a narrow size distribution	
4	Easy to scale-up	
5	Satisfying reproducibility	
6	Formulations with good stability	
7	Preparation of nanospheres and nanocapsules	

and (6) Low energy input (7) (Miladi et al., 2016). Table 6. depicts major advantages and drawbacks of this method.

Nanoprecipitation has become an important strategy in pharmaceutical, agricultural, food and cosmetic industry. In agricultural industry, the need of a controlled release system is crucial to reduce the environmental and health problems associated with the use of pesticides. Possibly the most investigated field is medicine and encapsulated actives for application in pharmaceutical industry attract a special attention. Based on nanoprecipitation simplicity and versatility, it is a potential preparation method of polymeric nanosystems for pre-clinical and clinical studies.

12. Conclusion

Several drugs could present bioavailability, stability or taste limitations. Encapsulation of such molecules in NPs could be a relevant alternative to circumvent such problems. This contributes to the enhancement of the efficacy of actives and promotes patient compliance. Nanoprecipitation is a simple and reproducible technique that has been widely used for the preparation of polymeric NPs intended for several biomedical applications. Operating conditions management is a key point to obtain NPs with suitable properties. Several research works have been carried out to use nanoprecipitation in a conventional way while other works focused on the enhancement of its scalability, reproducibility and safety *via* scale-up. Tee mixer and flash nanoprecipitation are among the techniques that were introduced to achieve such purposes. Advantages of submicron carriers prepared by nanoprecipitation in the biomedical and agricultural fields have been confirmed by numerous studies. These achievements include enhanced bioavailability, better targeting and tolerance, sustained release and enhanced absorption of the drug through biological barriers. Nanoprecipitation has been widely used to prepare NPs. Although several advances have been recorded, more *in vivo* testing in human is needed. Such investigations along with scale-up approach would be highly relevant to promote the clinical applications of nanoprecipitation technique.

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Part III
EXPERIMENTAL STUDIES

Summary

In the experimental part of this PhD thesis, PCL based nanoparticles loaded with indomethacin and *Nigella Sativa L.* Seeds Essential Oil (NSSEO) were prepared by nanoprecipitation method. A set of experiments were performed: (1) for studying formulation and process related parameters effect on the properties of the provided particles. (2) to encapsulate alone indomethacin within PCL based nanoparticles and consecutively its characterization in terms of size, surface charge, morphology, stability, and *ex vivo* skin penetration. (3) for encapsulation of indomethacin and NSSEO within PCL, characterization in terms of particle size, zeta potential, morphology, stability, and *ex vivo* skin penetration, (4) To assess *in vivo* anti-inflammatory activity of prepared nanoparticles containing indomethacin and NSSEO. In the first step, a systematic study has been done in order to adjust the formulation and process related parameters for obtaining nanoparticles with good properties such as size, surface charge and so on. Throughout this part of experiment it was figured out that in formulation related parameters polymer concentration, and aqueous phase volume had a paramount impact on colloidal particles properties. In operating conditions agitation speed organic phase and injection rate are the key factors to be taken into consideration. The results of this study proved that systematic study is essential before investigation of any drug encapsulation in order to design the nanoparticles with proper characteristics for *in vivo* and *in vitro* applications. In the second step, the finalized formulation was employed to encapsulate indomethacin alone to see the indomethacin presence effect and to prepare nanoparticles for comparison in terms of skin penetration and *in vivo* anti-inflammatory activity with the nanoparticles loaded with indomethacin and NSSEO together, which have prepared in the consecutive experiment. Along this study PCL based particles loading IND have been successfully prepared and characterized. The results obtained from DSC and FTIR studies of NPs indicated that no chemical interaction between drug and polymer in the formulation was occurred. The designed nanoparticles were pointed out stable for one month under storage temperatures of 4°C, RT, and 40°C. The pH of prepared colloidal dispersion was ranged between 4 and 6. This study supports indomethacin loaded nanoparticles penetration potential as a modern topical formulation that would decline frequency of administration, side effects and consecutively patients' compliance. In the third step, the essential oil of *Nigella Sativa* was extracted and analyzed. Indomethacin and NSSEO were successfully encapsulated within poly (ϵ -caprolactone) polymer by nanoprecipitation method. Designed nanoparticles were characterized by size and zeta potential measurement and stability study. In addition, designed nanoparticles were also characterized by fluorescent microscopy, TEM, FTIR, DSC techniques that all together confirm the encapsulation of indomethacin and NSSEO. The size and zeta potential of prepared particles were respectively (230 nm - 260 nm) and (-20 mV and -30 mV). Images obtained from TEM analysis shown that prepared nanoparticles have a spherical and regular form. Encapsulation efficiency of nanoparticles for indomethacin and NSSEO were correspondingly 70 % and 84 %. In conclusion, poly (ϵ -caprolactone)

based nanoparticles were elaborated, which could be loaded with indomethacin and NSSEO in the next experimental part of this thesis. In the fourth step, skin penetration of obtained nanoparticles was investigated. *In vivo* anti-inflammatory activity of poly(ϵ -caprolactone) based nanoparticles loaded with indomethacin and NSSEO that were already prepared, has assessed as well. In fact, these prepared nanoparticles were also characterized in our last experiment in terms of fluorescent microscopy, TEM, FTIR, DSC studies. Consequently, four formulations with different compositions were prepared and their anti-inflammatory activity was assessed on the xylene induced mice ear edema. The inflammation inhibition was evaluated based on the thickness (μm), and weight (mg) measurement of mice ear after and before formulations application. Furthermore, the quantification of anti-inflammatory activity was also taken place by histology microscopic analysis and immunohistochemistry study. This study firstly reports that PCL based nanoparticles loaded with NSSEO can significantly improve cutaneous penetration of indomethacin as a noninvasive approach. Furthermore, this study reinforces the anti-inflammatory activity enhancement of indomethacin by NSSEO within the polymeric nanoparticles. Nanoparticles loaded with indomethacin and NSSEO had skin better penetration than nanoparticles loaded with indomethacin alone that was confirmed with CLSM. Consequently, for providing the same efficacy taken dose of indomethacin can be decreased in order to reduce its side effects throughout the digestive system. Indeed NSSEO anti-inflammatory is mainly associated with presence of thymoquinone in its composition. Prepared particles as noninvasive penetration enhancement technique have upgraded the skin penetration of indomethacin, and can reduce its systemic concentration and side effects.

III.1. Effect of process and formulation parameters on polycaprolactone nanoparticles prepared by solvent displacement

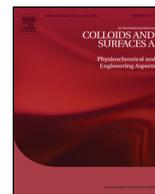
Summary

Recently nanotechnology in pharmaceuticals attracted a huge attention in order to deal with the conventional dosage forms drawbacks. Indeed, encapsulation opened the avenue to encapsulate active molecules, to target the interested area by drugs, to provide the appropriate drug release rate and to cover the unpleasant organoleptic properties of drugs. For the encapsulation of drugs, mostly biodegradable and biocompatible polymers are employed. These polymers can modify the physicochemical characteristics, biological properties, and release profile of drugs by encapsulation. Polymer selection for the encapsulation of drug molecules is based on the polymers toxicity and intended particles application. In fact, natural and synthetic polymers can be used for the drug molecules encapsulation. However, thanks to the purity and reproducibility properties synthetic polymers are better than natural polymers. Poly- ϵ -caprolactone is a biocompatible, biodegradable, semicrystalline and hydrophobic polymer. There are six methods that can be used for the preparation of particles from the already prepared polymers. These methods are including: nano-precipitation, emulsion-diffusion, emulsion-coacervation, double emulsification, polymer-coating and layer-by-layer. For elaboration of suitable nanoparticles for *in vitro* and *in vivo* applications, a proper encapsulation technique should be selected. In nanoprecipitation method that called solvent displacement or interfacial deposition, for preparation of particles two solvent (organic) and non-solvent (aqueous) phases are required. Nanoprecipitation method possesses advantages as particles production with high reproducibility in nano-scale range employing of low toxic ingredients, water, time and energy lower consumption, procedure and set-up simplicity. Thus, it is a good technique to prepare polymer based particles. For efficient encapsulation of drug in this technique active molecule solubility properties is needed that is a drawback of this method. In this work, different formulation and process related parameters such as evaporation technique, organic phase injection method and rate, stabilizers nature and concentration, polymer concentration, stirring speed, organic phase and aqueous phase volume, effect on the particle size and zeta potential and morphology were investigated. The uniqueness of this work is the complete studying of all process and formulation associated parameters from the beginning up to the end of nanoparticles preparation process which is of paramount importance for the prepared nanoparticles characteristics. Nanoprecipitation was used as the method of particles preparation and Poly- ϵ -caprolactone was used as polymer in this study. It was found that in formulation related parameters polymer concentration, and aqueous phase volume had a paramount impact on colloidal particles properties. In operating conditions agitation speed organic phase and injection rate are the key factors to be taken into consideration. The results of this study confirmed that systematic study is essential before investigation of any drug encapsulation in order to design the nanoparticles with proper characteristics for *in vivo* and *in vitro* applications.



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Effect of process and formulation parameters on polycaprolactone nanoparticles prepared by solvent displacement



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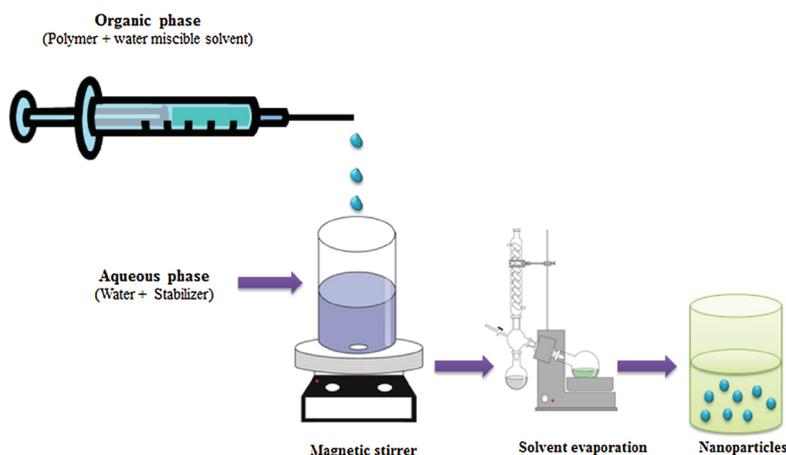
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HIGHLIGHTS

- To obtain polymer based particles with suitable properties, formulation and operating conditions have been investigated.
- Nanoprecipitation has been used as the method of particles preparation.
- In formulation related parameters polymer concentration, and aqueous phase volume had a crucial impact on colloidal particles properties.
- In operating conditions organic phase injection rate and agitation speed are the key factors to be taken into account.

GRAPHICAL ABSTRACT

Polycaprolactone based nanoparticles were prepared. Furthermore, in order to optimize the present formulation, process and formulation related parameters influence on obtained nanoparticles has been assessed and pointed out.



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ABSTRACT

Solvent displacement in comparison with other polymeric particles preparation technique provides certain crucial advantages such as water, time and energy lower consumption, procedure and set-up simplicity in nanoparticles preparation. The objective of this study was optimization of formulation in terms of the particle size, size distribution, zeta potential and morphology. In this study polycaprolactone based nanoparticles were prepared by solvent displacement or nanoprecipitation method. To prepare nanoparticles firstly, polycaprolactone was dissolved in acetone that form organic phase, secondly, for aqueous phase preparation, Tween[®] 80 and polyvinyl alcohol as stabilizer of the system, were dissolved in the water. Consecutively, under magnetic agitation organic phase was injected through a syringe to the aqueous phase. Acetone was removed under reduced pressure by rotavapor. Furthermore, different formulation and process related variables such as evaporation technique, organic phase injection method and rate, stabilizer nature, polymer concentration, Tween[®] 80 and polyvinyl alcohol

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concentration, stirring speed, organic and aqueous phases volume, were studied. To conclude, systematic study is indispensable before investigation of any drug encapsulation.

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1. Introduction

Researchers make special focus on tackling limitations associated with conventional drug delivery system. Biomedicine and biotechnology are the areas in which colloidal carriers are broadly applied. Medically used carriers comprise dendrimers, block ionomer complexes, polymeric biodegradable nanoparticles (NPs), polymeric micelles, liposomes, nanotubes, nanorods and quantum rods [1]. Encapsulation techniques are divided into two major groups; preformed polymer based and polymerization based processes. Both techniques provide us solid colloidal particles [2]. One of nanoparticles basic characteristics is their size, which usually reported to be around 5–10 nm with a superior size limit of ~1000 nm, even though the range generally obtained and used is 100–500 nm [3]. For the aim of active substances efficacy enhancement, their physicochemical properties could be changed via encapsulation [2,4,5]. For better membrane absorption and target drug delivery, particulate carriers could be employed. Moreover, encapsulation can mask unpleasant organoleptic properties of certain drugs and control release of active ingredients [6–12]. Encapsulation has emerged during the last decades as a promising and attractive option for miscellaneous biomedical applications. With the development of many biological drugs as nanomedicines, biotechnology has become one of the most interesting perspectives [13,14]. Encapsulation interest has been also proven for many other biomedical fields such as, cancer therapy [15], autoimmune diseases [16], infectious diseases [17] and theranostics [18]. To obtain these carriers various polymers with different physicochemical properties are used. However, mainly these polymers are biodegradable and biocompatible. Indeed, to make active substances stable, non-toxic and noninflammatory, they should be encapsulated in biodegradable polymers. Nanoparticle physicochemical properties (e.g. colloidal stability, hydrophobicity), biological behavior (e.g. cellular uptake, bioadhesion) and drug release characteristics (e.g. prolonged, delayed, triggered) could be modified through using these polymers [19–21]. Particles application and polymer toxicity are the issues that must be taken into account in polymer selection for polymer based particles preparation [22]. Poly- ϵ -caprolactone (PCL), poly (lactide) (PLA) and poly (lactide-co-glycolide) (PLGA) are the frequently utilized biodegradable polyesters polymers. Synthetic polymers are better than natural polymers in term of purity and reproducibility. PCL, which is generally used, is among the semicrystalline, hydrophobic polymers that are biocompatible and biodegradable [23]. High drug permeability and slow *in vivo* degradation properties of PCL make it a suitable polymer for the design of implant delivery device and so on. Furthermore, PCL micro and nano-scale carriers have attracted significant attention of pharmaceutical researchers. In addition, polymers such as poly (lactic-co-glycolic acid) (PLGA) and poly-D,L-lactic acid (PDLLA) can be mixed or copolymerized with PCL. Other fields of PCL applications include tissue engineering, scaffolds for bone, ligament, cartilage, skin, nerve and vascular tissues regeneration and interfacial tissue engineering as recently reported [24].

From the synthetic point of view, particles in general could be prepared through six classical techniques such as nanoprecipitation, emulsion-diffusion, emulsion-coacervation, double emulsification, polymer-coating and layer-by-layer [2]. Encapsu-

lation methods differ via their principles or the active substance nature to be encapsulated. To provide a formulation bearing appropriate properties of *in vitro* and *in vivo* applications, right selection of the encapsulation method is crucial. Nanoparticles preparation method should be selected according to concerned polymer and active ingredient physical and chemical properties. Nanoparticles should be prepared by a technique that does not destroy the active ingredient because most of encapsulation methods are employing various organic solvents, ultra-sonication, temperature and agitation [25]. Nanoprecipitation method called solvent displacement or interfacial deposition has been developed for first time by Fessi et al. in 1998 [26]. In the case of solvent displacement method, for particle preparation two solvent (organic) and non-solvent (aqueous) phases are necessary. Basically, organic phase is composed of drug and polymer while, aqueous phase includes water and stabilizing agents (synthetic or natural). After addition of the organic phase to the aqueous phase, submicron particles could be formed spontaneously upon organic solvent evaporation under reduced pressure. Through nanoprecipitation technique, nucleation, growth and aggregation are the stages by which polymer based particles are formed [2]. Nanoprecipitation method has several advantages such as production of particles with high reproducibility in nano-scale range using of low toxic ingredients (surfactants or organic solvents). Therefore, it represents a good approach to design polymer based particles. In spite of these advantages, nanoprecipitation technique has a few drawbacks which are attributed to the active molecule solubility properties needed for efficient encapsulation [27].

In the present work, it was focused on the study of different formulation and process related parameters such as evaporation technique, organic phase injection method and rate, stabilizer (Tween[®] 80 and polyvinyl alcohol) nature and concentration, polymer concentration, stirring speed, organic phase and aqueous phase volume, effect on the particle size and zeta potential and morphology. The complete studying of all process and formulation associated parameters from the beginning up to the end of nanoparticles preparation process which is of paramount importance for the obtained nanoparticles characteristics, add to the novelty of the current research.

2. Materials and methods

2.1. Materials

Acetone that is employed as solvent was provided by Laurylab, France. Poly- ϵ -caprolactone (PCL) which is used like wall material (Mw = 14,000 g/mol), polysorbate 80 (Tween[®] 80) (Mw = 1310 g/mol) and polyvinyl alcohol (PVA) (Mw = 31,000 g/mol) that are utilized such as stabilizer were purchased from Sigma-Aldrich, Germany.

2.2. Methods

2.2.1. Nanoparticles preparation

Polycaprolactone (PCL) based particles were formulated using the solvent displacement technique, which is described by Fessi et al. in 1989 [26]. Aqueous and organic phases were provided respectively. Firstly, for aqueous phase preparation PVA was dis-

solved in Milli-Q water and then mixed with Tween[®] 80. Secondly, with dissolution of PCL in acetone, organic phase was obtained. Subsequently, organic phase was injected dropwise to aqueous phase under continuous agitation.

2.2.2. Reference nanoparticles: composition – preparation

The amounts of formulation ingredients that have been used and the operating condition under which reference PCL based nanoparticles were prepared, shown in Table 1. In fact, for optimization of formulation, a series of experiments on process and formulation related variables were carried out in order to find out each parameter relation with obtained particles hydrodynamic particle size, zeta potential and morphology.

2.2.3. Nanoparticles characterization

The mean size (Z-average) and zeta potential of PCL based particles were determined employing Malvern particle size analyzer (Model–Nano ZS, Malvern instruments limited, UK). The prepared particles dispersion was diluted before measurement in 1 mM NaCl solution. All measurements were carried out in triplicate at 25 °C temperature. The morphology and appearance of the obtained nanoparticles were also assessed by transmission electron microscopy (TEM) apparatus Philips CM-120 at an accelerating voltage of 100 kV. To provide TEM photos, through micropipette a drop of suspension containing nanoparticles was put on the carbon-coated copper grid. Then the deposit droplet was left to dry before analysis. The extra volume of suspension was discarded from carbon-coated copper grid via blotting. In this study, at each set of investigation, just one operating condition or formulation related variable was altered and assessed while other variables were kept stable (see Table 1).

2.2.4. Statistical analysis

In tables and figures all data were shown like mean \pm standard deviation. Statistical analysis was performed employing the one-way analysis of variance (ANOVA) and Student's tests. For statistical significance $p < 0.05$ was selected such as criterion.

3. Results and discussion

In this work, most critical parameters were investigated and special attention was devoted to the effect of each parameter on the physical and colloidal properties of the obtained particles such as size, zeta potential and morphology. Generally, the average size of obtained particles from preformed polymers is in between 250 and 500 nm. From the other hand, chemical structure of used polymer and stabilizing agent are the governing parameters of the polymeric particles zeta potential [2]. Furthermore, medium pH is also a critical factor in nanoparticles zeta potential.

3.1. Acetone evaporation technique effect

To investigate the evaporation method effect on particle size and zeta potential, employed volume of organic solvent was removed through two different approaches of rotavaporation: (i) as usual, used acetone was removed from the product by rotavapor under decreased pressure and (ii) the employed acetone was removed under horizontal laminar flow with continuous magnet stirring and ambient temperature during 5 h (Büchi Rotavapor R-124) (25–30 °C). Consecutively, particles size was measured. The particles size was significantly enlarged from 175 to 297 nm by altering the acetone evaporation method from rotavaporation to ambient temperature ($p < 0.05$). From one hand, the size of obtained particles through ambient temperature evaporation was larger. From the other hand, this method of organic phase or acetone evapora-

tion took more time. It was pointed out that because of these two reasons rotavapor is a suitable method to remove acetone in order to obtain particles with smaller size. Furthermore, for acetone evaporation in ambient temperature with comparison to the rotavapor more time is needed.

The zeta potential of prepared particles was also determined and examined after acetone evaporation through rotavapor and ambient temperature with magnetic stirring as explained. Particles zeta potential when rotavapor was used was found -6.86 mV and while ambient temperature was employed was -7.07 mV ($p < 0.05$). Therefore, it could be concluded that acetone evaporation technique in opposite to the particle size, has no significant effect on particles zeta potential.

3.2. Organic phase addition behavior effect

To study addition method of organic phase influence on particle size, three methods were employed for the preparation of particles. Once, the organic phase was added directly at once on aqueous phase. Then, instead of disposable syringe for organic phase injection burette was also used. It should bear in mind that before starting of the experiment, debit of burette was adjusted with syringe, number of drops flow per minute was counted and that was the same with used syringe for injection (50 drops per minute or 2.5 ml/min). Here, 4 samples were prepared respectively with each method; at the end size of the obtained particles was examined and evaluated. Then, the particle size and zeta potential of the obtained particles via all three methods was measured. It was observed that the size of particles obtained through the direct addition of organic phase technic was significantly larger than the particles which were obtained by the organic addition via syringe. Particles prepared through direct addition of organic phase had a size of 280 nm. While the particle size was 175 nm when syringe process was used, while particles size was 253 nm when burette was used organic phase addition, which shows a significant particle size increase ($p < 0.05$). In comparison with syringe, burette use for addition has its own drawbacks such as complicate adjustment of precise addition debit and possibly air bubble existence in the burette which leads to select preferably syringe for organic phase injection than burette. In addition, through syringe the prepared particles size was smaller. According to our best of knowledge when organic phase add at once or by burette the speed of organic phase diffusion toward aqueous phase would be slowly than adding of organic phase addition by syringe. It should point out that there was not considerable change in provided particles zeta potential.

3.3. Stabilizers nature effect

To assess stabilizer nature influence on prepared particle size, 3 types of formulations were employed in order to obtain PCL based nanoparticles. PVA and Tween[®] 80 each one separately and PVA and Tween[®] 80 together were used in order to stabilize optimally the dispersion system and consecutively size of the obtained particles was evaluated to point out the best type of stabilizer. To see their synergistic effect and to find the proper stabilizer type, 4 samples firstly, just with PVA secondly with Tween[®] 80 and at the end with both of these stabilizer particles were prepared. The smallest particles were obtained by usage of stabilizers mixture. In the case of PVA alone, particles with 248 nm diameter, with Tween[®] 80 alone particles size around 191 nm diameter but with both PVA and Tween[®] 80 particles with 175 nm diameter, were obtained. Particle size reduction by employing both stabilizers PVA and Tween[®] 80 in this study, was significant ($p < 0.05$).

As PVA and Tween[®] 80 each one separately and PVA and Tween[®] 80 together were employed to stabilize the dispersion during the formation. It is interesting to notice that without any stabilizer

Table 1
Reference PCL based nanoparticles composition and operating condition.

Organic Phase		Aqueous phase			Stirrer speed (rpm)	Organic phase injection rate (ml/min)
PCL concentration (mg/ml)	Acetone volume (ml)	Tween® 80 concentration (mg/ml)	Milli-Q water volume (ml)	PVA concentration (mg/ml)		
8	25	10.6	50	5	500	2.5

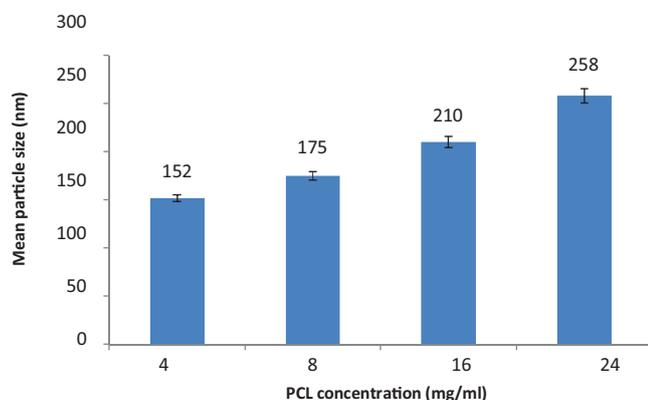


Fig. 1. PCL concentration relation with particle size.

agent, the used PCL polymer is not able to form precipitated polymer under spherical form. This is necessary to use stabilizer with well-defined ratio in order to obtain sterically stabilized particles or particles stabilized via depletion. Regarding the zeta potential of the prepared particles, it was found to be in between -2 and -8 mV irrespective of used non charged stabilizer agent.

3.4. The influence of polymer concentration

The influence of PCL amount in the used formulation on the final particle size, size distribution and zeta potential was investigated. Obviously, without taking into account the other operating parameters such as polymer nature and other used ingredients of formulation, solvent displacement technique is extremely sensitive to changes in polymer concentration. Furthermore, polymer concentration has a critical effect on the encapsulation efficiency as well. However, in the present research, blank particles were assessed so just particle size was studied. To this end, various concentrations of PCL such as 4 mg/ml, 8 mg/ml, 16 mg/ml and 24 mg/ml were employed to prepare PCL based nanoparticles. Polymer concentration effect is shown in Table 2. In this study particle size was respectively increased from 152 nm to 258 nm as reported in Fig. 1. Obtained data showed that increase of PCL amount increased significantly particle size ($p < 0.05$).

The findings of this investigation are in accordance with what reported by Chorny et al. in 2002 [27] and PCL concentration increase in particles preparation could enlarge particles size through boosting the organic phase viscosity [27]. Moreover, with modification in PCL concentration particles size standard deviation values did not enlarge significantly that shows the less polydispersity among particles.

In this part, for the study of polymer concentration influence on particles zeta potential, all parameters were fixed and only the amount of polymer used in the formulation was varied. The zeta potential of the obtained particles was investigated after following the same process such as for PCL concentration influence study on the particle size. Particles zeta potential of prepared particles with changes of PCL concentration from 4 mg/ml to 24 mg/ml was altered as well from -7.0 mV to -6.51 mV which was insignificantly changed ($p < 0.05$). Thus zeta potential change by PCL concentration

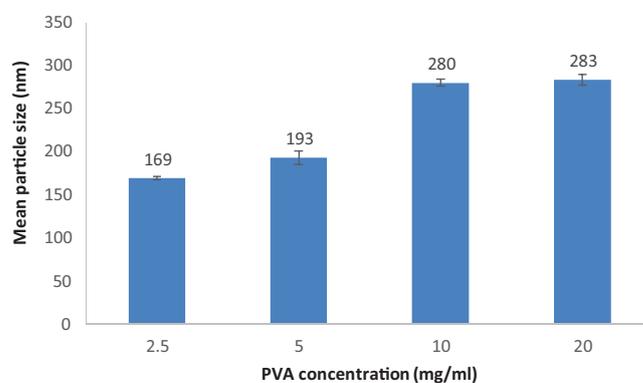


Fig. 2. PVA concentration effect on particle size.

alteration could be negligible in polymer based particles preparation.

3.5. Stabilizer (Tween® 80) concentration effect

In this work, in order to have stable particles dispersion, two stabilizers which include PVA and Tween® 80 were used. In fact, used stabilizers concentration influence on particles size was examined for each one separately. For the study of this parameter 4 samples with different concentrations of Tween® 80 (2.65 mg/ml, 5.3 mg/ml, 10.6 mg/ml and 21.2 mg/ml) were prepared. It was found that as Tween® 80 concentrations increased from 2.65 mg/ml up to 10.6 mg/ml the particle size gradually decreased from 224 nm to 185 nm. Prepared particles size could decrease because of aqueous phase viscosity augmentation by Tween® 80 and also due to the fast stabilization of the particles during the nucleation step. It should bear in mind that this particles size decreasing was going up to the 10.6 mg/ml concentration of Tween® 80. Contrary, if Tween® 80 concentrations cross this limit particles size could also increase. So, obtained results allow us to declare that Tween® 80 high concentrations improving particles size decrease to the certain limits while Tween® 80 higher concentrations than this limit, would have the opposite effect on the particle size.

To figure out Tween® 80 concentration and zeta potential relation, the same procedure such as for particle size study was followed. An increase of Tween® 80 amounts led insignificant zeta potential changes means from -7.95 mV to -6.73 mV ($p < 0.05$).

3.6. Stabilizer (PVA) concentration effect

The effect of PVA concentration ranging from 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml was investigated while other formulation ingredients concentration was stable in all experiments (see Table 1). The obtained results are reported in Fig. 2. Size of prepared particles was assessed. It was found that increasing PVA amount in the formulation increased significantly particle size from 169 nm to 283 nm ($p < 0.05$). The findings of this investigation are in agreement with the results tendency reported recently by Conrado et al. in 2013 [28] which could be attributed to the deposition of PVA on the surface of nanoparticles.

Table 2
Process and formulation parameters effect on particles size. Changes in formulation or process are shown in bold values.

Investigated parameters	Organic phase		Aqueous phase			Stirrer speed (rpm)	Organic phase injection rate (ml/min)	Mean particle size (nm)	Standard Deviation (SD)
	PCL concentration (mg/ml)	Acetone volume (ml)	Tween® 80 concentration (mg/ml)	Milli-Q water volume (ml)	PVA concentration (mg/ml)				
Stirring speed	8	25	10.6	50	5	75	2.5	211	1.27
	8	25	10.6	50	5	150	2.5	198	2.32
	8	25	10.6	50	5	300	2.5	194	1.49
	8	25	10.6	50	5	600	2.5	172	2.25
Organic phase injection rate	8	25	10.6	50	5	500	1	195	1.34
	8	25	10.6	50	5	500	5	194	1.05
	8	25	10.6	50	5	500	9	189	1.37
	8	25	10.6	50	5	500	13	197	3.62
PCL concentration	4	25	10.6	50	5	500	2.5	152	1.15
	8	25	10.6	50	5	500	2.5	175	1.15
	16	25	10.6	50	5	500	2.5	210	1.97
	24	25	10.6	50	5	500	2.5	258	2.50
PVA concentration	8	25	10.6	50	2.5	500	2.5	169	1.72
	8	25	10.6	50	5	500	2.5	193	3.08
	8	25	10.6	50	10	500	2.5	280	2.12
	8	25	10.6	50	20	500	2.5	283	4.75
Tween® 80 concentration	8	25	2.65	50	5	500	2.5	224	1.96
	8	25	5.3	50	5	500	2.5	210	2.80
	8	25	10.6	50	5	500	2.5	185	1.58
	8	25	21.2	50	5	500	2.5	210	3.79
Acetone volume	8	12.5	10	50	5	500	2.5	199	3.21
	8	25	10	50	5	500	2.5	198	1.50
	8	50	10	50	5	500	2.5	216	1.87
	8	75	10	50	5	500	2.5	2377	8.91
Milli-Q water volume	8	25	10	25	5	500	2.5	345	3.95
	8	25	10	50	5	500	2.5	194	2.98
	8	25	10	75	5	500	2.5	189	2.75
	8	25	10	100	5	500	2.5	181 ± 2	1.11

Table 3
Effect of PVA concentration on particles Zeta potential.

PVA concentration (mg/ml)	2.5	5	10	20
Zeta potential (mV)	-7.38	-6.75	-5.92	-4.45

To point out the PVA concentration effect, as Tween® 80 effect study on particles zeta potential, different quantities of PVA was employed for the preparation of 4 samples PCL based particles (see Table 3). According to the found results, while PVA concentration gradually increased from 2.5 mg/ml to 20.0 mg/ml prepared particles zeta potential was also insignificantly decreased from -7.38 mV to -4.45 mV ($p < 0.05$). This change is related to the PVA adsorption on PCL based nanoparticles surface that reduce negative charge numbers. The same observations have been reported by Lankveld and Lyklema in 1972 [29], which are important to take into account. The reason of the fact that obtained nanoparticles had a negative charge is the presence of Tween® 80 as surfactant in the formulation.

3.7. The influence of organic phase injection rate

To assess the organic phase injection rate, 4 samples with organic phase injection rate of 1 ml/h, 5 ml/h, 9 ml/h and 13 ml/h were prepared. The size of all obtained particles was measured in order, to find out the effect of this parameter. It was found that with increase of organic phase injection rate from 1 ml/h to 9 ml/h particle size was respectively decreased from 195 nm to the 189 nm. It should point out that the decreasing of particle size was going to the 9 ml/h organic phase injection rate after this limit, for 13 ml/h particle size was increased as well which is not interesting in the present study. Moreover, 13 ml/h organic phase injection rate could

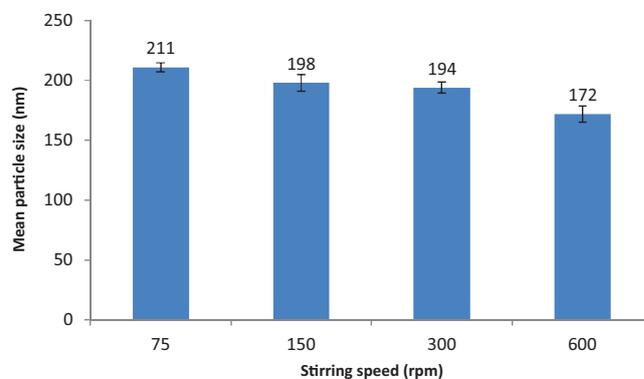


Fig. 3. Effect of stirring speed on particle size.

throw water outside of beaker in which there was aqueous phase while in normal preparation process organic phase was flowing by syringe directly to the aqueous phase.

The obtained particles zeta potential with organic phase injection rate of 1 ml/h, 5 ml/h, 9 ml/h, 13 ml/h in 4 samples were determined. Zeta potential of the prepared particles was changed from -8.03 to 1 ml/h flow to -7.77 with 13 ml/h debit. It could be concluded, that organic phase injection rate variation cannot change significantly particles zeta potential ($p < 0.05$).

3.8. The stirring speed effect

To study influence of stirring speed on the size of obtained particles, 4 samples under different stirring speed (75 rpm, 150 rpm, 300 rpm and 600 rpm) were made (see Fig. 3). At 75 rpm, the aver-

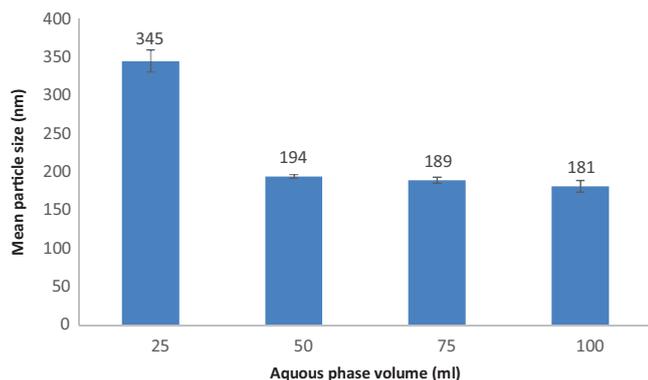


Fig. 4. Effect of aqueous phase volume on particle size.

age particle size was 211 nm while at 600 rpm stirring rate, they were significantly reduced to 172 nm ($p < 0.05$). It seemed, that mass transfer and diffusion degree, which persuade too homogeneous supersaturation and fast nucleation for smaller particles production, improved via high stirring speed as reported by Ali et al. in 2009 [30]. According to Ali et al. [30], up to a specific limit particle size could increase with increasing stirring speed and above that stirring rate won't be able to increase prepared particle size. Nevertheless, in our case there was more foam formation because of further stirring speed which could disturb the evaporation process of acetone.

Furthermore, as for the particle size study, zeta potential of all 4 samples under different stirring speed (75 rpm, 150 rpm, 300 rpm and 600 rpm) were determined. The zeta potential of particles in stirring rate of 75 rpm to 600 rpm was almost constant from -6.83 mV to -6.18 mV. In brief, zeta potential of prepared particles was insignificantly increased with higher stirring rates ($p < 0.05$).

3.9. The organic phase volume effect

For investigation of organic phase volume influence on the particle size, the volume of acetone (containing constant PCL polymer amount) was changed from 12.5 ml to 75 ml into the preparation of 4 samples. Particle size of all four samples has been examined. According to the obtained results particles size was also significantly increased from 199 nm to 2377 nm ($p < 0.05$). Particle size augmentation reason could be large drop formation which changes subsequently to the particle. Furthermore, in the study of acetone volume effect, particles size had a high standard deviation values in comparison with all other parameters that were studied in this work. This could figure out the polydispersity significance of particles.

For study of organic phase volume effect on zeta potential, as for particle size study volume of acetone was changed from 12.5 ml

to 75 ml into the preparation of 4 samples. Subsequently, particles zeta potential was determined and it was found that with mentioned volume changes respectively particles zeta potential changed insignificantly from -5.81 to -6.46 mV ($p < 0.05$).

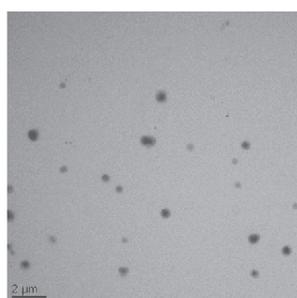
3.10. The aqueous phase volume effect

To assess the impact of aqueous phase volume on the particles size, 4 preparations with various volumes of 25 ml, 50 ml, 75 ml and 100 ml Milli-Q water were provided (see Fig. 4). In this study, it was found that particle size was, decreased significantly from 345 nm to 181 nm ($p < 0.05$). The reasons of obtaining smaller particles with high aqueous volume are the water-miscible solvent rapid diffusion and nucleation. The found results of this parameter study is in accord with was obtained by Miladi et al. in 2015 [31].

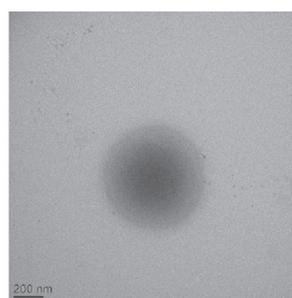
To assess aqueous phase volume effect on the particles zeta potential, 4 preparations with various volumes of 25 ml, 50 ml, 75 ml and 100 ml Milli-Q water, were provided. In this study, it was found that with increasing of aqueous phase from 25 ml to 100 ml particles zeta potential was insignificantly decreased from -5.46 mV to -7.05 mV ($p < 0.05$). Thus, aqueous phase volume has not an important effect on the zeta potential of PCL based particles through solvent displacement method. According to the findings of this research through transmission electron microscopy (TEM) images, obtained nanoparticles by the optimized formulation have spherical and regular form (See Fig. 5).

4. Conclusion and perspectives

Solvent displacement method is an efficient approach for particles preparation, thanks to several critical advantages: production of particles with high reproducibility in nano-scale range using of low toxic ingredients (surfactants or organic solvents). Prepared particles properties such as size and zeta potential could play important role in the particles stability and biological activity that is the reason why nanoparticles formulation should be optimized. In the present study, authors focused on the study of the variables attributed to the formulation and the process of nanoparticles preparation. The investigated parameters are evaporation technique, organic phase addition method, stabilizer nature, polymer concentration, Tween[®] 80 and PVA concentration, organic phase injection rate, stirring speed, organic and aqueous phases volume. In brief the principal results of this investigation are: (a) acetone evaporation is better by rotavapor than through ambient temperature under magnetic stirring, (b) organic phase injection via syringe could provide particles with better properties than organic phase addition by burette and syringe technique is more simple than burette, (c) it was found to use both PVA and Tween[®] 80 together as stabilizers but with lower quantities in order to obtain more stable particles, particles size could increase significantly through



(A). The optimized formulae, scale bare 2 μ m.



(B). The magnification of one particle, scale bare 200 nm.

Fig. 5. TEM images of PCL particles.

PCL concentration increasing, (d) augmentation of PVA concentration also significantly increase the size of the particles. In addition, PVA increase has opposite influence on particles zeta potential, (e) with higher rate of organic phase injection obtained particles was decreased. The stirring higher speed also significantly decreased the particles diameter but could not have any effect on particles zeta potential. Moreover, large aqueous phase volumes could significantly decrease prepared particles size. Further studies could complete this work, with adding active molecule into the blank nanoparticles which have prepared here and to investigate the relation of studied parameters here with active ingredient existence in blank optimized formulation.

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III.2. Polycaprolactone Based Nanoparticles Loaded with Indomethacin for Anti-Inflammatory Therapy: From Preparation to *Ex Vivo* Study

Summary

Non-Steroidal Anti-inflammatory Drugs (NSAIDs) as a mostly used drugs category bring together all the drugs that reduce fever, pain and inflammation. Indeed, indomethacin (IND) is a poorly water-soluble NSAID that is a weak acid. In addition, IND is a potent anti-inflammatory drug that has been shown a satisfied efficiency for an external application. Nevertheless, IND because of its mechanism of action (Cox enzymes inhibition) induces certain gastro-intestinal side effects such as gastrointestinal ulcers or bleeding. To overcome the side effects associated with indomethacin usage, its taken amount can be reduced target drug delivery to the interest area can be designed. For the protection of drug molecules from the liver first pass effect and control of drug delivery ratio, skin can be employed for the drug administration. However, skin permeability towards drug molecules is lower than digestive system mucous membrane. Therefore, skin despite of its important role in the protection of body from the outside can play a role of a barrier for the delivery of drug. A number of chemical and physical cutaneous penetration enhancement strategies are studied for the tackling of this hurdle. Though mentioned techniques irritate, damage, and disorder the function of skin. Hence, to keep skin status and functions normal nanoparticles based drug delivery to and through the skin would be the most appropriate option. In nanoprecipitation method that was described in earlier experiment part certain polymers especially biodegradable polyesters such as polylactide (PLA), polylactide-co-glycolide (PLGA) and polycaprolactone (PCL) are used. As in the previously carried out experiment part formulation and process related parameters have been studied. Therefore, this research is targeted for the preparation of polycaprolactone based nanoparticles containing indomethacin to offer the topical effective anti-inflammatory therapy of inflammatory diseases. Nanoparticles were prepared under the same condition as optimized in early carried out experiment, for formulation here just indomethacin has added else it is the same formulation as finalized formulation. The similar formulation is employed, this work can be explained in three points: (a) preparation of nanoparticles loaded with indomethacin, (b) characterization of prepared nanoparticles in terms of size, surface charge, morphology, encapsulation efficiency, stability and physical state, (c) evaluation and to confirmation of skin penetration ability of prepared nanoparticles, an *ex-vivo* study was performed on fresh human skins. Oppositely to the already works, in this research non-invasive method has been used for the enhancement of indomethacin dermal drug penetration. *Ex-vivo* skin penetration study was carried out on fresh human skin and Core Shell Evidots (CSE) was used as fluorescent agent. In fact, this fluorescent agent in biomedical applications are superior to traditional organic dyes since CSE can emit the whole spectrum, are brighter and have little degradation over time. In brief, PCL based particles loading IND have been successfully prepared and characterized. The results provided by DSC and FTIR studies of NPs show that no chemical interaction between drug and polymer in the formulation was occurred. The designed nanoparticles were pointed out stable for one month under storage temperatures of 4°C, RT, and 40°C. The pH of prepared colloidal dispersion was ranged

between 4 and 6. This study reinforces indomethacin loaded nanoparticles penetration potential as a modern topical formulation that would decline frequency of administration, side effects and consecutively patients compliance.

Polycaprolactone Based Nanoparticles Loaded with Indomethacin for Anti-Inflammatory Therapy: From Preparation to *Ex Vivo* Study

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ABSTRACT

Purpose This work focused on the preparation of polycaprolactone based nanoparticles containing indomethacin to provide topical analgesic and anti-inflammatory effect for symptomatic treatment of inflammatory diseases. Indomethacin loaded nanoparticles are prepared for topical application to decrease indomethacin side effects and administration frequency. Oppositely to already reported works, in this research non-invasive method has been used for the enhancement of indomethacin dermal drug penetration. *Ex-vivo* skin penetration study was carried out on fresh human skin.

Methods Nanoprecipitation was used to prepare nanoparticles. Nanoparticles were characterized using numerous techniques; dynamic light scattering, SEM, TEM, DSC and FTIR. Regarding *ex-vivo* skin penetration of nanoparticles, confocal laser scanning microscopy has been used.

Results The results showed that NPs hydrodynamic size was between 220 to 245 nm and the zeta potential value ranges from -19 to -13 mV at pH 5 and 1 mM NaCl. The encapsulation efficiency was around 70% and the drug loading was about 14 to 17%. SEM and TEM images confirmed that the obtained nanoparticles were spherical with smooth surface. The prepared nanoparticles dispersions were stable for a period of 30 days under three temperatures of 4°C, 25°C and 40°C. In addition, CLSM images proved that obtained NPs can penetrate the skin as well.

Conclusion The prepared nanoparticles are submicron in nature, with good colloidal stability and penetrate the stratum corneum layer of the skin. This formulation potentiates IND skin penetration and as a promising strategy would be able to decline the side effects of IND.

KEY WORDS anti-inflammatory · indomethacin · nanoparticles · polycaprolactone · skin penetration

ABBREVIATIONS

COX enzyme	Cyclooxygenase enzyme
CLSM	Confocal laser scanning microscopy
DSC	Differential scanning calorimeter
EE	Encapsulation efficiency
FTIR	Fourier transform infrared spectroscopy
IND	Indomethacin
NPs	Nanoparticles
NSAIDs	Non-Steroidal Anti-inflammatory Drugs
PCL	Polycaprolactone
PVA	Polyvinyl alcohol
RT	Room temperature (25°C)
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

INTRODUCTION

Non-Steroidal Anti-inflammatory Drugs (NSAIDs) are from the most used drugs category in the world. Indomethacin (IND) as a NSAID decreases fever, pain and inflammation. IND is a poorly water-soluble drug which is a weak acid like the majority of NSAIDs (Fig. 1). Moreover, IND is a potent anti-inflammatory drug which has been shown an acceptable efficiency for an external application (1,2). However, IND mechanism of action is associated with the presence of gastro-intestinal side effects (2). Indeed, these adverse effects

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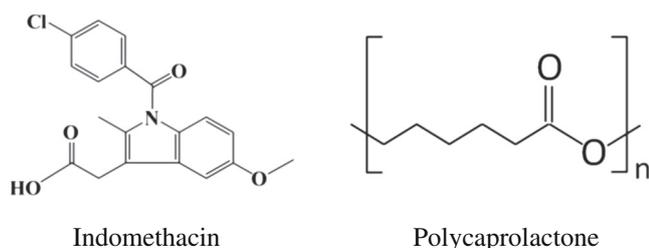


Fig. 1 Chemical structures of Indomethacin and Polycaprolactone.

are due to COX-I enzymes inhibition (3,4). IND such as most of NSAIDs either causes or intensifies gastrointestinal ulcers or bleeding. Furthermore, IND has a short half-life (4–5 h) (5). In fact, to decrease the side effects of Indomethacin (e.g. stomach or intestinal bleeding, ulcers or renal function impairment), it is possible to reduce the amount to be taken and to provide target drug delivery to the interest area (6). Skin as a route of drugs administration could be used to control the delivery ratio of active agents for a long period and decline the dosage of drugs thanks to the safety of drugs from liver first pass effect (7). Nevertheless, drugs dermal permeability in comparison with mucous membrane of digestive tube is low therefore; skin plays a role of biologic protector against foreign substance entrance to the body. Stratum Corneum, due to its drug molecules low permeability, could be an obstacle for the development of transdermal drug delivery systems. In order to overcome this hurdle, several chemical and physical cutaneous penetration enhancement approaches were known; which is associated with the skin irritation, damage and consecutively dermal barrier function disorders. Therefore, it would be more interesting to deliver a drug and meanwhile maintain the skin barrier function normal. For this reason skin drug delivery with aid of NPs could pave the way for obtaining this objective (8). Since long time before nanotechnology has attracted a considerable attention towards drug delivery application (9). Encapsulation plays an important role in overcoming the obstacles such as low bioavailability, poor stability, and unpleasant organoleptic properties of several drugs (10). This reality of tackling the constraints that faced conventional dosage forms attracted a special attention. The hydrophobicity or hydrophilicity properties of drugs are the important criteria for the selection of a suitable encapsulation technique (11). Nanoprecipitation is an encapsulation technique named also solvent displacement or interfacial deposition. Nanoprecipitation method, which was first developed by Fessi *et al.* (12), has been widely used for the encapsulation of hydrophobic drug molecules (nanocapsule or nanosphere forms). The solvent phase mainly involve film-forming substances, drug, lipophilic surfactant, oil and a drug molecule solvent or oil solvent (if it is needed). Moreover, it is possible to use natural, synthetic or semi-synthetic polymers as film-forming materials. To prevent prepared nanoparticles aggregation, surfactants have to be added to the formulation (13). In this technique certain polymers especially biodegradable polyesters such as polylactide (PLA),

polylactide-co-glycolide (PLGA) and polycaprolactone (PCL), have been used. To obtain nanoparticles by nanoprecipitation method, solvent and nonsolvent phases are needed (12). Furthermore, in this technique ethanol, acetone, hexane, methylene chloride and dioxane are from the mostly employed solvents. Generally, solvent and nonsolvent phases are called respectively organic and aqueous phases (13,14). Polycaprolactone (PCL) is a biocompatible and biodegradable polymer which has been employed to encapsulate certain drugs for the purposes of bioavailability improvement, targeting and sustained delivery. Biodegradable polymers do not require to be removed from the body after application (15). Moreover, PCL has been widely used for drug delivery purposes (16).

Thus, in this study efforts were made to design a new drug delivery system in order to afford an effective local anti-inflammatory therapy by the aid of nanotechnology possessing the already mentioned advantages. The aim of the preformed work could be explained from three different points of views. To optimize formulation and operating procedure for PCL based blank nanoparticles preparation a systematic study has already been done by Badri *et al.* (17). Firstly, PCL based NPs loaded with IND for topical application, have been prepared. Secondly, obtained nanoparticles were characterized in terms of size, surface charge, encapsulation efficiency, morphology, physical state and stability. Finally, to assess and to prove the skin penetration capacity of prepared nanoparticles, an *ex vivo* study was performed on fresh human skins.

MATERIALS AND METHODS

Materials

Polycaprolactone ($M_w = 14,000$ g/mol) that was used as wall material, polyvinyl alcohol (PVA) ($M_w = 31,000$ g/mol) and polysorbate 80 (Tween® 80) ($M_w = 1,310$ g/mol) which were employed as stabilizing agent were purchased from Sigma-Aldrich, Germany. IND such as core materials was supplied by VWR. Acetone that was used as solvent for PCL and IND was provided by Laurylab, France.

Methods

Nanoparticles Preparation

Indomethacin loaded NPs were obtained via nanoprecipitation method as described by Fessi *et al.* (12). The encapsulation was performed in two separate steps. In fact, for organic phase preparation, 200 mg PCL with the aid of mild heat and magnetic agitation were dissolved in 25 ml acetone then 40 mg IND was also added. From the other hand, 5 mg PVA was dissolved with the aid of mild heat in 50 ml Milli-Q water and then mixed by magnetic agitation with 135 mg Tween-80® (see

Table I). Consecutively, the organic phase containing IND, PCL and acetone was added dropwise to the aqueous phase that was comprises PVA, Tween – 80® and Milli-Q water. The evaporation of acetone was carried out subsequently via Buchi Rotavapor R-124® (under high temperature and reduced pressure conditions).

Characterization of Nanoparticles

Nanoparticles size, zeta potential, morphology; physical status and interaction of drug and polymer with FTIR, DCS, and stability and encapsulation efficiency were assessed.

Particle Size and Zeta Potential Measurement

To measure colloidal dispersions size and zeta potential, Malvern particle size analyzer using dynamic light scattering (Zetasizer –Nano ZS, Malvern instruments limited, UK) was

used. The NPs were dispersed in a 1 mM NaCl solution previous to each measure. All measurements were performed in triplicate at room temperature (25°C).

Encapsulation Efficiency and Drug Loading

In order to determine the encapsulation efficiency, encapsulated IND (within carrier) and free IND should be separated. Thus, obtained dispersion was subjected to centrifugation at 15, 000 rpm for 30 min. The quantity of IND loaded in NPs was determined by ultraviolet spectrophotometer UV-1800 (Shimadzu, Japan) via direct method. IND amount was measured at $\lambda = 318$ nm wavelength following of analytical method validation. The quantity of encapsulated drug was determined by dissolution of precipitated NPs in acetone. Drug loading was indicated as the encapsulated drug amount to the polymer quantity ratio. The following equations were used to calculate the encapsulation efficiency and drug loading:

$$\text{Encapsulation efficiency}\% = \frac{\text{Amount of encapsulated drug}}{\text{Initial amount of drug used in formula}} \times 100$$

$$\text{Drug loading}\% = \frac{\text{Added drug} - \text{Free (Untrapped drug)}}{\text{Polymer amount}} \times 100$$

Particles Morphology and Surface Characteristics

To examine nanoparticles surface morphology, shape and appearance, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) have been used. Scanning Electron Microscopy study of NPs morphology assessment was performed with a FEI Quanta 250 FEG microscope at the “Centre Technologique des Microstructures” (CT μ) under a 10 kV accelerating voltage (Claude Bernard University Lyon 1, France). For the preparation of SEM samples NPs suspension was diluted in 1:1, 1:2, and 1:4 proportions with *Milli-Q water*. Subsequently, diluted suspension containing NPs was dropped by a micropipette on a flat metallic holder and left to dry in ambient temperature for 12 h. The coating of samples has been carried out under vacuum via cathodic sputtering in the presence of platinum (10 nm). However, for NPs shape evaluation TEM has been performed

via Philips CM-120 Transmission electron microscope (CMEABG, Claude Bernard University Lyon 1, France) by 120 kV accelerating voltage. By micropipette a drop of NPs suspension was diluted in 2 ml of *Milli-Q water* and then one drop of it placed on carbon-coated copper grid. The over needed NPs suspension has been thrown out via blotting the grid with filter paper and posed NPs suspension on the grid dried before TEM study at room temperature.

Fourier Transform Infrared Spectroscopy (FTIR)

For the chemical description of drug molecule structure, polymer and NPs FTIR analysis was done. Attenuated total reflection (ATR) FT-IR (IS50, Thermofisher) was used to study pure powder of IND, PCL and loaded NPs in a comparative manner.

Table I Nanoparticles Preparation Ingredients and Operating Condition

Organic phase			Aqueous phase			Operating condition	
PCL concentration (mg/ml)	Acetone volume (ml)	Indomethacin concentration (mg/ml)	Tween – 80 concentration (mg/ml)	Milli – Q water volume (ml)	PVA concentration (mg/ml)	Stirrer speed (rpm)	Organic phase injection speed (ml/min)
4	25	0,8	2,7	50	0,1	300	9

Differential Scanning Calorimeter (DSC) Analysis

To evaluate the physical status and interaction of drug and polymer in formulation DSC study was performed. For this end, colloidal dispersion was centrifuged to obtain two phases of supernatant and precipitation. Then supernatant part has been removed and NPs (precipitation) were dried in hold and consecutively subjected to thermal analysis. Drug loaded NPs, blank NPs and drug alone were taken as analytical samples for the DSC study. Indeed, IND DSC scan was employed as a control. Differential scanning calorimeter Q200 (TA instruments, USA) was utilized as instrument for test. A nitrogen purge of 50 mL/min was employed for all measurements. The heating rate was 10°C/min and the temperature range was 20–300°C. Hermetic aluminium alloy pans were used.

Nanoparticles Skin Penetration Study

The confocal laser scanning microscopy (CLSM) is a non-invasive imaging approach that could be employed to provide images based on the fluorescence excitation. In addition, CLSM would be used to visualize the distribution of NPs containing drug molecules in different biological tissues (18,19). To obtain CLSM images, a light with a precise wavelength should excite fluorophore (fluorescent agent), endure its excited state only for a few nanoseconds and soon via emitting of longer wavelength drops back to the ground state. Fluorescent agent value of quantum efficiency value (QE) could explain the intensity of emitted energy *via* fluorescent agent at its optimum wavelength of excitation (18,20).

$$\text{Quantum efficiency value (QE)} = \frac{\text{Emited energy}}{\text{Absorbed energy}}$$

To assess nanoparticles skin penetration, freshly excised abdominal human skins were used. The skin surface was cleaned with water, and 100 µl of obtained NPs dispersion that contained 10 µl of Core shell Evidots in Toluene, were topically applied. After 24 h of application at room temperature, biopsies of skin (3 mm in diameter) were realized, frozen and embedded in Tissue-Tek O.C.T. Cryostat sections (10 µm) perpendicular to the skin surface were prepared and mounted on poly-lysine coated slides. Skin sections were stained with Dapi to visualize cell nuclei. The tissue was observed by confocal laser scanning microscopy (FV1000 Olympus). Laser at 525 nm (green) was used to observe the localization of Core shell Evidots labeled NPs in the skin. Dapi stained slides were examined with a 405 nm diode laser. Green fluorescence intensity and localization of CLSM images were the two indicators of NPs penetration assessment through the skin in a comparative manner between fresh human skins treated with NPs and control fresh human skins (untreated with NPs).

Stability Study of Nanoparticles

The colloidal stability of dispersions has been studied from physicochemical and physical aspects. For this end, obtained NPs were stored for a period of one month in 4°C, RT and 40°C temperature degrees. For the evaluation of physicochemical stability, nanoparticles EE % and pH under already mentioned storage condition was measured while NPs physical stability was assessed via size, size distribution and zeta potential measurement. In fact, EE % and colloids dispersion of NPs was determined once just after NPs preparation and secondly after 30 days of storage in 3 different already mentioned storage conditions in order to observe the stability of encapsulated IND beside the colloidal stability of NPs in the dispersion. The measurements were carried out after each 10 days. Indeed, chemical structure of polymer, used surfactant and the pH of the medium play a crucial role in NPs surface charge or NPs zeta potential (14).

STATISTICAL ANALYSIS

The study data are expressed as mean ± standard deviation (SD) and analyzed statistically. The values have been analyzed by ANOVA Analysis of variance. For statistical significance $p < 0.0001$ (***), $p < 0.001$ (**) and $p < 0.01$ (*) were selected such as criteria. *P*-value less than $p < 0.01$ considered significant.

RESULTS

Particle Size and Zeta Potential Measurement

Nanoparticles size and zeta potential have been measured in diluted samples and all measurements were carried out in triplicate at room temperature (25°C). The size of NPs was ranged in between 220–245 nm (± 79, 75) and NPs zeta potential at pH 5 and 1 mM NaCl was found to be in between -13 and -19 mV (± 4, 61) (Figs. 2 and 8). Formulated

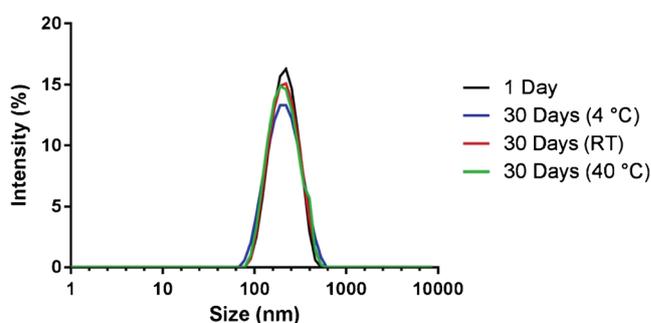


Fig. 2 Colloidal size dispersions, RT = room temperature (25°C).

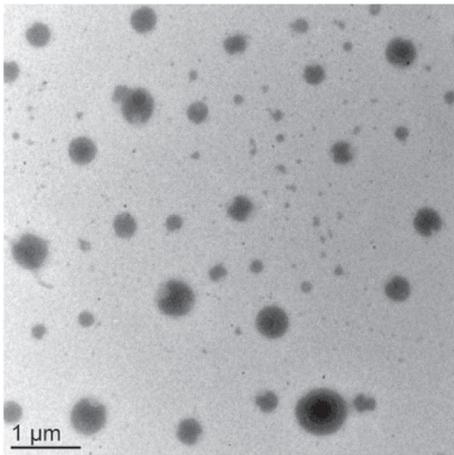


Fig. 3 Transmission Electron Microscopy image of PCL based nanoparticles loaded with IND, The scale bar represents 1 μm .

dispersions stability is mainly due to the presence of stabilizer PVA which induces good depletion stabilization. Negative zeta potential of NPs could be originated from the carboxylic end group of PCL which is used as a polymer in the formulation.

Encapsulation Efficiency and Drug Loading

In this study, nanoparticles encapsulation efficiency was measured by direct method. Thus, firstly encapsulated IND (within carrier) and free IND were separated. Therefore, prepared dispersion was centrifuged at 15,000 rpm for 30 min. The quantity of IND loaded in NPs was determined by ultraviolet spectrophotometer UV-1800 (Shimadzu, Japan). The quantity of encapsulated drug was determined by dissolution of precipitated NP in acetone. The encapsulation efficiency of IND loaded nanoparticles was ranged between 70–76% while its drug loading was limited between 14–17%. It means

that 14 to 17% of nanoparticles weight is composed of Indomethacin.

Particles Morphology and Surface Characteristics

Indomethacin loaded nanoparticles morphology was visualized by Scanning Electron Microscopy (SEM) and prepared NPs were assessed in terms of surface texture, shape, inter-particulate bridging presence and smoothness. In addition, to evaluate obtained nanoparticles shape and appearance TEM analysis was carried out. It was confirmed by TEM images that prepared NPs were spherical, properly isolated and had not inorganic impurities (see Figs. 3 and 4).

Fourier Transform Infrared Spectroscopy (FTIR)

The Fourier transform infrared spectroscopy analysis was also employed for the characterization of NPs. Usually; the interaction of drug with polymers could lead to identifiable changes in FTIR spectra (21). Indomethacin, PCL and loaded NPs FTIR patterns have been shown in Fig. 5. The FTIR spectrum of pure IND could be seen in Fig. 5(a). In fact, the characteristic bands of IND revealed free acid carbonyl groups (C = O) stretching band at 1711 cm^{-1} , C = O amide at 1688 cm^{-1} , phenolic carbon stretching vibration (C = C stretch vibration) at 1587 cm^{-1} , O-H stretch vibration at 3380 cm^{-1} and C-Cl stretch at 839 cm^{-1} . On the other hand, the characteristic bands of PCL appeared at 2865 and 2942 cm^{-1} which is related to the symmetric and asymmetric aliphatic stretching, respectively (Fig. 5(b)). In addition, the strong characteristic carbonyl (C = O) stretching band raised at 1721 cm^{-1} . The C-O and C-C stretching vibration bands of PCL appeared at 1292.9 cm^{-1} . Also, the symmetric and asymmetric C-O-C vibration bands appeared at 1162 and 1238 cm^{-1} , respectively.

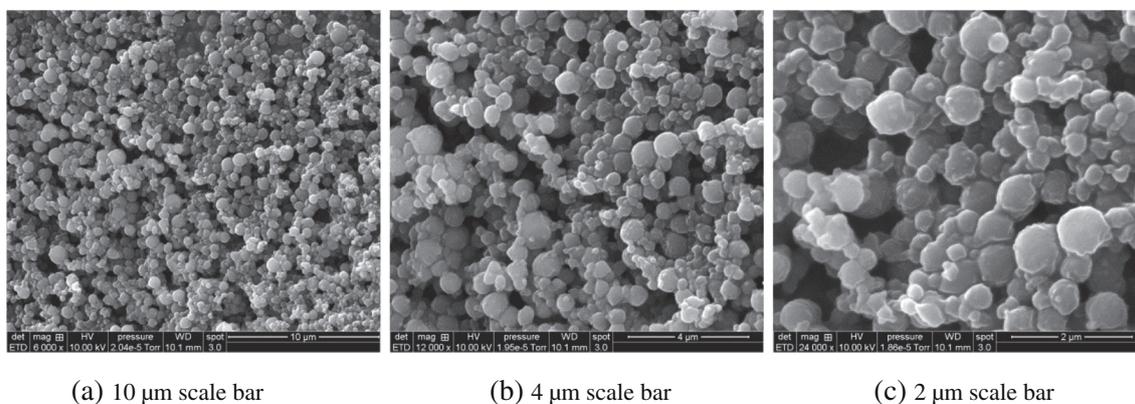
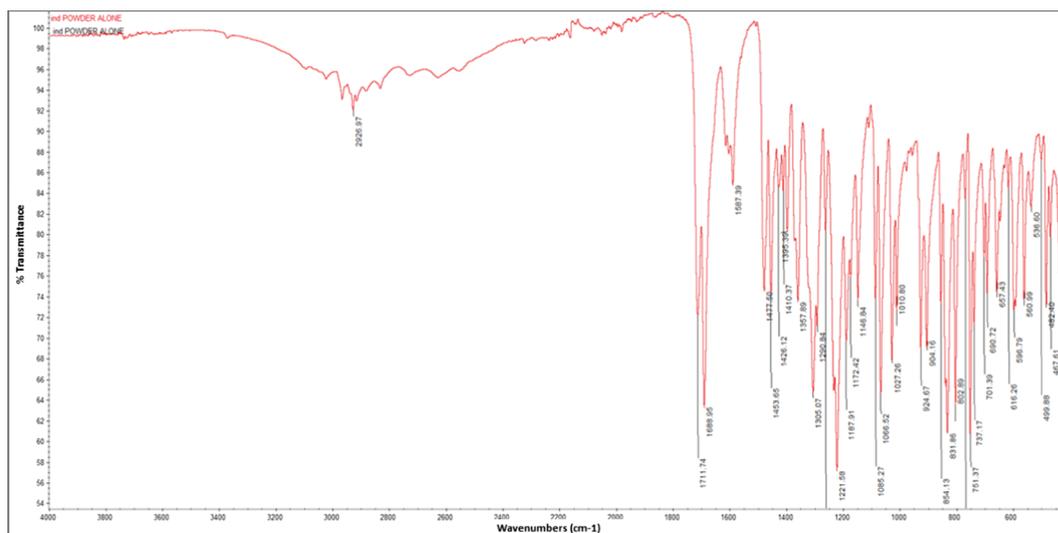
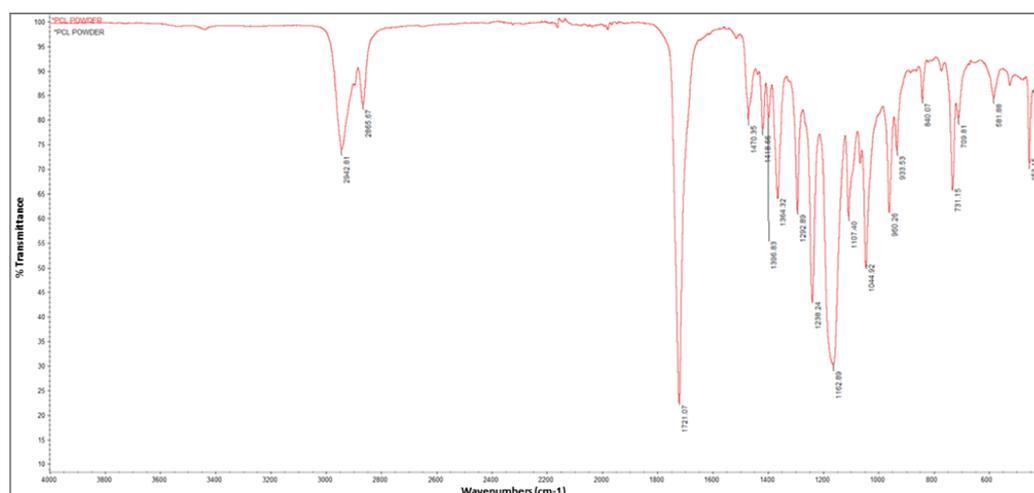


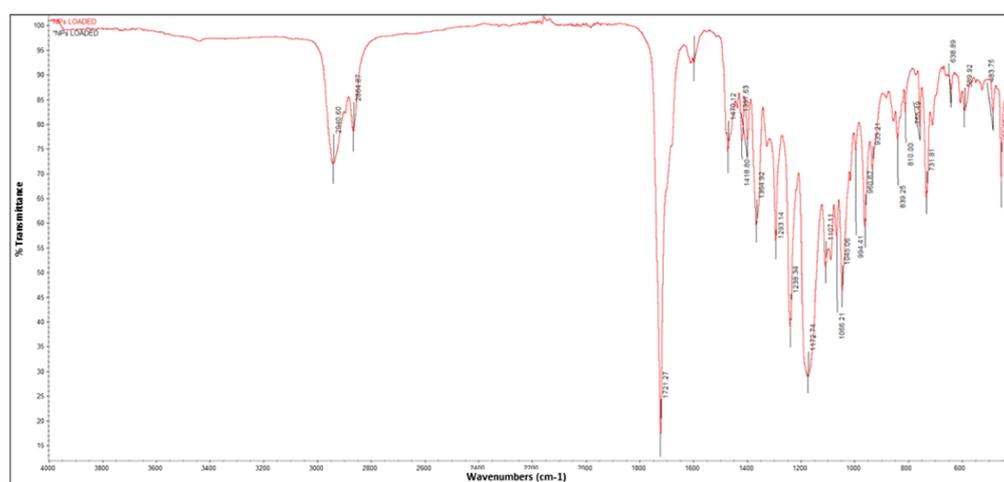
Fig. 4 Scanning Electron Microscopy images of PCL based nanoparticles loaded with IND.



(a) Fourier transform infrared spectroscopy spectrum of IND powder



(b) Fourier transform infrared spectroscopy spectrum of PCL powder.



(c) Fourier transform infrared spectroscopy spectrum of loaded NPs.

Fig. 5 The fourier transform infrared spectroscopy spectra representation. (a) IND powder; (b) PCL powder; and (c) loaded nanoparticles.

Differential Scanning Calorimeter (DSC) Analysis

In the present research, DSC study was took place in order to define the physical state of the formulation (drug and polymer). The obtained DSC curves of loaded NPs, drug-free NPs and IND powder are represented in Fig. 6. Indeed, IND and PCL are both hydrophobic substances therefore, it seems more feasible that IND is either encapsulated inside NPs or adsorbed on to the surface of NPs.

Nanoparticles Skin Penetration Study

Nanoparticles penetration ability through the skin has been proved by numerous studies (19,22) and found to be size, surface charge nature and density and softness dependent. The cutaneous penetration and localization assessment of PCL based NPs containing IND were performed by CLSM. From the other hand, encapsulation of IND has already confirmed by the evaluations of DSC, FTIR analyses and EE % determination. Furthermore, PCL based NPs skin penetration could be observed by comparison of the green fluorescence intensity of treated skins with NPs and control skins CLSM images (see Fig. 7). Confocal images revealed higher green fluorescence intensity in the various epidermal and dermal layers after application of PCL based NPs. Nevertheless, they shown a low background autofluorescence linked to keratin and collagen.

Stability Study of Nanoparticles Dispersion

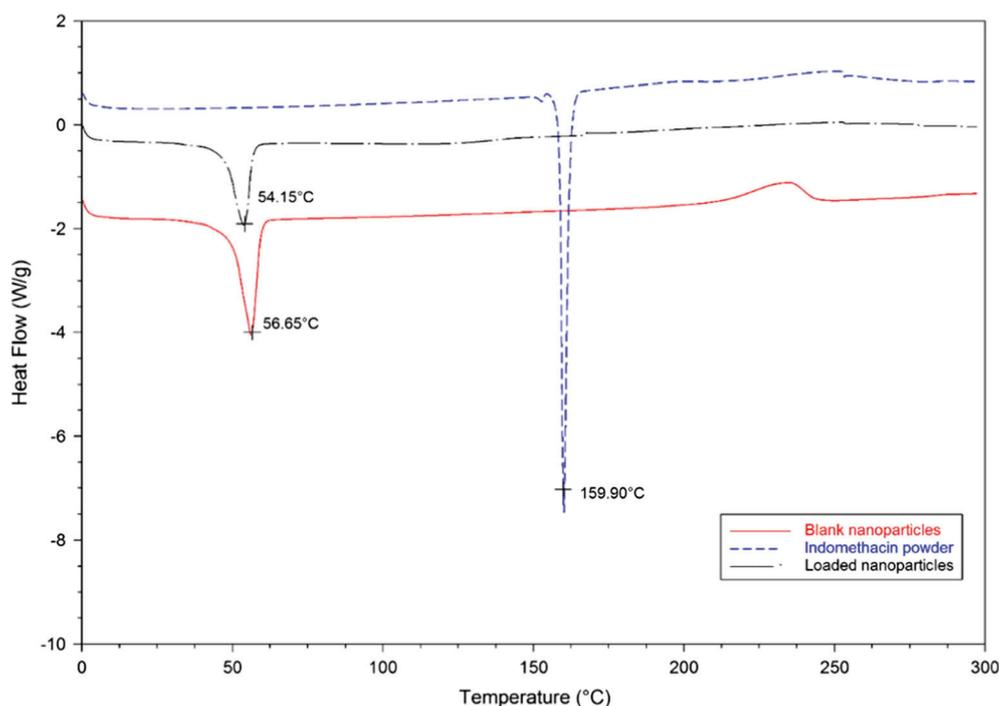
The colloidal stability of prepared nanoparticles suspension was evaluated by measurement NPs size, size distribution, zeta potential, pH and EE% as a function of storage period (30 days) and temperature (4°C, RT and 40°C) (see Fig. 8).

The results in terms of obtained NPs size and zeta potential were shown that no significant changes have been seen in prepared NPs size, Zeta potential, pH and EE%. Prepared NPs EE % and drug loading % which have been measured after preparation were respectively 75% and 15%. Obtained NPs EE % and drug loading % after 30 days, were respectively (72–74%) and (14–15%). Additionally, pH of colloidal dispersions was observed during 30 days within each 10 days measurement in all three different conditions of 4°C, RT and 40°C (Fig. 9).

DISCUSSION

NSAIDs are among the mainly prescribed drugs classes for the treatment of different inflammatory diseases. In fact, NSAIDs consist of 8 groups of drugs. IND is a potent anti-inflammatory drug molecule which has an adequate efficiency by the external route. However, IND, via its mechanism of action and chemical nature, could provoke several side effects like gastrointestinal ulcers, bleeding and impairment of kidney function (1–8). This fact underlines the demand for design of new dermal formulations to tackle such drawbacks and

Fig. 6 DSC thermograms of IND powder, blank and loaded nanoparticles.



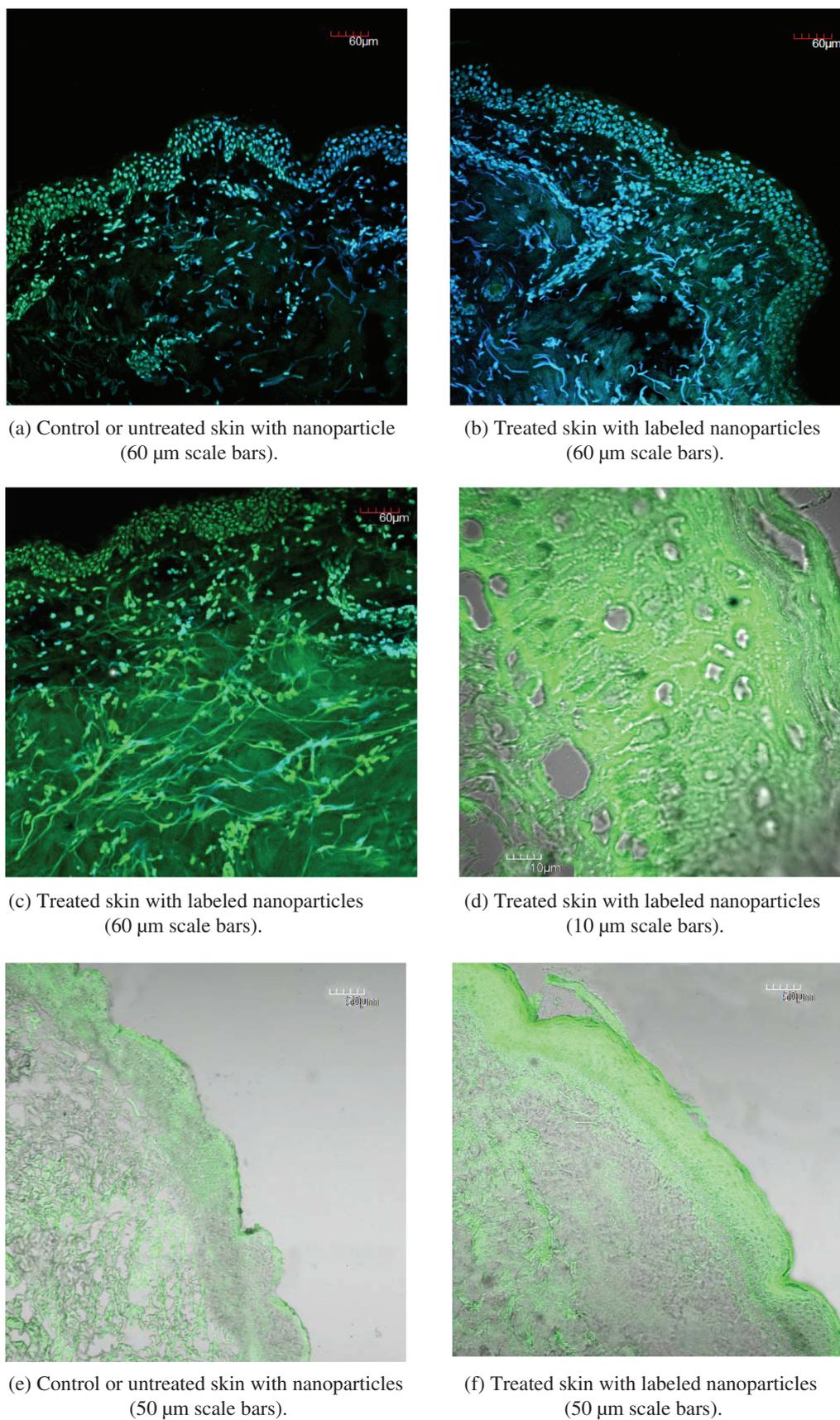


Fig. 7 Polycaprolactone based IND loaded nanoparticles CLSM depictive images of control and treated fresh human skins with fluorescently labeled nanoparticles.

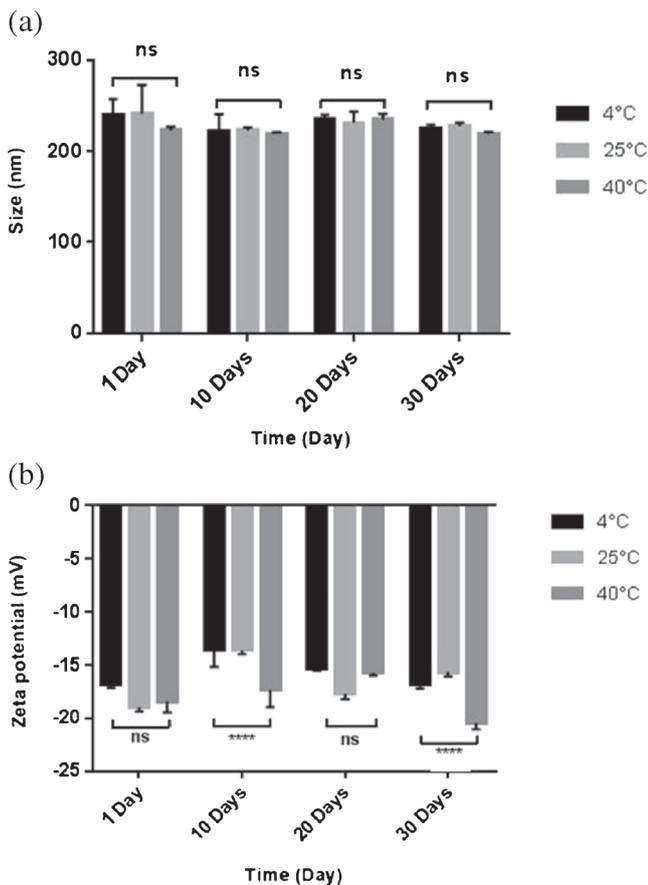


Fig. 8 Stability of PCL based nanoparticles containing IND in terms of size (a) and zeta potential (b) during one month (30 days) under three different temperatures of 4°C (black bars), RT (light gray bars) and 40°C (dark gray bars). The taken results are the average of three measurements, (ns = non-significant). Statistical analysis: $p < 0.0001$ noted ****, $p < 0.001$ noted ** and $p < 0.01$ noted *.

decrease administration frequency. Therefore, to provide topical analgesic and anti-inflammatory effect for the symptomatic treatment of inflammatory diseases, polycaprolactone based NPs loaded with IND were prepared. Indomethacin

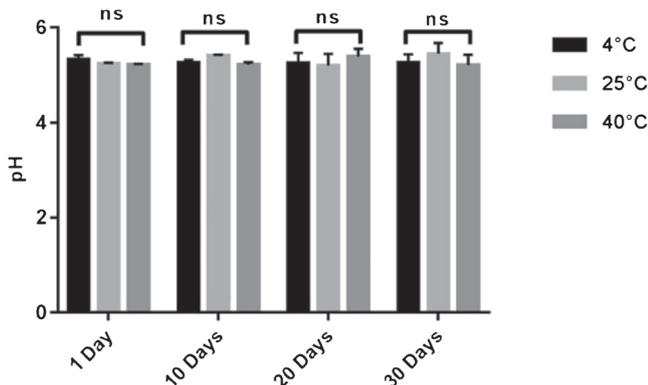


Fig. 9 Indomethacin loaded NPs pH during one month (30 days) storage under three different temperatures of 4°C (black bars), RT (light gray bars) and 40°C (dark gray bars). The represented data are the average of three measurements, (ns = non-significant).

loaded NPs are expected to be used for topical application. The zeta potential values of nanoparticles in this study were not in agreement with what was reported by Tomoda *et al.* (8) which could be attributed to the carboxylic end group of PCL. Moreover, the obtained nanoparticles size was larger than the nanoparticles size which was prepared by Tomoda *et al.* (8). However, this study pointed out that nanoparticles with a size range of 100 nm - 400 nm had the same potential of dermal penetration. In addition, Indomethacin suspension as a conventional dosage from particles had considerably larger size than the prepared nanoparticles in this study (23). However, Indomethacin suspension was intended for the ocular application. The results showed that prepared NPs were suitable in terms of size, size distribution and zeta potential. Hydrophobic nature of Indomethacin and PCL could increase the encapsulation efficiency due to affinity between these materials which is not the case in already performed research regarding Indomethacin encapsulation (8). Therefore, prepared NPs encapsulation efficiency and drug loading were considerable. The prepared NPs based on SEM evaluation, possess submicron size and smooth surfaces (see Fig. 3). According to the SEM images, some NPs had light bridges with each other which could be attributed to the PVA presence in the formulation. In fact, PVA has a sticky nature and it is crucial to completely remove it from formulation in order to have NPs clear SEM images. SEM and TEM images together shows that obtained NPs are spherical, completely separated and had smooth surfaces. After encapsulation, the common characteristic FTIR bands of both IND and PCL were appeared in Fig. 5(c) which confirms the successful encapsulation of IND in PCL based NPs. From the matching of the IND, PCL and loaded PCL based NPs spectra, it can be pointed out that the IND spectrum is present in loaded NPs spectrum in the wavelength of 1587 cm^{-1} and 839 cm^{-1} . All these results together bring us to the conclusion of the successful encapsulation of IND in PCL (24–26). Indomethacin DSC curve revealed only one endothermic peak, which is corresponding to the melting point of IND (159.90°C), the same results was found by Lin *et al.* (27). In addition, thermal behavior of blank and loaded NPs was characterized by an endothermic peak at respectively 56.65°C and 54.15°C that figure out the PCL melting point. In this work according to the thermal studies IND powder, blank and loaded NPs show a simple eutectic phase diagram. In fact, as shown in Fig. 6, upon putting IND inside of NPs its endothermic melting peak was reduced. The melting peak endothermic reduction of loaded NPs by IND presence could be thanks to either IND solubilization within carrier and/or heat induced solid state interaction as well as polymer dilution effect. DSC study allowed concluding that IND and PCL did not interact with each other which is demonstrated via FTIR analysis as well. The pH of the colloidal dispersions was stable during 30 days and it was found to be in between 4 and 6. The observed

changes in pH value during 30 days were not significant. The results showed that there was not significant change in encapsulated IND quantity during 30 days of storage. The skin penetration study of NPs has been done by CLSM on fresh excised human skins. It is indicated that NPs penetrate through skin when they are directly deposited on the surface of the *stratum corneum*. In opposite to the majority of previously performed studies on the Indomethacin cutaneous formulation, here in this work, non-invasive dermal penetration enhancement technique was employed and fresh human skin was used for *ex vivo* skin penetration capacity assessment. This mimics better physiological conditions. Consequently, neither physical nor chemical enhancement approaches was used in this study in order to prevent disruption of skin normal function. It is revealed that prepared NPs are capable of penetrating skin barrier; since dermally applied drugs are safe from the first pass effect so plasma peak concentration would be increased. Thus, needed dose of indomethacin to be taken by patient could be decreased (1–7) (28). In addition, gastric side effects due to the direct contact of Indomethacin (weak acid) with gastric mucus beside their mechanism of action can be prevented by dermal application of Indomethacin. The dermal application of such type of formulation is one of the promising options towards Indomethacin usage in therapy.

CONCLUSIONS

To conclude, PCL based particles loading IND have been prepared by nanoprecipitation technique with EE 70–76% and drug loading 14–17%. In addition, prepared NPs have a size of 220 nm – 245 nm, and Zeta potential of –13 mV up to –19 mV which could increase the permeability of IND into the skin. Furthermore, DSC and FTIR studies of NPs show that no chemical interaction between drug and polymer in the formulation has been occurred. The prepared NPs were found stable for one month under storage temperatures of 4°C, RT, and 40°C. From the other hand the pH of the obtained colloidal dispersion was at the interval of 4 and 6. This study revealed Indomethacin NPs penetration potential as a modern topical formulation which would reduce administration frequency, side effects and consecutively compliance of patients.

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III.3. Poly (ϵ -caprolactone) nanoparticles loaded with indomethacin and *Nigella Sativa* L. essential oil for the topical treatment of inflammation

Summary

The annual herbaceous plant of *Nigella Sativa* (black seed or black cumin) that belong to the Ranunculaceae family is native to Southwest Asia, North Africa, and Southern Europe. *Nigella Sativa* L. as medicinal plants is considered to be safer for treatment of various diseases. *Nigella Sativa* L. Seeds Oil was used since long time for treatment of diseases in the world. The biological properties of *Nigella Sativa* L. Seeds Essential Oil (NSSEO) have well known. Thymoquinone is the most abundant element of *Nigella Sativa* that is typically responsible of NSSEO therapeutic effects. Indomethacin as non-steroidal anti-inflammatory drug (NSAID) externally shown a satisfactory efficiency; is a highly anti-inflammatory potent drug. However, indomethacin has numerous drawbacks. To deal with these drawbacks it is important to decrease the employed indomethacin dosage and accordingly its side effects. On the other hand, indomethacin has a poor bioavailability because of its hydrophobic nature and water insolubility at skin pH. All above described reasons turned indomethacin a good applicant molecule for the encapsulation. In this research nanoprecipitation was used as the encapsulation method to design the nanoparticles and poly (ϵ -caprolactone) was chose as a polymer. Indomethacin has previously encapsulated in our already performed experiment. The presence of NSSEO in the nanoparticles formulation made this work unique. This research was targeted to extract NSSEO and elaborate nanoparticles loaded with indomethacin and NSSEO in order to boost indomethacin bioavailability and anti-inflammatory activity and to decline indomethacin taken dosage and its side effects. Firstly, Essential Oil of *Nigella Sativa* has extracted that was then encapsulated with indomethacin. Studies such as TEM, DSC and FTIR were performed to find out and confirm the structures changes and successful encapsulation of indomethacin and NSSEO accordingly. TEM analysis of obtained nanoparticles showed the spherical and regular form of designed nanoparticles, these particles revealed a good size distribution. Nanoparticles DSC and FTIR results proved the presence of indomethacin and NSSEO within nanoparticles. The size of prepared nanoparticles was found to be between (230 nm) and (260 nm) while their zeta potential was ranged from (-20 mV) up to (-30 mV). Encapsulation efficiency of designed nanoparticle for indomethacin and NSSEO were respectively 70 % and 84 %.



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Poly (ϵ -caprolactone) nanoparticles loaded with indomethacin and *Nigella Sativa* L. essential oil for the topical treatment of inflammation



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ABSTRACT

Indomethacin has a high anti-inflammatory potential and an acceptable efficiency for external application. However, indomethacin can cause several side effects. It is crucial to reduce the employed indomethacin dosage and accordingly associated side effects. Therefore, topical delivery of indomethacin is an interesting strategy. Thus, we designed poly (ϵ -caprolactone) based nanoparticles loaded with the *Nigella Sativa* L. Seeds Essential Oil (NSSEO) and indomethacin to enhance analgesic and anti-inflammatory effects of indomethacin. Nanoparticles were prepared by nanoprecipitation method. Prepared nanoparticles pre-stability study was also carried out. Nanoparticles size was ranged between 230 nm and 260 nm and zeta potential of nanoparticles was between – 20 mV and – 30 mV at pH around 6. Encapsulation efficiency of indomethacin and NSSEO within nanoparticles was respectively 70% and 84% while drug loading of indomethacin and NSSEO were 14% and 5.63% respectively. The size and zeta potential of nanoparticles was not changed significantly within 30 days of pre-stability investigation. Nanoparticles had a size in nano scale with round shape and a proper stability. Indomethacin and NSSEO were successfully encapsulated that would boost the anti-inflammatory and analgesic effects of indomethacin.

1. Introduction

Nigella sativa L. (black seed or black cummin) belong to the Ranunculaceae family. *Nigella Sativa* L. as an annual herbaceous plant is native to Southwest Asia, North Africa, and Southern Europe. In comparison with modern medicines, *Nigella Sativa* L. as other medicinal plants is considered to be safe for treatment of various diseases. Globally, *Nigella Sativa* L. seeds essential oil was used since the centuries for treatment of different diseases. *Nigella Sativa* L. Seeds Essential Oil (NSSEO) biological properties such as: anti-inflammatory, analgesic, immunomodulatory, spasmolytic, and anti-oxidant has been well studied. Therefore, the treatment of different diseases such as rheumatism, bronchitis, asthma etc., are being taken place by the NSSEO [1]. The main phytochemicals of NSSEO are thymoquinone, p-cymene, α -pinene, thymohydroquinone, dithymoquinone, linoleic acid and nigellone [2] [3]. Thymoquinone is the most abundant component of *Nigella Sativa* L., which is mostly the responsible of NSSEO therapeutic properties [4]. Based on a research, NSSEO and thymoquinone

strongly inhibited nitric oxide (NO) production and repressed NO synthase (iNOS), tumor necrosis factor (TNF)- α , cyclooxygenase (COX)-2, interleukin (IL)-6, and IL-1 β expression in lipopolysaccharide (LPS)-activated RAW264.7 cells [5]. A gold standard non-steroidal anti-inflammatory drug (NSAID) molecule named indomethacin is used to lessen fever, pain, swelling, and stiffness. In fact, indomethacin with a satisfactory efficiency for usage into the exterior part of the body; has a high anti-inflammatory potential [6] [7]. In a research it was found that Aspirin, indomethacin, and ibuprofen were more potent inhibitors of COX-1 than COX-2 [8]. Nevertheless, indomethacin could raise several side effects such as gastric ulcer, bleeding at the digestive system. Therefore, it is crucial to avoid indomethacin (weak acid) stomach mucosal contact, to reduce employed indomethacin dosage and consequently its side effects [9]. For instance it was figured out by a study that gastrointestinal (GI) tract and central nervous system (CNS) complaints were prominent after systemic administration (oral) than topical application of indomethacin. In addition, indomethacin topical administration has higher efficacy for

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athletes in superficial injuries treatment of athletes [10]. For this end, indomethacin topical delivery could be taken into account as an approach [11]. Indomethacin is unstable under UV light. Furthermore, indomethacin is poorly water soluble (~ 0.01 mg/ml) and is only soluble at basic pH (12) at which drug significantly hydrolysis take place. Therefore, indomethacin poor bioavailability can be attributed to indomethacin solubility in the skin pH [12]. These reasons together made indomethacin a good candidate for the encapsulation. Skin has been studied as an administration site of drug for its systemic effects, since systemic therapeutic agents can be delivered for long time with a controlled ratio. In addition, administered drug on the skin is escaping and safe from the liver first pass effect, which can decrease the dosage of intended drug molecule [13]. The low permeability of drug molecules through the stratum corneum has been the limiting factor toward the development of transdermal delivery system of therapeutic agents. To tackle this challenge many studies have been reported and certain cutaneous penetration improvement approaches were found out that can trigger irritation, damage and successively barrier function complaints in the skin. However, nanotechnology can improve drug skin penetration without facing skin to the above mentioned problems [14,15]. Thanks to a study it was found that PCL polymer is a biocompatible, and safe polymer for *in vivo* application [16]. In this study, in contrary to the majority of previously performed researches, firstly to figure out the effect of formulation and operating condition related parameters, a systematic study has been done [17]. Afterwards, within a separate study indomethacin has encapsulated alone into the polymeric carriers [18] in order to compare its characteristics with the formulation that contained both indomethacin and NSSEO. On the other hand, in contrary to the most of previously performed studies, here noninvasive approaches such as nanotechnology was employed. These two factors together made this study more valuable in which it was hypothesized. *Nigella Sativa* L. Seeds Oil encapsulation together with indomethacin within the polymeric nanoparticles would enhance anti-inflammatory activity of indomethacin and decrease indomethacin side effects. Therefore, here this work is focused on the design and characterization of PCL based nanoparticle loaded with indomethacin and NSSEO, for the cutaneous application.

2. Materials and methods

2.1. Materials

Poly(ϵ -caprolactone) (PCL) ($M_w = 14,000$ g/mol), polyvinyl alcohol (PVA) ($M_w = 31,000$ g/mol) and polysorbate 80 (Tween[®] 80) ($M_w = 1,310$ g/mol) were purchased from Sigma-Aldrich, Germany. Indomethacin was provided by George Van Waters and Nat Rogers laboratory products distributor (VWR), acetone was purchased from Laurylab, France. In this work, deionized water was used.

2.2. Methods

2.2.1. Plant material selection

Seeds of Moroccan *Nigella Sativa* L. (black seed) were obtained from the local market of Agadir City (Southwest of Morocco). Seeds and plants were provided following the germination of an aliquot of seeds that were taxonomically identified and authenticated and a voucher specimen was deposited at the herbarium of Faculty of Sciences, of Ibn Zohr University, Agadir, Morocco. Consecutively, seeds were carefully selected and screened manually, cleaned and washed with water to remove dust and impurities and then were immediately dried in a ventilated oven at a temperature not exceeding 40 °C. After drying, seeds were stored in a sealed jar away from light and moisture.

2.2.2. Extraction of *Nigella Sativa* L. Seeds essential oil

Dried seeds of *Nigella Sativa* L. were grounded into fine powder and immediately subjected to extraction by conventional steam diffusion

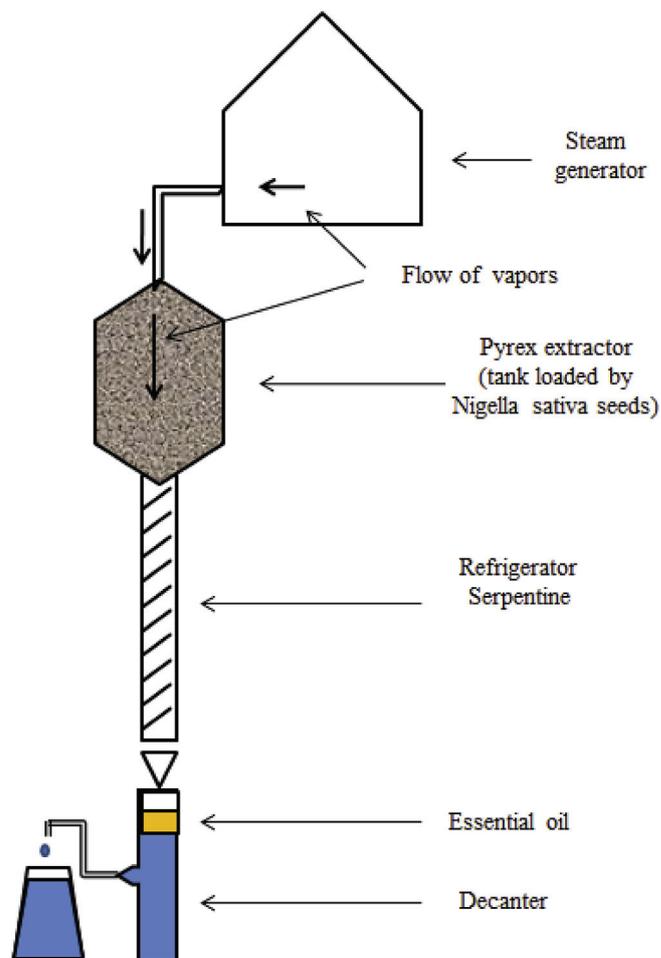


Fig. 1. Schematic diagram of steam diffusion and gravity extraction process of *Nigella sativa* L. seeds essential oil (NSSEO).

(hydrodiffusion and gravity technique) without use of microwave energy [19] [20] [21]. Briefly, 1 L Pyrex extractor (tank) was loaded with *Nigella sativa* L. seeds powder. It was then simply traversed by a continuous flow of water vapors, coming from a steam generator, at top to bottom direction. At the bottom of the extractor, a serpentine refrigerant allows the condensation of the exiting vapors of water. Finally, after passing through the serpentine refrigerant, condensed vapors were collected in a decanter, allowing the accumulation of NSSEO (see Fig. 1). The extraction was continued up to providing of a constant volume of NSSEO. Extracted essential oil was immediately dried over the anhydrous disodium sulfates and kept in an amber flask at 4 °C temperature until used.

2.2.3. Gas chromatography analysis of *Nigella Sativa* L. Seeds essential oil

Analysis of NSSEO was carried out by successive use of two kinds of gas chromatography (GC) apparatus, equipped with two types of capillary columns:

a Gaz chromatography – flame ionization detector (GC-FID)

The analysis was conducted on an Agilent 7890 GC apparatus equipped with a split/splitless injection and a FID detector. Injector and detector temperatures were both maintained at 250 °C. NSSEO 1 μ L volume was injected in split mode (1/120) firstly to the HP-1 GC column (50 m \times 0.320 mm \times 0.50 μ m) under a constant pressure (3 psi) of helium as carrier gas; applied oven temperature program was as follows: 8 min at 80 °C, increasing to 220 °C at 2 °C/min, from 220 °C to 310 °C at 10 °C/min and finally held isothermally 10 min at 310 °C. A

second injection was performed on a HP-Innowax GC column (60 m × 0.320 mm × 0.50 μm) under a constant flow of helium (1.3 mL/min rate) and the temperature program was 2 min at 60 °C temperature, and then increased to 245 °C at 2 °C/min and then held isothermally for 30 min.

b) Gas Chromatography-Mass Spectroscopy (GC-MS)

This analysis was performed on an Agilent 5975C inert XL MSD equipped with triple axis detector and an autosystem sampler operated by MSD Chemstation G1701EA software. NSSEO 0.2 μL volume was injected (in the split-ratio 1/120) and on the previous HP-1 and HP-Innowax capillary columns, under the same conditions for the GC-FID. Mass Spectroscopy (MS) energy ionization was set at 70 eV; electron ionization mass spectra were acquired in scan mode over the mass ranged 35–450 uma. Injector, interface and ion-source temperatures were kept at 250 °C degree.

c) Identification of NSSEO components

Percentages of NSSEO components were determined based on their GC-FID peak areas obtained from two capillary columns, without use of FID response factor correction. Chemical identification of NSSEO components was firstly started by comparing their GC retention indices (RI) on non-polar and polar columns (determined by linear interpolation relatively to the retention time of a series of n-alkanes (C5–C30) injected in the same conditions on the two capillary columns with that of literature data and those of the add-in-house database of the Institut des Sciences analytiques (ISA), CNRS, at Villeurbanne, of France. Secondly, chemical identification was completed and finally confirmed by computer matching with three commercial mass spectral libraries (NIST data gateway database 2005; Arômes CNRS version 2012; Wiley) and with the SCA add-in-house library.

2.3. Nanoparticles preparation

Nanoprecipitation that has developed by Fessi et al. [22], was used as a method for nanoparticles preparation in this research. A protocol, which was already provided and studied systematically throughout the blank nanoparticles preparation [17] has been employed.

2.4. Experimental protocol

The encapsulation of indomethacin and *Nigella sativa L.* seeds oil was performed in two separate phases. Indeed, to prepare organic phase, 200 mg PCL under the mild heat and magnetic stirring were dissolved in 25 ml acetone then 40 mg indomethacin and 300 mg NSSEO was also added into the solution. To provide the aqueous phase, 5 mg PVA was dissolved in 50 ml Milli-Q water with the aid of mild heat and mixed consecutively under magnetic agitation after PVA complete dissolution 135 mg Tween-80[®] was also added. Successively, organic phase containing NSSEO, indomethacin, PCL and acetone was added dropwise to the aqueous phase (see Fig. 2). Acetone evaporation was carried out afterwards by Buchi Rotavapor R-124[®] (under reduced pressure and high temperature conditions).

2.5. Nanoparticles characterization

The size, zeta potential, morphology, physical status and interaction of drug and polymer via DSC, FTIR, and pre-stability of nanoparticles were studied. Furthermore, nanoparticles, fluorescence microscopy, and encapsulation efficiency were also carried out.

2.5.1. Particle size and zeta potential

For the measurement of nanoparticles size and zeta potential, Malvern particle size analyzer using dynamic light scattering (Zetasizer

- Nano ZS, Malvern instruments limited, UK) was employed. Nanoparticles were dispersed in 1 mM NaCl solution previous to each zeta potential measurement in order to provide a conducting medium for electrophoresis mobility. However, nanoparticles size measurement was taken place after colloidal dispersion dilution within a 1 mL of distilled water. All measurements were carried out in triplicate at room temperature (25 °C) and their average was taken as the result.

2.5.2. Fluorescence microscopy study

Fluorescent images were taken using a fluorescence microscope (Zeiss Axioplan 2 Imaging apparatus, equipped with 10 × and 40 × lenses and a monochrome camera). To qualitatively assess NSSEO encapsulation in PCL based nanoparticles, fluorescence microscopy was utilized. Thus, prior to the encapsulation, fluorescent agent named quantum dots (QDs-PPs) was added into the organic phase of both formulations in order to visualize the encapsulated NSSEO in nanoparticles during observation. Then, a droplet of 5 μL from each samples (nanoparticles with and without NSSEO) was deposited onto glass slide, dried at room temperature and observed by fluorescence light. Prepared samples were excited firstly with a 550 (± 25) nm band-pass filter (green light) and fluorescence from the sample (red color) was observed with a 605 (± 70) nm band-pass filter. The fluorescence of obtained images using the same focus was compared.

2.5.3. Encapsulation efficiency (EE)

To measure the encapsulation efficiency % and drug loading %, encapsulated indomethacin and NSSEO into the carriers should be separated within the colloidal dispersion. Therefore, provided dispersion was centrifuged at 14,000 round per minute (rpm) for 40 min. As obtained nanoparticles containing NSSEO and indomethacin, thus for determination of nanoparticles EE %, both encapsulated NSSEO and indomethacin were quantified separately. The loaded indomethacin and NSSEO quantity in nanoparticles was respectively determined by high performance liquid chromatography (HPLC) and gas chromatography (GC).

2.5.3.1. a). Indomethacin determination. In first time, indomethacin amount was determined by High Performance Liquid chromatography (Agilent 1200 series) following the validation of method, which was proposed by Kwong et al., in 1981. However, proposed method was modified in terms of mobile phase composition and UV- wavelength. A Kinetex C 18 column (4, 6 × 100 mm, 2.6 μ particle size) was used and the mobile phase composed from methanol and water (55:45% v/v). The conditions were such as flow of mobile phase (1 ml/min), volume of injected sample (5 μl), fixed UV wavelength for indomethacin detection (230 nm). Indomethacin stock solution (2000 μg/ml) was prepared in a mixture of methanol and acetonitrile (75:25). To determine the EE %, direct method was employed and colloids dispersion was centrifuged at a speed of 14000 rpm for 40 min. Supernatant was removed by micropipette and the quantity of encapsulated indomethacin was measured following total nanoparticles dissolution in methanol and acetonitrile. Then, it was filtered and transferred to the vial of HPLC. As shown in the following equation obtained nanoparticle EE %, was determined based on the ratio of initial drug quantity and drug encapsulated into the nanoparticles.

2.5.3.2. b). *Nigella sativa L.* Seeds essential oil determination. Since thymoquinone play a principal role to exercise anti-inflammatory activity within NSSEO. Therefore, for NSSEO EE % determination, quantification of thymoquinone was carried out by GC equipped with flame ionization detector (GC-FID). Shimadzu GC 2010 plus equipped with split/splitless injector and capillary column Equity-5[™] (30 m × 0.25 mm, 0.25 μm) that was already conditioned at 280 °C for 30 min, was used for the analysis. The carrier gas was helium (He) at a constant flow rate of 1 mL/min with a pressure of 65.3 kPa while H2

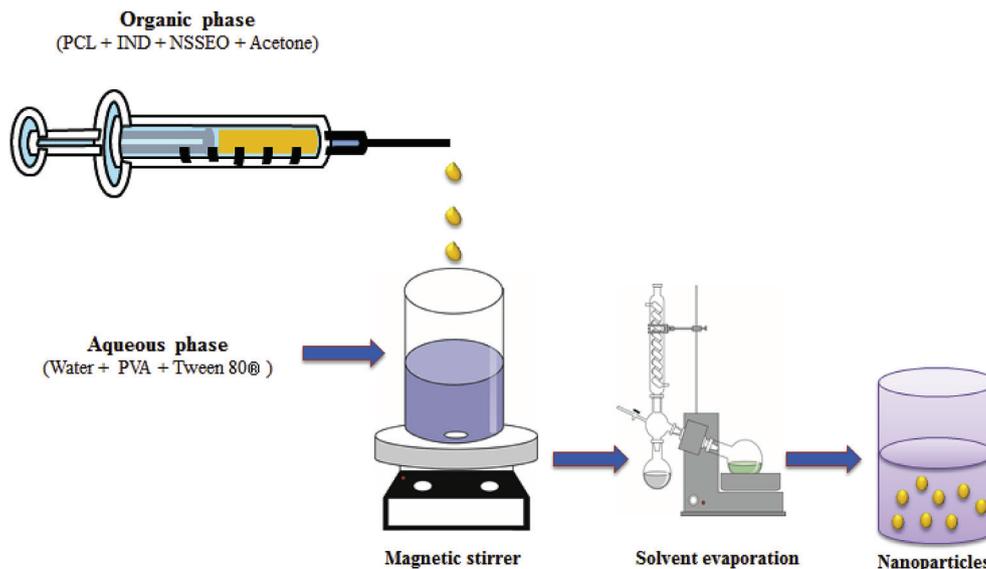


Fig. 2. Illustration of nanoparticles preparation methodology.

Flow was 30/30 ml/min and the air flow was 300/300 ml/min. The FID detector temperature was maintained at 300 °C and injected sample volume was 2 µL. For analysis, the oven temperature was increased from 100 °C to 190 °C at 2.5 °C/min, the total program time was 35 min.

To determine the EE % and drug loading % the following equations were employed:

$$\text{Encapsulation efficiency \%} = \frac{\text{Amount of encapsulated drug}}{\text{Initial amount of drug used in formula}} \times 100 \quad (1)$$

$$\text{Drug loading \%} = \frac{\text{Added drug} - \text{Free (Untrapped drug)}}{\text{Polymer amount}} \times 100 \quad (2)$$

In order to measure the EE % and drug loading % of NSSEO in nanoparticles, precipitated nanoparticles suite to the centrifugation was dissolved in acetone. Drug loading has defined as the ratio of encapsulated drug quantity vice-versa to the used polymer amount. Prior to determination of nanoparticles EE %, both analytical methods were validated in terms of linearity, precision, specificity and accuracy.

2.5.4. Nanoparticles morphology

To study nanoparticles shape and appearance, Transmission Electron Microscopy (TEM) was employed. Nanoparticles TEM has been carried out by Philips CM-120 Transmission electron microscope (CMEABG, Claude Bernard University Lyon 1, France) by 120 kV accelerating voltage. For this aim a drop of nanoparticles suspension was diluted in 2 ml of *Milli-Q water* and consecutively one drop of this dilution was placed on the carbon-coated copper grid. Supplementary amount of nanoparticles suspension was removed by blotting the grid through filter paper and instilled nanoparticles (NPs) suspension on the grid dried prior to TEM analysis at room temperature.

2.5.5. Fourier transform infrared spectroscopy (FTIR) study

To evaluate the encapsulation and distribution of indomethacin and NSSEO in nanoparticles, FTIR analysis was done on Thermo Scientific Technologies FTIR spectrometer with IR MONIC Solution software. For samples preparation a volume of colloidal dispersion was taken and centrifuged under the speed of 14000 rpm for 40 min. Consecutively, supernatant was removed and the precipitated part was dried at room temperature after 72 h. In this study, to prepare samples no initial preparation was taken place and samples FTIR spectrum was reported between 650 cm⁻¹ and 4000 cm⁻¹. Prior to each acquisition blank background had been adjusted. Samples for this study were: pure

Nigella Sativa L. Seeds Essential Oil, pure indomethacin, Blank NPs and NPs loaded with indomethacin and NSSEO.

2.5.6. Differential scanning calorimeter (DSC) study

In order to study the physical status and interaction of drug and polymer within the formulation, DSC analysis was carried out. Therefore, centrifugation was taken place on the colloidal dispersion. Following of supernatant phase removal, precipitated nanoparticles have dried in a hold and subsequently exposed to the thermal study. Samples for this study were including drug loaded NPs, blank NPs, indomethacin and NSSEO. To have a control, indomethacin, NSSEO, and polycaprolactone DSC scans were used. Differential scanning calorimeter Q200 (TA instruments, USA) has employed for the study. For all measurements, a nitrogen purge of 50 mL/min was utilized. The temperature range was 20–300 °C and the heating rate was 10 °C/min. The thermal analysis was done by heating of approximately 10 mg samples in a covered hermetic aluminum alloy sample pan under dry nitrogen atmosphere.

2.5.7. Pre-stability study of nanoparticles

The pre-stability of nanoparticles from physicochemical points of view was studied via colloidal dispersion EE % and pH measurement. In addition, for pre-stability study of nanoparticles from physical points of view the size, and zeta potential of prepared nanoparticles was measured as well. In fact, to evaluate the pre-stability of encapsulated indomethacin beside dispersed nanoparticles, the EE % of nanoparticles was determined once upon nanoparticles preparation and once after one month of preparation in four different temperatures. All measurements were carried out after each 10 days within one month under three different conditions (4 °C, 25 °C and 40 °C temperature degrees). However, EE % was just determined after preparation of nanoparticles and at the end of the study period (after one month).

2.5.8. Statistical analysis

The data are expressed as mean ± standard deviation and analysis was carried out statistically by GraphPad Prism 7.0 software. The analysis of data was taken place via one-way analysis of variance (ANOVA). The criteria for statistical significance were p < 0.0001 (***), p < 0.001 (**), and p < 0.01 (*) and P-value less than p < 0.01 considered significant.

3. Results and discussion

In this study efforts were made to extract NSSEO and to encapsulate the extracted NSSEO within the polymeric nanoparticles together with indomethacin.

3.1. Extraction of *Nigella Sativa L. Seeds essential oil (NSSEO)*

The hydro-diffusion method was chosen for the extraction [19,20] due to its performances compared to classic methods such as hydro-distillation. This technique is based on the same principles of steam distillation except that plant material (the ground seeds) is crossed from top to bottom by a continuous flow of water vapors coming from a steam generator. Under the effect of this descending flow of vapors, there is an instantaneous and rapid release of the volatile substances, which remain trapped initially in the plant, resulting in an acceleration of the extraction process that is also facilitated by the effect of gravity [19]. Grinding of *Nigella Sativa L.* seeds will facilitate release and extraction of volatile compounds that may be imprisoned inside plant.

3.2. Gas chromatography analysis

Gas chromatography is the technique of choice for the analysis and chemical identification of essential oils. In this work, two complementary techniques for the chemical characterization of *Nigella Sativa L.* essential oil components, namely gas chromatography coupled to the flame ionization detector (GC-FID) and gas chromatography coupled to the mass spectrum detector (GC-MSD) were used. Two different types of capillary columns were used as well: HP-1 of apolar nature and the polar HP-Innowax one in order to facilitate the separation of different constituents and to resolve the problem of co-elution of some compounds for a better and exact identification of the chemical composition. We have already investigated on these two complementary techniques of gas chromatography to study the chemical composition of several essential oils [23,24]. By combining the results obtained by GC-FID and GC-MSD and based on the reference data relating to the retention indexes of the various volatile compounds [25–27] and respectively Nist Data Gateway Libraries, the add-in-house database of the CNRS, ISA- Villeurbanne related to Retention indexes and data from data bank mass spectrum (respectively Nist 2005; Arômes CNRS version 2012 and Wiley libraries). The following results related to the constituents of NSSEO were achieved (Table 1).

NB: Percentages of individual compounds were calculated based on the GC-FID peak area obtained after separations on apolar and polar columns. RRIa/p show retention indices on apolar and polar columns, respectively, RIA and RIP, indicate the retention indices obtained experimentally on the HP-1 apolar and HP-Innowax polar column, determined relatively to the retention times of C5–C30 n-alkanes injected in the same conditions.

This investigation that was carried out by GC-FID and GC-SM has enabled us to determine the contents of more than 25 different terpenic compounds, which are hydrocarbons (monoterpenes and sesquiterpenes) or oxygenated hydrocarbons and have different functional groups (alcohol, esters, ketones, ethers or phenols). The essential oil contains various potentially active terpenic compounds that exhibit important biological activities. Moreover, the content of monoterpene hydrocarbons is the highest (84.70%) with *para*-cymene as the major compound (56.67%) followed by *alpha*-thujene (16.35%) and *alpha* and *beta*-pinene with a content of (3.58%) and a quantity of phenolic compounds (about 9.15%). Previously carried out studies confirm that gamma terpinene is the biochemical precursor of *para*-cymene, which is the precursor of thymol [28] while, thymol itself is the precursor of thymoquinone. In fact, thymoquinone and thymohydroquinone are among the main compounds in the essential oil of *Nigella Sativa L.* that has shown too crucial biological and pharmacological activities. Clinical studies proved that NSSEO and its components exhibit anti-

Table 1

Nigella sativa L. essential oil chemical composition as determined by gas chromatography - flame ionization detector (GC - FID) and gas chromatography - mass spectrometry (GC-MS).

N°	Compound	RRI ap/p	RI ap	RI p	Content (%)
1	α -Thujene	932/1023	925	1021	16.35
2	α - Pinene	936/1022	934	1025	3.58
3	Camphene	950/1066	949	1070	0.054
4	Sabinene	974/1120	970	1125	1.49
5	β - Pinene	978/1110	976	1113	3.58
6	Myrcene	987/1159	986	1166	0.02
7	<i>p</i> - cymene	1015/1268	1020	1275	56.67
8	Limonene	1025/1199	1024	1205	2.41
9	1,8-Cineole	1025/1224	1028	1214	0.05
10	γ - Terpinene	1051/1243	1049	1247	0.46
11	<i>trans</i> - Thujanol-4	1068/1465	1065	1469	0.03
12	<i>para</i> - cymenene	1079/1452	1075	1447	0.05
13	Fenchone	1080/1402	1078	1410	0.02
14	Terpinolène	1082/1285	1081	1289	0.03
	Unidentified	-	-	-	0.82
15	β - Thujone	1108/1451	1103	1457	0.02
	Unidentified	-	-	-	4.71
16	Camphor	1122/1521	1123	1517	0.045
17	Terpinene-4-ol	1160/1600	1178	1610	0.31
	Unidentified	-	-	-	0.37
18	Thymoquinone	1244/nf	1240	1249	4.47
19	<i>E</i> - Anethole	1279/1845	1275	1847	0.056
20	Bornyl acetate	1283/1575	1281	1578	0.12
21	Carvacrol	1288/2219	1287	2227	1.68
22	Eugenol	1344/2155	1341	2165	0.6
23	α - Longipinene	1350/1482	1351	1476	0.16
24	Longifolene	1400/1574	1398	1583	0.79
25	(<i>E</i>) β - Caryophyllene	1415/1587	1422	1606	0.085
26	2-Tridecanone	1478/1806	1487	1810	0.034
27	Eugenyl acetate	1486/2263	1485	2273	0.029
28	Thymohydroquinone	1554/2178	1553	2193	0.31
30	Total				99.40%

Monoterpene hydrocarbons: 84.694%, Sesquiterpene hydrocarbons: 1.035%, Total Oxygenated compound compounds: 7.729% and unknowns: 5.9%.

inflammatory, antioxidant, antibacterial, antiproliferative, apoptotic, antitumor, antiepileptic and antidiabetic properties [29].

3.3. Nanoparticles characterization

3.3.1. Particle size and zeta potential

The size and zeta potential of prepared nanoparticles were measured by Malvern particle size analyzer using dynamic light scattering (Zetasizer –Nano ZS, Malvern instruments limited, UK). Pre-stability study of colloidal dispersion was carried out via prepared nanoparticles size and zeta potential evaluation. In fact, previous to each zeta potential measurement, nanoparticles were dispersed in 1 mM NaCl solution, pH was 5. However, to measure the size of prepared nanoparticles, colloidal dispersion was diluted in 1 mL of distilled water before to each measurement. As can be seen in Fig. 3 and Fig. 8, nanoparticles size was $230\text{--}260 \pm 12.47$ nm and zeta potential of nanoparticles was between (-20 mV) and (-30 mV) ± 4.082 . All aforementioned measurements were performed in triplicate at room temperature (25 °C). The stability within this formulation is due to the PVA (stabilizer) presence that provides depletion stabilization for the obtained colloidal dispersion. Indeed, nanoparticles negative zeta potential can be attributed to the used polymer (PCL) carboxylic group. In addition, colloidal dispersion pH was about 6 ± 0.82 (see Fig. 9).

3.3.2. Fluorescence microscopy study

To qualitatively observe the encapsulation of NSSEO, fluorescence microscopy was used. Therefore, prior to encapsulation fluorescent agent was added into the NSSEO in order to visualize encapsulated NSSEO within nanoparticles during observation by fluorescence microscopy (see Fig. 4).

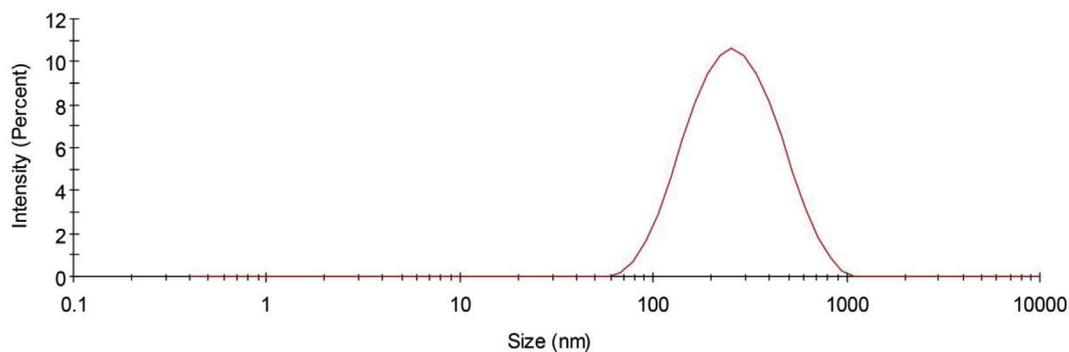


Fig. 3. Nanoparticles size and size distribution by intensity.

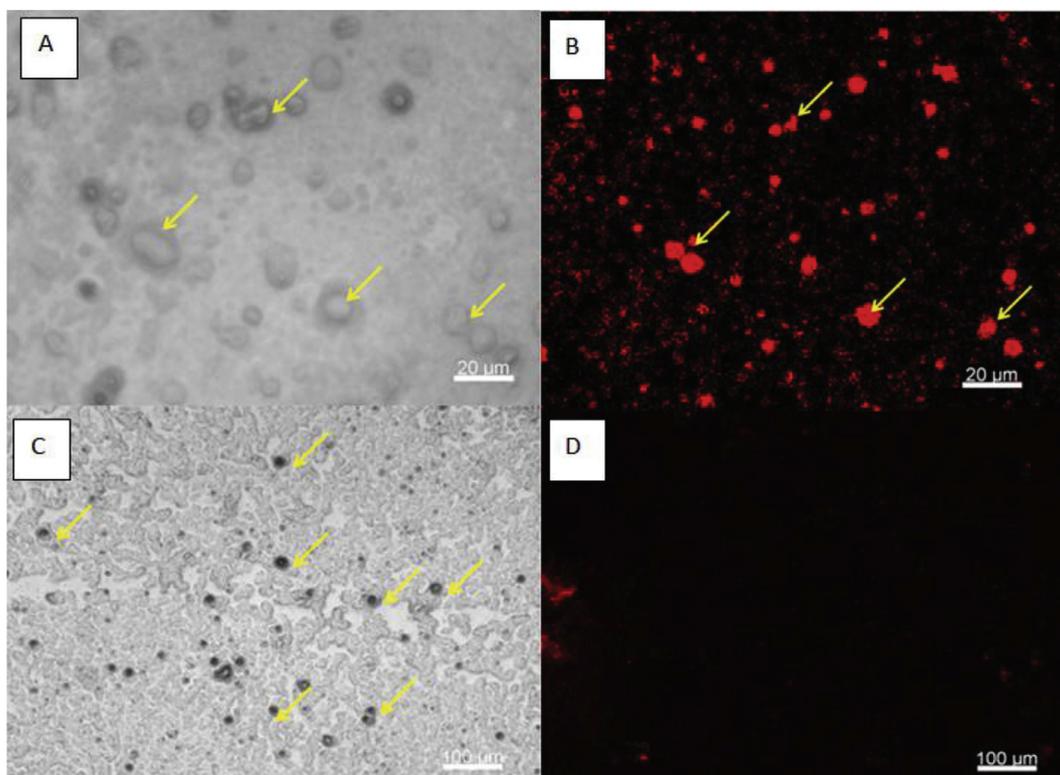


Fig. 4. Fluorescence microscope images of fluorescent loaded nanoparticles (A). *Nigella sativa* L. Seeds essential oil and indomethacin loaded nanoparticles with white light (B). *Nigella sativa* seeds essential oil and indomethacin loaded nanoparticles with green light (C). Indomethacin loaded nanoparticles with white light (D). Indomethacin loaded nanoparticles with green light.

NB: The fluorescent agent was added in the organic phase to visualize loaded NSSEO within the nanoparticles under light during study with fluoresce microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

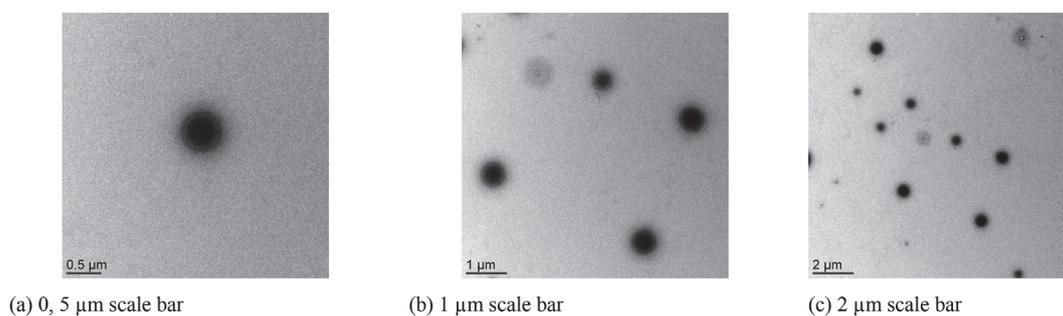


Fig. 5. Transmission Electron Microscopy images of PCL based nanoparticles loaded with indomethacin and NSSEO, (a). 0.5 µm scale bar, (b). 1 µm scale bar, and (c). 2 µm scale bar.

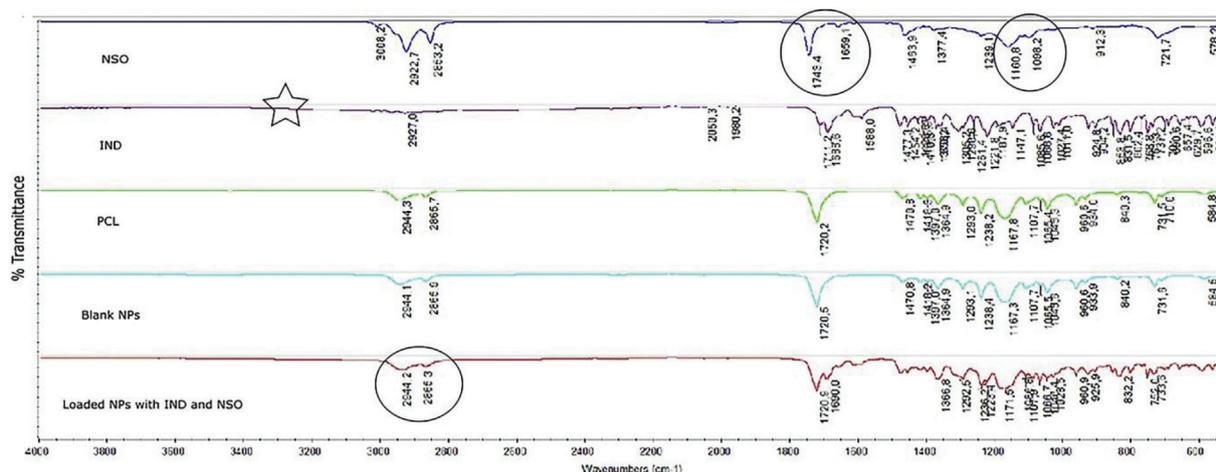


Fig. 6. Fourier transformed infrared spectroscopy (FTIR) spectrum noted between 650 cm^{-1} and 4000 cm^{-1} . NSO: Nigella Seeds Essential Oil, IND: Indomethacin, PCL: Poly (ϵ -caprolactone), Blank NPs: Unloaded nanoparticles, Loaded NPs with IND and NSO: nanoparticles containing Indomethacin and *Nigella Sativa L.* Seeds Essential Oil.

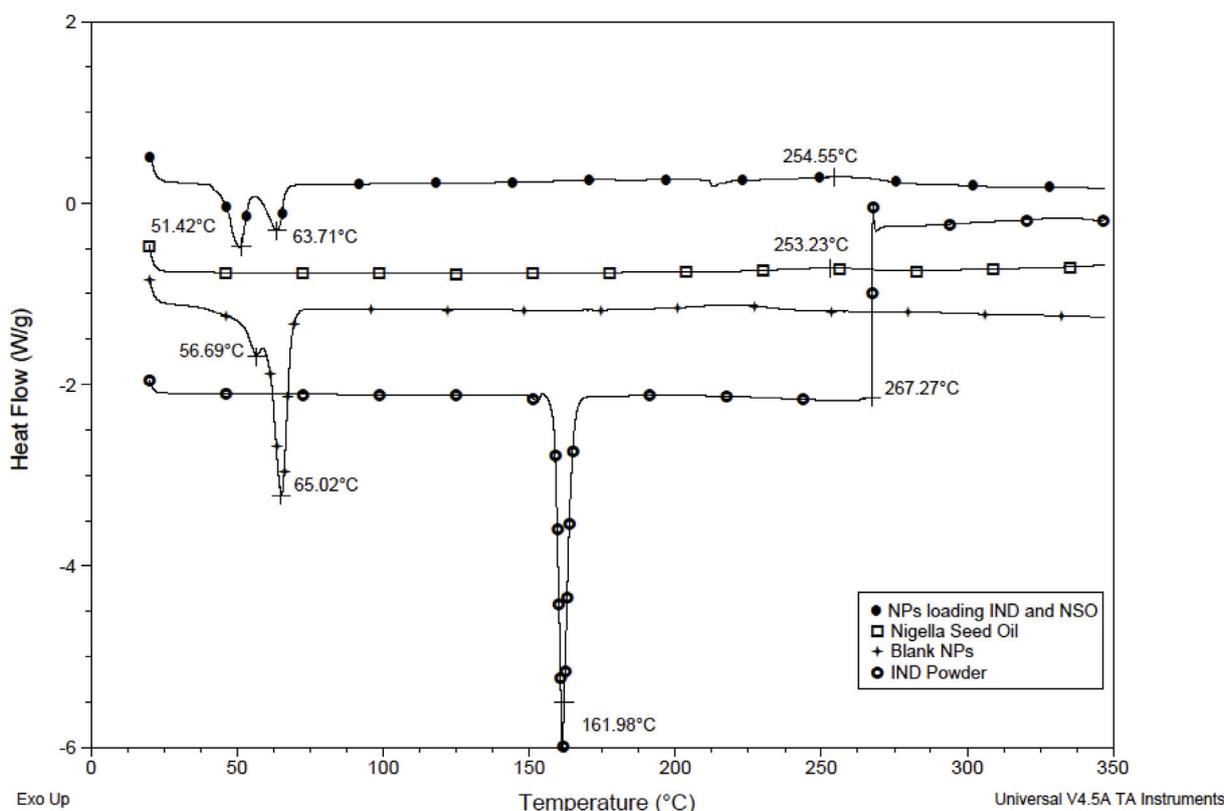


Fig. 7. Blank nanoparticles, nanoparticles loaded with indomethacin and NSSEO, indomethacin, and NSSEO DSC thermograms.

Fig. 4A shows NSSEO encapsulation inside of particles under white light. Then, band-pass filter was changed to 550 (± 25) nm filters while keeping the same focus. The designed particles were observed with high intensity indicating the encapsulation of NSSEO within nanoparticles. The same optical characterization was made for using only nanoparticles without NSSEO (see Fig. 4C). Here nanoparticles were well presented and were observed under white light. When the filter was changed to 550nm, there was no fluorescence observed (see Fig. 4D) which confirm NSSEO successful encapsulation.

3.3.3. Encapsulation efficiency

Encapsulation efficiency of indomethacin and NSSEO were

respectively 70% and 84%. Encapsulation efficiency of NSSEO of our work was higher than the study that was done by Rushmi et al. [30]. The drug loading of indomethacin and NSSEO were 14% and 5.63% respectively.

3.3.4. Nanoparticles morphology

In order to study the morphology of provided nanoparticles, Transmission Electron Microscopy (TEM) analysis was carried out. TEM images showed that nanoparticles have a spherical and regular form (See Fig. 5).

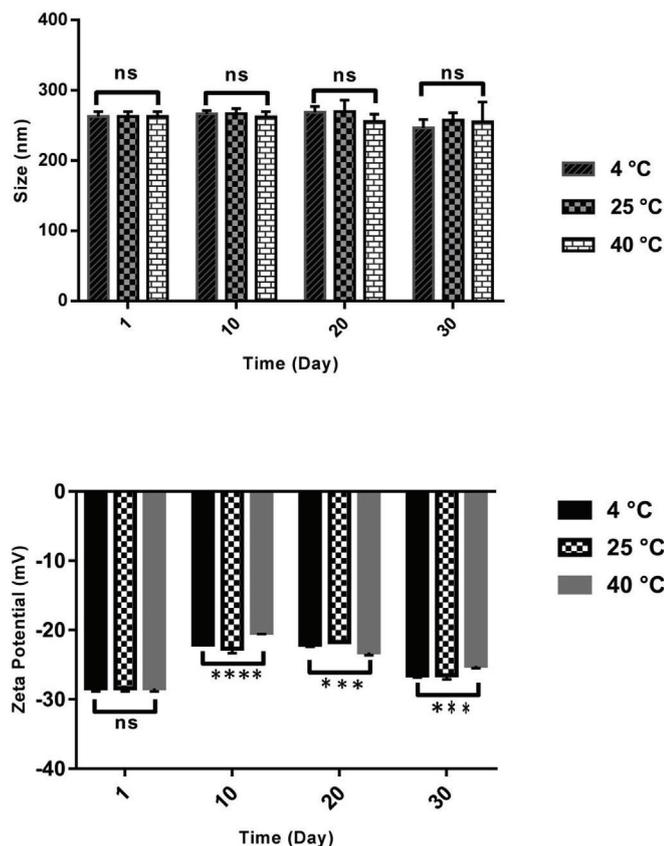


Fig. 8. Indomethacin and *Nigella Sativa* L. Seeds essential oil loaded NPs stability during one month (30 days) storage in three different temperatures of 4 °C, 25 °C and 40 °C. P-value less than 0.01 was considered significant noted *, P < 0.001 noted *** and P < 0.001 noted ****. The shown data are the average of three measurements, (ns = non-significant).

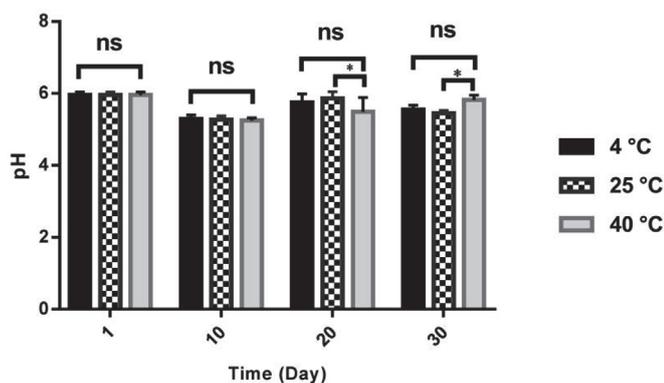


Fig. 9. Indomethacin and *Nigella Sativa* L. seeds essential oil loaded NPs pH through one month (30 days) storage in three different temperatures of 4 °C (black bars), 25 °C (checked bars) and 40 °C (light gray bars). The shown data are the average of three measurements, (ns = non-significant). P-value less than 0.01 was considered significant noted *, P < 0.001 and noted **.

3.3.5. Fourier transform infrared spectroscopy (FTIR) study

To chemically define the prepared nanoparticles, indomethacin, NSSEO and polymer, Fourier transform infrared spectroscopy analysis was performed. Generally, drug-polymer interaction leads to identifiable changes in FTIR spectra [31]. The FTIR patterns of NSSEO, indomethacin, PCL, blank and loaded NPs were shown in Fig. 6. Indeed, indomethacin characteristic bands presented free acid carbonyl groups (C=O) stretching band at 1711 cm^{-1} , C–Cl stretch at 839 cm^{-1} , C=O amide at 1688 cm^{-1} , and phenolic carbon stretching vibration (C=C

stretch vibration) at 1587 cm^{-1} . Additionally, PCL characteristics bands revealed at 2865 and 2942 cm^{-1} that is respectively attributed to symmetric and asymmetric aliphatic stretching (Fig. 6). Moreover, carbonyl (C=O) strong characteristic stretching band presented at 1721 cm^{-1} and PCL C–O and C–C stretching vibration bands revealed at 1292.9 cm^{-1} . Furthermore, vibration bands of symmetric and asymmetric C–O–C seemed at 1162 cm^{-1} . The C–O stretching vibration band of NSSEO revealed at the intervals of 1749–1659 cm^{-1} and 1128–1084 cm^{-1} that is in accordance with what was reported by Abdul Rohman and Rizka Ariani [32,33]. In addition, the aliphatic stretching vibration band of NSSEO appeared at 2929–2863 cm^{-1} . As can be seen in Fig. 6, FTIR bands of indomethacin, NSSEO and PCL all together were appeared in the same spectra following encapsulation. Based on matching of indomethacin, PCL, and loaded PCL based nanoparticles spectra, it could be draw a conclusion that NSSEO and indomethacin were successfully encapsulated within polymeric nanoparticles [34–37].

3.3.6. Differential scanning calorimeter (DSC) study

To describe the physical status of encapsulated compounds and polymer DSC analysis was performed. Thermograms of blank nanoparticles, nanoparticles loaded with indomethacin and NSSEO, indomethacin, and NSSEO alone are presented in Fig. 7. The thermograms of blank nanoparticles and loaded nanoparticles are matching with the endothermic peaks at 56.69 °C and 51.42 °C respectively that correspond to the used polymer (PCL) melting point. Indomethacin endothermic peak at 161.98 °C that relate to the loss of crystalline water was not appear neither in blank nanoparticles thermogram nor in loaded nanoparticles thermogram. In this case, indomethacin is dispersed homogeneously throughout the polymeric matrix [38]. As indomethacin and NSSEO are both hydrophobic, thus indomethacin and NSSEO could disperse along polymeric matrix. Therefore, the drawn conclusion by Yadav and Sawant looks more feasible in this research [38]. However, the endothermic peak of NSSEO at 253.23 °C was slightly appeared within loaded nanoparticles thermogram that figures out the encapsulation of NSSEO as well (See Fig. 7).

From the comparison of DSC curves obtained from blank nanoparticles, NSSEO, indomethacin, nanoparticles containing indomethacin and NSSEO, it can be easily figured out that NSSEO is successfully encapsulated within polymeric nanoparticles.

3.3.7. Pre-stability study of nanoparticles

Nanoparticles pre-stability study was performed by physical and physicochemical points of view through the measurement of the prepared nanoparticles size, zeta potential, pH, and encapsulated indomethacin and NSSEO stability within one month. In fact, measurement of these criteria was performing after each 10 days for a period of 30 days of storage under 3 different temperatures (4 °C, 25 °C and 40 °C) condition. The surface charge of nanoparticles can be influenced by factors such as: used polymer, and surfactant chemical structure and pH of medium [39].

As depicted in Fig. 8 (a), the size of prepared nanoparticles was not changed significantly within 30 days of stability investigation (change interval was 250–270 \pm 12.47 nm). Furthermore, zeta potential of prepared nanoparticles throughout the period of stability study was changed from (–22 mV) up to (–28 mV) \pm 4.082 at pH around 6 \pm 0.82 that was non-significant as well.

Throughout 30 days of the stability study, pH of colloidal dispersion was stable, which was ranged between 5 and 6. The changes in pH values during this study were non-significant. In addition, for pre-stability study of encapsulated indomethacin and NSSEO within nanoparticles, encapsulation efficiency of prepared nanoparticles was determined at the end of study period (one month) and it was the same (69.5% and 83.3% respectively).

4. Conclusion

The essential oil of *Nigella Sativa* L. was effectively extracted and analyzed. Indomethacin and NSSEO were successfully encapsulated within poly (ϵ -caprolactone) polymer by nanoprecipitation method. Designed nanoparticles were characterized by size and zeta potential measurement and pre-stability study. Furthermore, prepared nanoparticles were also characterized by fluorescent microscopy, TEM, FTIR, DSC techniques that all together confirm the encapsulation of indomethacin and NSSEO. The size and zeta potential of prepared particles were respectively $(230\text{--}260 \pm 12.47 \text{ nm})$ and (-20 mV) up to $(-30 \text{ mV}) \pm 4.082$. Prepared nanoparticles TEM images shown that designed nanoparticles have a spherical and regular form. Encapsulation efficiency of nanoparticles for indomethacin and NSSEO were correspondingly 70% and 84%. In addition, drug loading of designed nanoparticles for indomethacin and NSSEO was 14% and 5.63% respectively. In brief, poly (ϵ -caprolactone) based nanoparticles were elaborated that could be loaded successfully with indomethacin and NSSEO. Such prepared particles as noninvasive penetration enhancement approach can improve skin penetration, reduce systemic concentration and side effects of indomethacin accordingly. To confirm this, further studies are required to perform.

Declaration of interest

No conflicts of interest is declared by authors.

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III.4. Topical co-delivery of indomethacin and *Nigella Sativa L. Essential oil* in poly- ϵ -caprolactone nanoparticles: *In Vivo* study of anti-inflammatory activity

Summary

Inflammation protects the body from the aggressive agents and other injuries via a natural defense cascade that include different interactions between cells and mediators. The signs of inflammation are: elevated blood flow, increased cellular metabolism, cellular influx, soluble mediators release, vasodilation and fluids extravasation. Acute inflammation is the first reaction which can be described through the raised plasma movement and indigenous immune cells as neutrophils and macrophage from the blood into the damaged tissues. In fact, inflammatory agents application cause cell membranes phospholipase A₂ activation that would trigger the release of arachidonic acid and inflammatory mediators that make easy the immigration of leukocytes to the inflammation site. Inflammation first phase differentiates itself by the release of products such as histamine, bradykinin, serotonin, and cyclooxygenase (COX) (0-1 h); whereas the late phase of edema can be known by prostaglandins release, oxygen-derived free radicals production and polymorphonuclear leukocytes (PMN) infiltration. Indomethacin as a potent Non-Steroidal Anti-inflammatory Drugs (NSAIDs) revealed adequate efficiency for the external application to decrease the fever, pain and inflammation. Indomethacin induces side effects within digestive tube that are related to its mechanism of action. The gastrointestinal ulcers or bleeding can be triggered or intensified by indomethacin usage. Indeed, indomethacin side effects can be reduced by decreasing of taken amount of drug and targeting drug delivery to the interest area. Skin could be used as an administration rout to control the drug delivery and to decrease the dosage of indomethacin. Nanoparticles in comparison with the other skin drug delivery approaches can deliver drugs without any skin function disturbance. Thus, it is possible to grasp this goal by nanoparticles application on the skin. Nanotechnology based drug delivery has attracted the attention since long time. Certain drugs drawbacks related to the bioavailability, stability, and organoleptic can be handled with the encapsulation. From the other hand, since long time *Nigella Sativa* Seeds Essential Oil (NSSEO) was used for the treatment of different diseases. Due to the biological properties of NSSEO, it is well known. The main phytochemicals of NSSEO are including thymoquinone, p-cymene, α -pinene, thymohydroquinone, dithymoquinone and nigellone. In fact, thymoquinone formed the main part of NSSEO to which mostly attributed NSSEO therapeutic usages. In addition, percutaneous absorption can be enhanced by NSSEO. In our previous experiment, poly(ϵ -caprolactone) based nanoparticles loaded with indomethacin and NSSEO were prepared by nanoprecipitation method and then characterized in terms of TEM, encapsulation efficiency, size, zeta potential, DSC, FTIR and skin penetration by Confocal Laser Scanning Microscopy (CLSM). Here in this study the *in vivo* anti-inflammatory activity of those nanoparticles was investigated. In fact, four formulations such as: indomethacin (1 %) gel, blank nanoparticles, nanoparticles load with indomethacin alone, and nanoparticles loaded with indomethacin and NSSEO were prepared. To this end, edema was induced in mice ears by xylene; consequently, the anti-inflammatory activity was studied in terms of mice ears thickness (μm) and weight (mg) measurement

that were compared to the positive control and pre-injection values. In addition, to assess and quantify the inflammatory inhibition, histology microscopic analysis and immunohistochemistry study were performed. Based on the found data, up to one hour prepared formulations application no significant differences were observed in mice ears thickness at the start of experiment. However, in 1st hour, mice ear thickness was decreased significantly via indomethacin gel whereas the thickness was stable for three other formulations. Moreover, in 2nd hour the thickness reduction by nanoparticles containing indomethacin and *NSSEO* in comparison with the blank nanoparticles was significant that was less than indomethacin gel. From the other hand in 3rd hour, thickness depression by nanoparticles loaded with indomethacin and *NSSEO* was highly significant in comparison with indomethacin gel. Furthermore, in 6th and 8th hours the declined thickness by nanoparticles loaded with indomethacin and *NSSEO* together, nanoparticles loaded with indomethacin alone and indomethacin gel was respectively significant. This inflammatory inhibition of nanoparticles containing indomethacin and *NSSEO* has reached its peak 8 h after xylene application. According to the found results the anti-inflammatory activity was time dependent. The left ear of mice within all four groups was considered as negative control. Prepared nanoparticles loaded with indomethacin and *NSSEO* had better skin penetration than nanoparticles loaded with indomethacin alone. These results proved that *NSSEO* possesses a significant anti-inflammatory activity and can be used as an enhancer for indomethacin anti-inflammatory activity in a topical application within the nanoparticles.



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RESEARCH ARTICLE

TOPICAL CO-DELIVERY OF INDOMETHACIN AND *NIGELLA SATIVA L. ESSENTIAL OIL* IN POLY- ϵ -CAPROLACTONE NANOPARTICLES: *IN VIVO* STUDY OF ANTI-INFLAMMATORY ACTIVITY.

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nanoparticles; anti-inflammatory

Abstract

Indomethacin is a potent, nonselective Non-steroidal Anti-inflammatory Drug (NSAID) but its low water-solubility precludes its use as topical dosage form. As with other NSAIDs, the systemic delivery is associated with high risk of serious gastrointestinal adverse events including bleeding, ulceration and perforation of stomach and intestines. Here we demonstrate a safer way of administration i.e via topical demonstrating synergistic effects when co-delivered with *Nigella sativa L.* seeds essential oil (NSSEO) in the form of co-encapsulated particles (~200 nm) of poly- ϵ -caprolactone. The particles showed penetrability across stratum corneum to dermis layer in *ex-vivo* human skin. Further study in the xyline-induced ear edema in mice was performed, and co-encapsulated particles demonstrated highest anti-inflammatory effect compared to indomethacin particles and indomethacin gels. Despite slower onset compared to indomethacin gels, the inflamed ear continued to show reduction in thickness over 8 hours of observation demonstrating synergistic and pro-longed effect contributed by NSSEO. In immunohistochemistry study of CD45+, the mice ears treated with co-encapsulated particles showed considerable reduction in lesions, epidermal-dermal separation and inflammatory cells (lymphocytes and neutrophils) infiltration as compared to other formulation. Based on microscopic evaluation, the anti-inflammatory inhibition effect of co-encapsulated particles is the highest (90%) followed by indomethacin particles (79%) and indomethacin gel (49%). The findings suggest not only skin permeability of indomethacin significantly improved but also the therapeutic effects, all provided by the presence of NSSEO in the particles. This study paves the way to

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more co-encapsulation of any other contemporary medicines in combination with this wholesome natural oil, NSSEO.

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Introduction:-

Acute inflammation is the first reaction that is identified through the increase in plasma movement and indigenous immune cells such as neutrophils and macrophages, from the blood into the damaged tissues (Ferrero-Miliani et al., 2007) (Medzhitov, 2008). Indeed, inflammation inducers cause cell membranes phospholipase A₂ activation that would trigger the release of arachidonic acid and inflammatory mediators (cytokines, serotonin, histamine, prostaglandin and leukotrienes), which facilitate leukocytes migration to the inflammation site (Sarkhel, 2016). The release of products such as histamine, bradykinin, serotonin, and cyclooxygenase (COX) is linked with the first phase of inflammation (0-1 h), whereas prostaglandins release, oxygen-derived free radicals production and polymorphonuclear leukocytes (PMN) infiltration is related to the late phase of oedema (Sadeghi et al., 2014). Globally, non-steroidal anti-inflammatory drugs (NSAIDs) are one of the mostly prescribed drugs classes. Indomethacin, being one of the NSAIDs first line non-opioid drugs prescribed for cancer pain. In addition, indomethacin is poorly water soluble (~ 0.01 mg/ml) and is only soluble at basic pH of 12 at which the drug significantly hydrolysis takes place (Lin et al., 1994). The intrinsic solubility of indomethacin was determined to be 8.8 µg/mL at pH of 9 (Comer et al., 2014). Thus, indomethacin poor skin bioavailability can be attributed to its insolubility in water for the skin pH. Various novel dosage forms (liposomes, nanospheres, nanoparticles etc.) have been formulated using indomethacin as the model drug to tackle its skin permeability problem but none of the studies use essential oil as means to increase permeability or penetrability via topical delivery. Here, we demonstrated the functional use of our selected essential oil, namely *Nigella sativa L.* seeds essential oil (NSSEO). We have observed at least two main functions this most studied oil provided in our study, namely as permeation enhancer and as efficacy modulator, both gave rise to synergistic effects. The Stratum Corneum (SC) acts as an obstacle towards efficient use of transdermal drug delivery systems development because of its low drug permeability. Therefore, numerous physical (sonophoresis, *microneedles*, and *iontophoresis*) and chemical [dimethyl sulphoxide (DMSO), ethanol, and Laurocapram (Azone[®])] skin penetration enhancement methods had been studied in order to mitigate this challenge (Benson, 2005) (Li et al., 2005). However, most of these methods cause skin irritation and damage, and ultimately disturbance of skin barrier function. Additionally, previous studies formulated indomethacin in various dosage forms like gels, cream, microparticles, nanospheres and nanoparticles. All these formulations utilized certain types of additives, either as co-solvent [propylene glycol (PG)] (Pinheiro et al., 2015), co-polymer [e.g. methoxy poly(ethylene glycol) in poly(ε-caprolactone)] (Kim et al., 2001), co-solubiliser (e.g. hydroxypropyl-β-cyclodextrin, methylcellulose) or as agent to improve encapsulation efficiency (Nagai et al., 2015). However all of the additives are lacking of evidence that can recognize them as agents that contribute to therapeutic efficacy unlike natural oil, NSSEO.

There are other studies that have also incorporated an ingredient to enhance therapeutic effect of indomethacin notably copper (Yassin et al., 2015). However, the enhanced inflammatory effects as observed in the copper-indomethacin topical delivery were thought to be attributed to the activation of copper-dependent opioid receptor. Any activation of opioid receptor will concomitantly increase risk of opioid-associated adverse effects such as sedation, dizziness, tolerance and respiratory depression.

Therefore, our main approach here was to select an agent that can confer modulation on skin permeability and penetrability of indomethacin while carrying other beneficial therapeutic efficacy. In our case, we had selected the ancient herb, most widely and thoroughly researched folklore and prophetic medicine i.e *Nigella Sativa L.* Seeds Essential Oil (NSSEO) as the co-therapeutic agent to be co-encapsulated with indomethacin in biodegradable poly-ε-caprolactone. Therapeutic efficacy of this ancient oil had been

stated in old medicinal manuscripts and its fame transgresses any nation, religion and borders. NSSEO has been used for the treatment of different diseases namely rheumatism, bronchitis, asthma and others for centuries. NSSEO efficacy may be due to its biological activities such as anti-inflammatory, analgesic, immunomodulatory, spasmolytic, and anti-oxidative properties (Ahmad et al., 2013). The principal phytochemicals of NSSEO are thymoquinone, p-cymene, α -pinene, thymohydroquinone, dithymoquinone and nigellone (Al Juhaimi et al., 2013). In fact, thymoquinone formed the main part of NSSEO to which mostly NSSEO therapeutic usages are attributed to it (Ravindran et al., 2010). It is reported that the production of inflammatory cytokines can be inhibited by thymoquinone in NSSEO (Ahmad et al., 2013). The uniqueness of this study in comparison to previous researches are: (a) the usage of xylene (topical application) instead of carrageenan (injection administration route), (b) anti-inflammatory activity study via evaluation and assessment of several parameters together such as mice ears thickness, weight, histology and immunohistochemistry (IHC), (c) obtaining of information from the combination of microscopy and IHC data that help to indicate overall tissue condition, (d) employing of nanoparticles as skin penetration non-invasive enhancement approach lead to maintenance of the skin's normal function as opposed to other physical and chemical techniques. Furthermore, in this research, the mouse was used as model, which is a well-known and well described model in the literatures. Moreover, male mouse was used that can avoid eventually the interference with hormonal cycle of female mice. Here in this work, edema was shown as the increase in mice ears thickness and weight that are consecutively compared with corresponding values pre-application.

The focus of this study is in the comparative evaluation of *in-vivo* anti-inflammatory activity of nanoparticles co-encapsulated with indomethacin and NSSEO and nanoparticles containing indomethacin alone using acute cutaneous mice inflammation model. Substantially, our data found that the co-encapsulation of indomethacin with NSSEO enhanced anti-inflammatory effect via topical treatment and suggest a possible decrease in indomethacin systemic concentration that can consequently reduce the indomethacin side effects.

Materials and methods:-

Materials:-

Nigella Sativa L. Seeds Essential Oil (NSSEO) was kindly provided by the Faculty of Sciences, Ibn Zohr University, Agadir, Morocco. Poly(ϵ -caprolactone) (PCL), polyvinyl alcohol (PVA) and polysorbate 80 (Tween[®] 80) were supplied by Sigma-Aldrich, Germany. Indomethacin was provided by George Van Waters and Nat Rogers laboratory products distributor (VWR) and acetone was purchased from Laurylab, France. Electronic balance, digital micrometer (model J15, BLET), isoflurane, triethanolamine (TEA), polyethylene glycol (PEG 300), polyvinyl pyrrolidone (PVP), Carbopol ETD 2001 (C2001), and Hexylene glycol (HG) were provided by one of the common chemical products suppliers in Europe.

Preparation of nanoparticles:-

Nanoparticles containing indomethacin and NSSEO were prepared in two separate phases by nanoprecipitation technique that was firstly designed by Fessi et al, (Fessi et al., 1989). To prepare the organic phase, 200 mg PCL was dissolved in 25 ml acetone under mild heat and magnetic stirring. Then 40 mg indomethacin and 300 mg NSSEO were added into the solution of PCL in acetone for co-encapsulation of indomethacin and NSSEO. For the aqueous phase preparation, 50 mg PVA was dissolved in 50 ml Milli-Q water at mild heat and mixed with 135 mg Tween-80[®] using magnetic agitation. Subsequently, the organic phase containing NSSEO, indomethacin, PCL and acetone was added dropwise by KDS 100 Legacy Single Syringe Infusion Pump operating at 220 volts alternating current (VAC) to the aqueous phase (Figure 1). Acetone evaporation was performed afterwards (Buchi Rotavapor R-124[®]) (under reduced pressure and high temperature conditions). Mostly the parameters of nanoparticles preparation process and formulation were inspired by a systematic study which was done under the same condition by Badri et al, (Badri et al., 2017). The composition and operating condition of blank and indomethacin nanoparticles are further shown in **Table 1**.

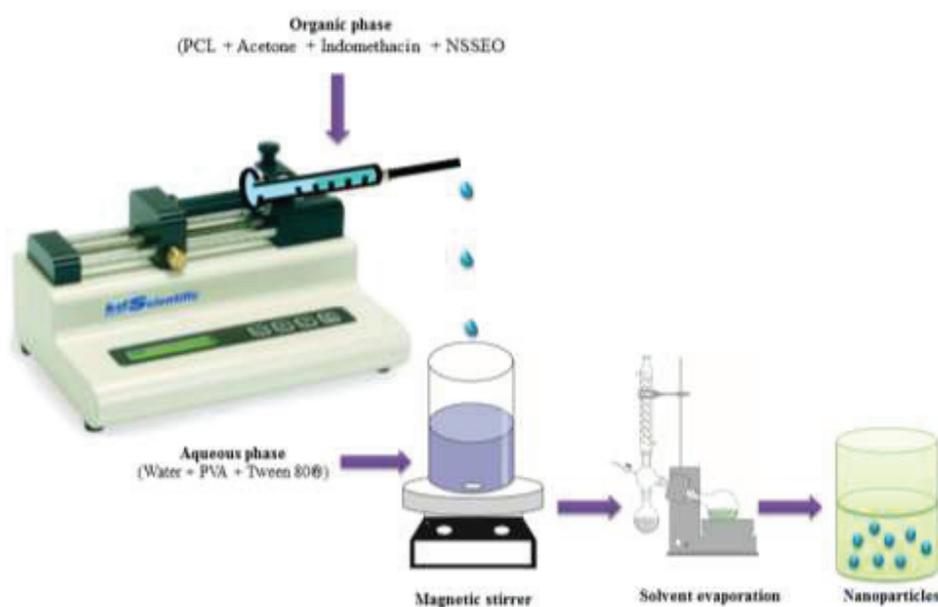


Figure1:-Schematic diagram of nanoparticles preparation by nanoprecipitation method.

Indomethacin gel preparation:-

To prepare 1% w/w indomethacin gel, 1% w/w of gel forming agent, C2001, was slowly added in small increments into a vortex of 57 % w/w sterile water under continuous magnetic agitation. Then, resin was added to prevent the entrapment of air, and stirring was continued at a reduced speed. The resulting dispersion was stored at rest mode in the dark for 24 h to obtain a homogenous solution. HG (30 % w/w) and PEG 300(10 % w/w) as solvents, were first mixed together and then added into the 1 % w/w indomethacin to dissolve indomethacin. The solution was then poured into the mixture in small portions with constant stirring until homogeneity was achieved. Afterwards, 1 % w/w of TEA as neutralizer was added to the mixture in order to increase the pH and trigger the formation of gel. The obtained gel packaging was carried out in amber glass containers and stored for 24 h in a dark place at room temperature (20 ± 2 °C) (Shawesh et al., 2003).

Table 1:-Nanoparticles composition and operating condition

(a). PCL based blank nanoparticles

Organic Phase		Aqueous phase			Operating condition	
PCL concentration (mg/ml)	Acetone volume (ml)	Tween® 80 concentration (mg/ml)	Milli-Q water volume (ml)	PVA concentration (mg/ml)	Stirrer speed (rpm)	Organic phase injection rate (ml/min)
8	25	2.7	50	1	300	9

(b). PCL based nanoparticles loaded with indomethacin

Organic Phase			Aqueous phase			Operating condition	
PCL concentration (mg/ml)	Acetone volume (ml)	Indomethacin concentration (mg/ml)	Tween® 80 concentration (mg/ml)	Milli-Q water volume (ml)	PVA concentration (mg/ml)	Stirrer speed (rpm)	Organic phase injection rate (ml/min)
8	25	1.6	2.7	50	1	300	9

(c). PCL based nanoparticles containing indomethacin and *Nigella Sativa* L. Seeds Essential Oil

Organic Phase				Aqueous phase			Operating condition	
PCL concentration (mg/ml)	Acetone volume (ml)	Indomethacin concentration (mg/ml)	NSSEO concentration (mg/ml)	Tween® 80 concentration (mg/ml)	Milli-Q water volume (ml)	PVA concentration (mg/ml)	Stirrer speed (rpm)	Organic phase injection rate (ml/min)
8	25	1.6	6	2.7	50	1	300	9

Nanoparticles characterization:-

To characterize the prepared nanoparticles, their size, zeta potential, morphology, and encapsulation efficiency studies were carried out.

Particle size and zeta potential:-

To measure nanoparticles size and zeta potential, Malvern particle size analyzer using dynamic light scattering (Zetasizer - Nano ZS, Malvern instruments limited, UK) was used. Prepared nanoparticles were dispersed in 1mM NaCl solution previous to each zeta potential measurement whereas nanoparticles size measurement was carried out after colloidal dispersion dilution within a 1 mL of distilled water. All measurements were taken place in triplicate at room temperature (25 °C) and their average was taken as the result.

Nanoparticles morphology:-

For nanoparticles shape and appearance assessment, Transmission Electron Microscopy (TEM) was employed. Nanoparticles TEM has been taken place by Philips CM-120 Transmission electron microscope (CMEABG, Claude Bernard University Lyon 1, France) by 120 kV accelerating voltage. To this end, a drop of nanoparticles suspension was diluted in 2 ml of *Milli-Q water* by micropipette and consecutively one drop of this dilution was placed on the carbon-coated copper grid. Supplementary amount of nanoparticles suspension was removed via blotting the grid through filter paper and instilled nanoparticles suspension on the grid dried prior to TEM analysis at room temperature.

Ex vivo skin penetration:-

Skin penetration study was carried out on the *ex vivo* human skin model by confocal laser scanning microscopy (CLSM). Indeed, CLSM is a non-invasive optical imaging technique. The distribution of applied nanoparticles that contain active ingredient and fluorescent agent (Core Shell Evidots) in skin can be visualized and inspected by CLSM (Pygall et al., 2007) (Alvarez-Román et al., 2004). Fresh excised human abdominal skin was obtained from plastic surgery. Samples for this study comprised of: (a) mixture of 25 µl Core Shell Evidots (CSE) and 50 ml distilled water liquids, (b) poly(ϵ -caprolactone) based NPs loaded with indomethacin and CSE, (c) poly(ϵ -caprolactone) based NPs loaded with indomethacin, CSE and NSSEO (preparation of samples b, and c described in Table 1, except that for *ex vivo* study, 50 µl CSE were added into the organic phase). Afterwards prepared formulations were topically applied at room temperature on a defined area of skin (100 µL/cm²) following a gentle massage for 30 seconds. A skin area remained untreated and served as control. After 1 h of CSE and NPs application, a 3 mm punch biopsy specimen was taken from each area. Skin biopsies were placed at - 80 °C until use for the study. The frozen skin samples were embedded in Tissue-Tek[®] and cut in 7 µm vertical cryostat sections. Sections were mounted using VECTASHIELD[®] mounting media and were imaged under a CLSM (Leica SP2 AOBS microscope with blue laser excitation at 405 nm, 63x oil immersion objective). Blue fluorescence intensity was the indicator of NPs penetration assessment through the skin in a comparative manner between treated and untreated (control) human skins.

In vivo anti-inflammatory activity study:-**Animals:-**

Adult male Swiss mice (6 - 8 weeks age) were housed and given diet at libitum under standard laboratory conditions of temperature, and relative humidity. On the day of the experiment, mice were individually weighed and tagged with a temporary tail marking. For each step of experiment, mice were anesthetized using isoflurane 2.5 %, with 1 L/min flow for induction, then isoflurane 1.5%, with 0.5 L/min flow for maintaining anesthesia. In this study, four groups of animals were used (n = 7 each) in accordance to the minimum number for a proper statistical analysis. Regular observation of the animals during the habituation period was performed. Experiments were in compliance with guidelines for study in animals' laboratory and were approved by the Ethics Committee of Animal Experimentation of French National Center for Scientific Research (CNRS).

Acute ear edema induction:-

In order to induce an acute edema in mice ears, xylene as a phlogistic agent was topically applied on the inner and outer surface of the right ear of mice (30 μ L/ear). The left ear was considered as negative control and received only distilled water (20 μ L/ear).

Application of designed formulations:-

To apply formulations separately and to study the anti-inflammatory effect of applied formulations comparatively, animals were randomly assigned into four groups (Figure2). **Group I** animals have employed to evaluate the absence of anti-inflammatory activity of the blank nanoparticles (20 μ L/ear). **Group II** animals were used to assess the anti-inflammatory activity of the nanoparticles containing indomethacin (20 μ L/ear). **Group III** animals have used to evaluate the anti-inflammatory activity of nanoparticles containing indomethacin and NSSEO (20 μ L/ear). **Group VI** animals were used as a positive control, and 1% indomethacin gel (100 mg/ear) was applied. The quantity of indomethacin in all indomethacin-containing formulations is constant (1 mg/ear), following Garrido et al, (Garrido et al., 2004). In addition, NSSEO quantity in the formulation that contained NSSEO was 2 mg/ear. The left ear of mice within all four groups was considered as negative control on which the distilled water (20 μ L/ear) was applied. Furthermore, formulations have topically applied after application of xylene on the inner and outer surface of the right ear of all four groups of mice.

Anti-inflammatory activity assessment:-

Experiments were carried out based on Oliveira et al, method (Liduina Maia de Oliveira et al., 2013). The thickness (μ m) of each ear was measured using a precise digital micrometer (model J15, BLET) that was put close the ear tip, just distal to the cartilaginous ridges. Measurement was performed prior to the application of xylene (0h) and then at 1h, 2h, 3h, 6h, and 8h time intervals after induction of an inflammatory response. The edema is evaluated based on the alteration of the thickness of mice right ear versus mice left ear. Anti-inflammatory activity of prepared formulations was assessed by comparing weight (mg) and thickness (μ m) reduction with respect to the positive control and pre-injection values (Figure 7).

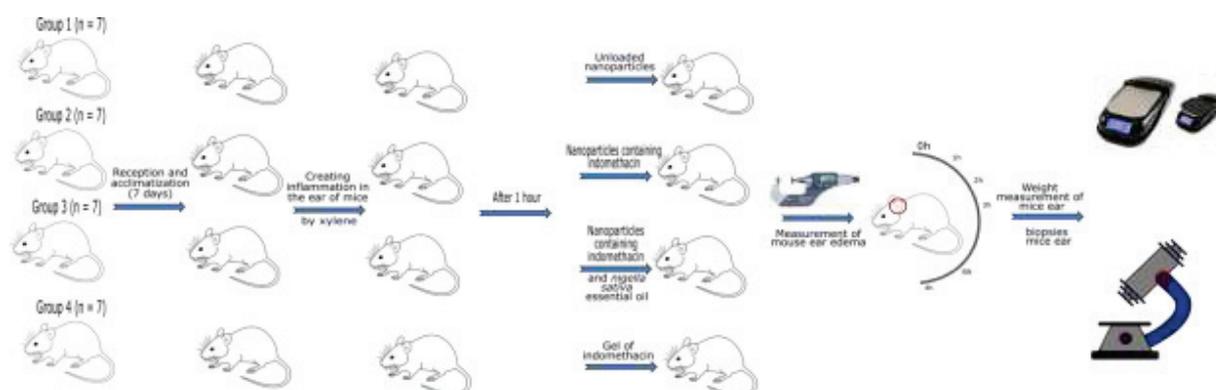


Figure 2:-*In vivo* study experimental design scheme, Four Groups (n = 7 each) of mice were employed, (Group 1 for anti-inflammatory activity assessment of the blank nanoparticles, Group 2 for evaluation of anti-inflammatory activity of nanoparticles containing indomethacin, Group 3 for the study of anti-inflammatory activity of nanoparticles containing indomethacin and NSSEO and Group 4 were used as a positive control, to investigate the anti-inflammatory activity of 1% indomethacin gel).

Histology analysis:-

Mice removed ears was studied from two aspects of simple histology and immunohistochemistry analysis. The induction of skin inflammation can take place in 1 – 2 h of exposure, which is described via increased blood circulation, vascular permeability, infiltration of leukocyte into the skin, degeneration of epidermis, boosted oxidative species levels, and DNA damage.

Mice were first euthanized and ears were cut and fixed (10 % formaldehyde for 24 h- 48 h) and processed by standard methods of histology. Afterwards, fixed mice ears were embedded in paraffin for 3 h, sliced into 3 μ m sections and stained by Hematoxylin-Phloxine. Mice ears process was performed in Leica ASP300 S. The samples preparation for simple histology microscopy observation and immunohistochemistry (IHC) followed the same procedure up to cutting of mice ears (1 single piece). Subsequently, samples were mounted on the Superfrost[®] gelatin-coated slides (SIGMA – ALDRICH). On the other hand, IHC Tissue prepared samples were mounted on the Superfrost[®] slides that was then coated with water, to remove background noise gelatin wasn't used in IHC. Tissues were observed by Axio Scan Z1 de Zeiss slide scanner at a magnification of 20X.

Immunohistochemistry (IHC) study:-

Primary monoclonal mouse antibodies (streptavidin peroxidase conjugates) raised against CD45 (clone PD7/26) was used to analyse leucocytes. Embedded tissues was first treated with Cell Conditioning Solution (CC2) before paraffin was removed using the absolute ethanol. Inflammation was detected using a biotinylated anti-mouse rabbit IgG secondary antibody, followed by colorimetric recognition using Streptavidin-biotin peroxidase detection system (DISCOVERY DAB Map Detection Kit (RUO)). The counterstaining of sections was performed with hematoxylin (4 min) and bluing reagent (4 min), and sections were then mounted under coverslips (Figure 3) to be viewed under a slide scanner (Axio Scan Z1 de Zeiss) at 20X magnification. Obtained images were processed with Fiji software (image processing package) and analysis was performed for epidermis integrity, dermis thickness, infiltration of leukocytes and edema.

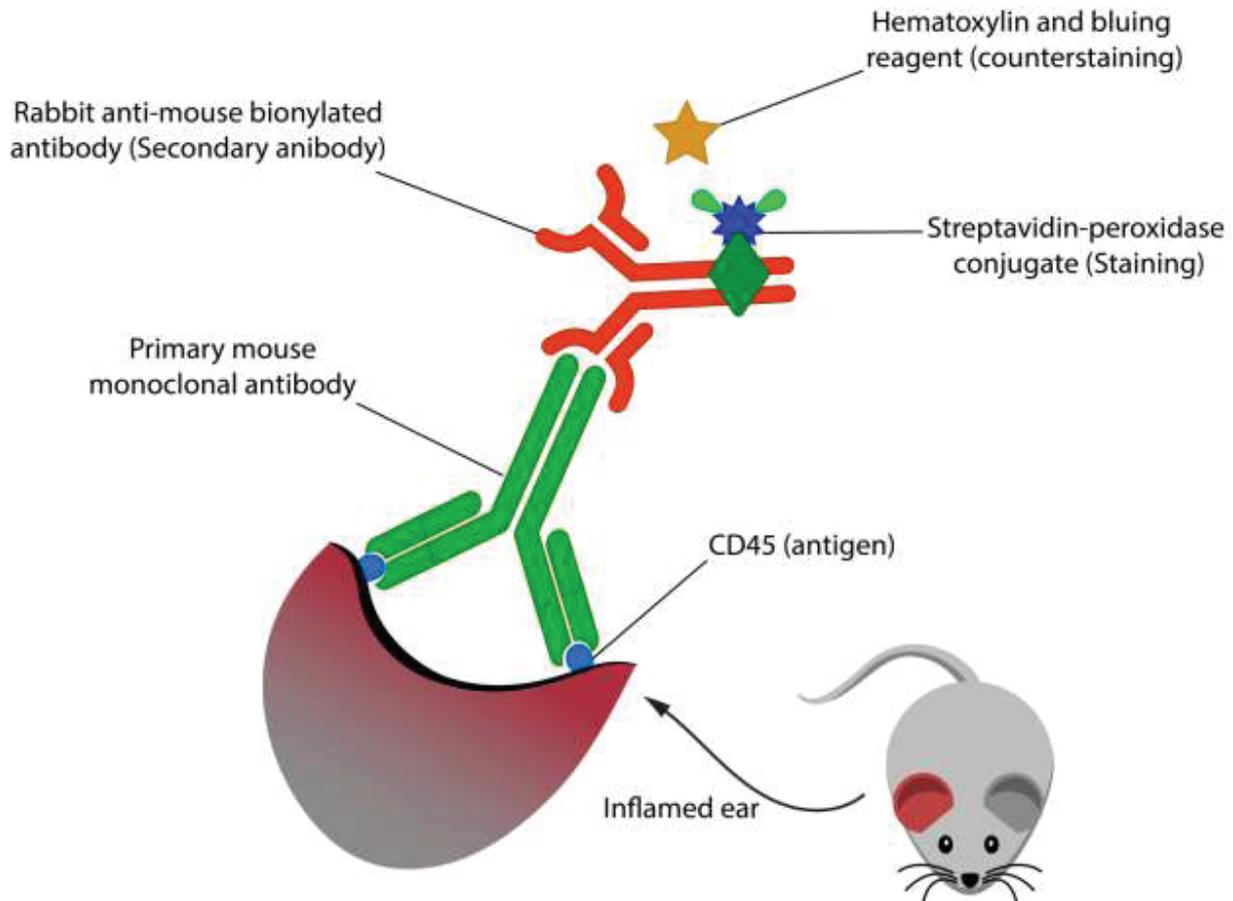


Figure 3:-Indirect enzyme linked immunohistochemistry (streptavidin peroxidase conjugated method) illustration.

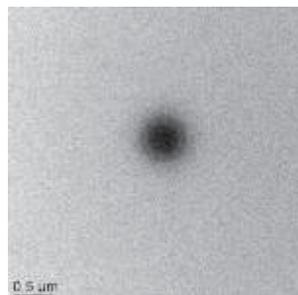
Statistical analysis:-

Data were analyzed by GraphPadPrism7.0 software and shown as mean \pm standard deviation. Multiple comparisons were carried out by one-way analysis of variance (ANOVA) at $P < 0.01$ significance.

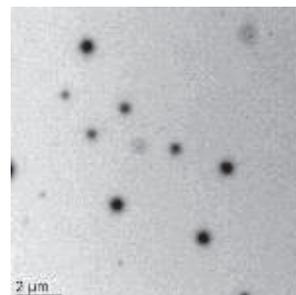
Results And Discussion:-

Nanoparticles characteristics:-

Nanoparticles size and zeta potential were ranged between $230 - 260 \pm 12.47$ nm and $(-20$ mV) up to $(-30$ mV) ± 4.082 , respectively. The pH of the colloidal dispersion was around 6 ± 0.82 , whereas the encapsulation efficiency of indomethacin and NSSEO within the designed nanoparticles was $70, 84 \pm 5.73$ % respectively. Prepared nanoparticles size distribution was about 0.166 ± 0.007 . Transmission Electron Microscopy (TEM) analysis showed that nanoparticles have a spherical and regular form (Figure 4).



(a) 0, 5 μ m scale bar



(b) 2 μ m scale bar

Figure4:-Transmission Electron Microscopy images of PCL based nanoparticles loaded with indomethacin and NSSEO, (a). 0.5 μm scale bar, and (b). 2 μm scale bar showing nanoparticles smooth surface and regular form.

Nanoparticles loaded indomethacin and NSSEO: are able to penetrate *ex vivo* fresh human skin:-

The skin plays principal functions such as barrier role, temperature control role and repair role that contribute to homeostasis process of human body (Sala et al., 2018). Skin has a potential application in drug delivery thanks to its large surface area. Prevention of first pass metabolism, minimization of pain and possible controlled release of drugs are from the advantages of the Topical or transdermal delivery over the conventional oral and intravenous dosage forms (Desai et al., 2010). Skin drug delivery (SDD) that is a smart method to the treatment of many diseases, cover in general dermal and transdermal drug delivery (Sala et al., 2018). Active molecules after topical application of nanoparticles can be absorbed via pathways such as transcellular, intercellular or transappendageal (Figure 5). It is possible that either topically applied nanoparticles place into the skin without degradation or with degradation nearby to the skin surface, consecutively loaded active molecule would penetrate into the layers of skin. Nanoparticles physicochemical properties including size, surface charge, used nanomaterials properties, and so on are governing the interaction of nanoparticles with skin (Desai et al., 2010).

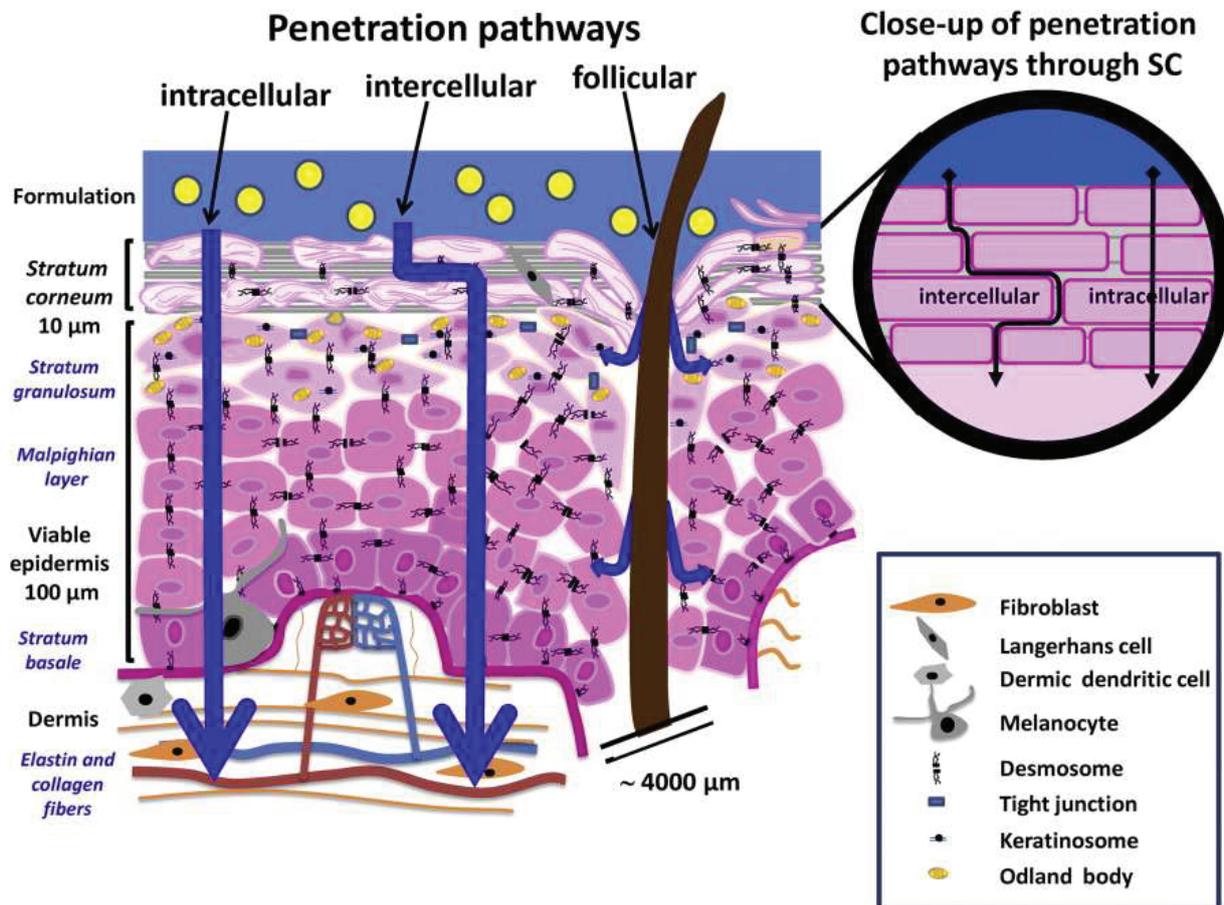


Figure 5:-Sketch of the three penetration pathways: transcellular, intercellular and follicular. The upper right inset is a close-up of the SC showing the transcellular pathway and the tortuous intercellular pathway (Bolzinger et al., 2012).

CLSM images of skin histological sections are shown in Figure 6 and Figure 7. The images taken from the control skin section demonstrated, in which an autofluorescence in the dermis can be observed coming from collagen and elastin fibrous structures. The image obtained from sample B (Fig. 6) showed the total

fluorescence comprised of the autofluorescence and the fluorescence of the CSE. For samples Fig. 6B and Fig. 6C, fluorescence was distributed throughout the *Stratum Corneum (SC)* and the dermis. For sample Fig. 6D, fluorescence was observed in the epidermis and dermis, fluorescence signal of the dermis appeared to be highest compared to control. The Figure C illustrated skin location of NPs, which are visible only through the epidermis. The fluorescence labeling by CSE confirmed that NPs could be observed using CLSM. Fluorescence emission provided semi-quantitative information on the skin penetration of NPs. CLSM images proved that NPs would penetrate the skin, reaching the dermis. As no fluorescence was observed in the *SC* after application of NPs with *Nigella Sativa L* Seeds Oil (Figure 7D), the relative accumulation of fluorescence in the *stratum corneum* after application of NPs without NSO as can be seen in Figure 6B indicate that NSO facilitates the penetration of NPs throughout the outermost layer of the epidermis. Nanoparticles were visualized across the epidermis but were hardly detectable in the dermis because of the autofluorescence of collagen and elastin fibers. These findings draw a conclusion that nanoparticles loaded with indomethacin and NSSEO can penetrate the *SC* barrier to improve the anti-inflammatory activity of indomethacin.

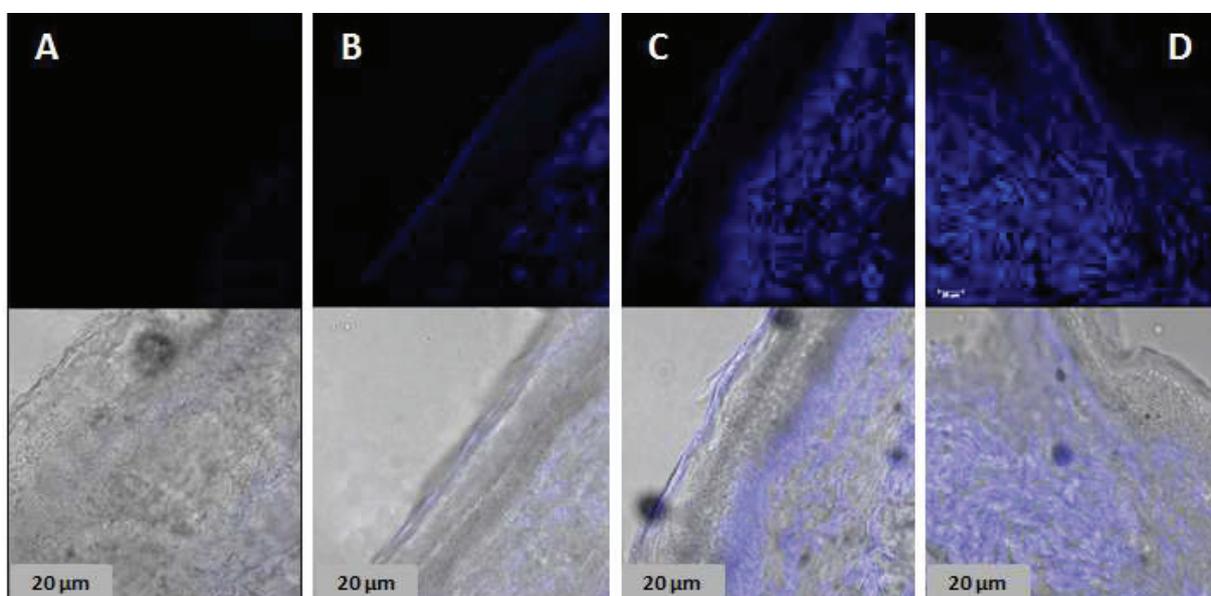


Figure 6:- CLSM images of NPs deposited on human skin, Scale bars are 20 μm . The CSE used in this study have an emission peak at 516 nm. Scale bars are 20 μm , (A): control or untreated skin with nanoparticles, (B): mixture of 25 CSE and distilled water, (C): nanoparticles loaded with indomethacin and CSE, and (D): nanoparticles loaded with indomethacin, NSSEO and CSE.

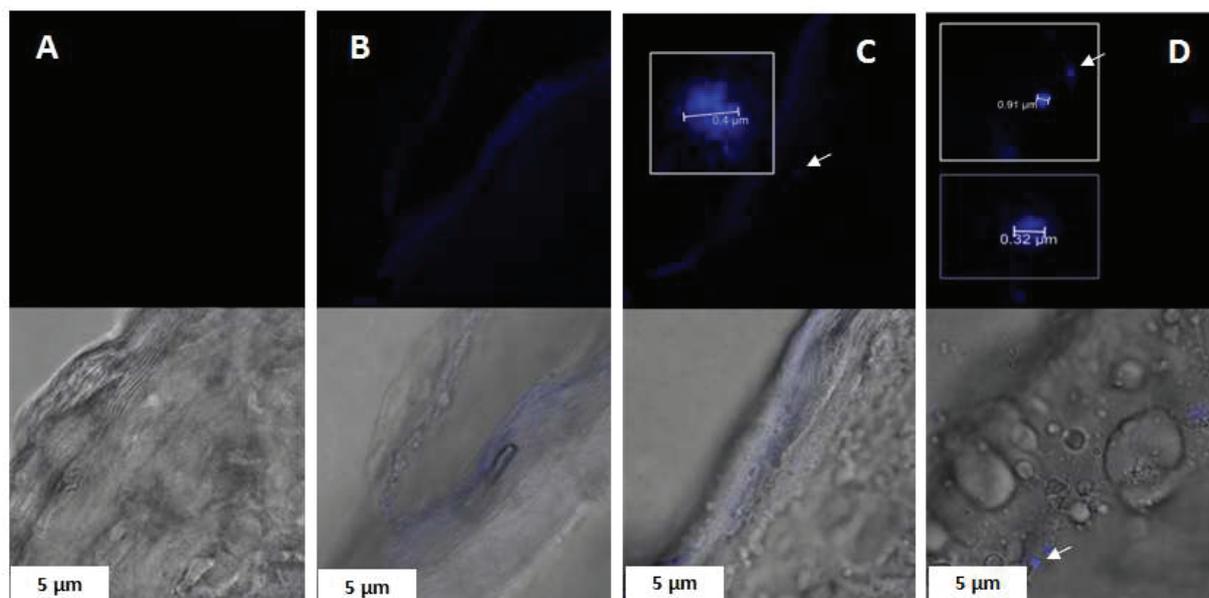


Figure 7:-CLSM images of NPs (arrow) through the epidermis, Scale bars are 5µm. (A): control or untreated skin with colloidal dispersion, (B): mixture of 25 CSE and distilled water, (C): nanoparticles loaded with indomethacin and CSE, and (D): nanoparticles loaded with indomethacin, NSSEO and CSE.

Since *ex-vivo* investigation results of this research were promising therefore it let us to go further and perform the *in-vivo* anti-inflammatory study as well.

Nanoparticles containing indomethacin and NSSEO: has higher anti-inflammatory efficacy:-

As can be seen in Figure 8, upon the application of all four formulations at the beginning of experiment (0 h), no significant differences were observed in mice ears thickness. This can be explained by the SC permeability barrier property towards xylene, which was applied for the creation of edema (thickness). However, in 1st hour the thickness decreased significantly by indomethacin gel treatment while the thickness was stable for three other formulations. Since polymer based nanoparticles and gel are two different forms therefore this can be indicated to the imprisoning of indomethacin and NSSEO within the polymeric nanoparticles that is not the case for indomethacin gel ($P < 0.01$) in other words release of indomethacin from nanoparticles take time to exert its anti-inflammatory activity. In addition, in 2nd hour the thickness of mice ears by nanoparticles containing indomethacin and NSSEO in comparison with the blank nanoparticles was significantly reduced as compared to the indomethacin gel ($P < 0.001$). On the other hand, in the 3rd hour, thickness depression by nanoparticles loaded with indomethacin and NSSEO was highly significant in comparison to indomethacin gel ($P < 0.0001$). Furthermore, in 6th and 8th hours the thickness reduction by nanoparticles loaded with indomethacin and NSSEO together, nanoparticles loaded with indomethacin alone and indomethacin gel was respectively significant ($P < 0.00001$). This inflammatory inhibition of nanoparticles containing indomethacin and NSSEO reached its peak 8 h after xylene application and showed the highest superiority in reducing mice ear thickness.

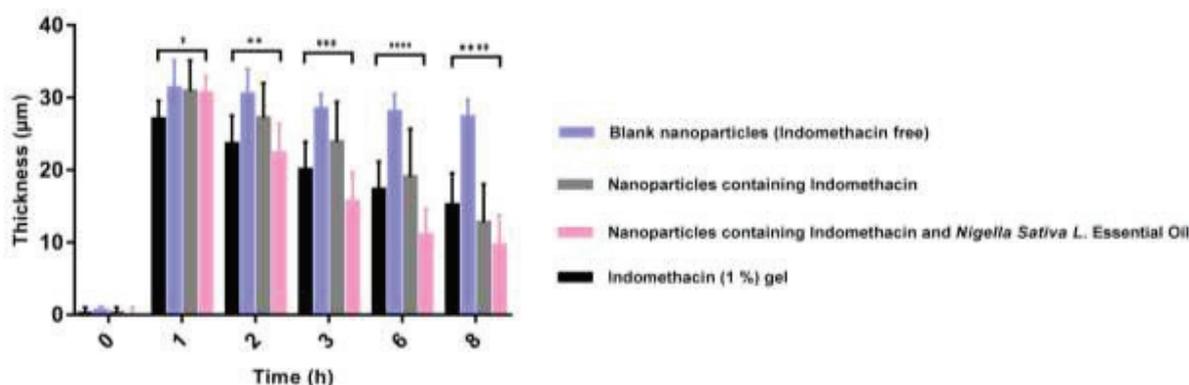
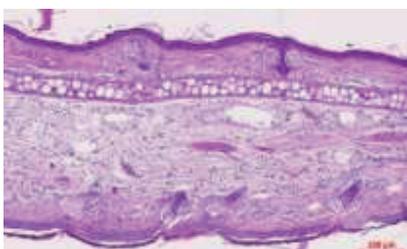


Figure 8:-Anti-inflammatory activity assessment is based on mice ear thickness measurement in 0h, 1h, 2h, 3h, 6h, and 8h time intervals. Mice ears thickness reduction by all four formulations is indicated by bars. Data described in Figure 8 shows that nanoparticle loaded with indomethacin and NSSEO has the highest anti-inflammatory activity among these formulations except 0h due to NSSEO enhancing effect. $P < 0.01$ is noted *, $P < 0.001$ is noted **, $P < 0.0001$ noted *** and $P < 0.00001$ noted ****. Multiple comparisons were carried out by one-way analysis of variance (ANOVA) at $P < 0.01$ significance.

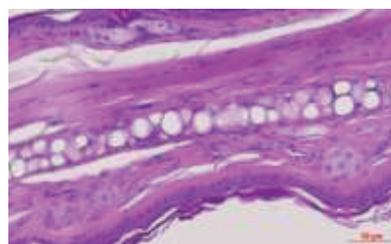
Xylene as phlogistic agent endorses neurogenic inflammation by acting on immune cells, mast cells, and vascular smooth muscle (Liduína Maia de Oliveira et al., 2013). The left ear of mice within all four groups was considered as negative control on which the distilled water (20 µl/ear) was applied. The experiments performing intervals were limited to the 8 h.

Tissue inflammation histopathological changes can be dealt with easier via nanoparticles loaded with indomethacin and NSSEO:-

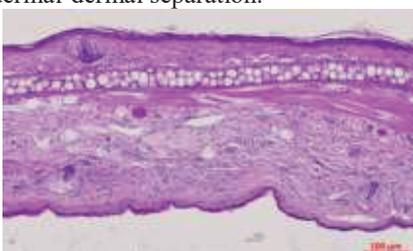
Histology and immunohistochemistry studies have shown that subsequent to the xylene contact, rat skin histopathological changes consist of epidermal-dermal layers separation and infiltration of granulocyte into the skin at 4 h and 6 h time points (Figure 9; Figure10).



(a). Xylene induced mice ear edema treated with blank nanoparticles denoting more lesions, epidermal-dermal separation.



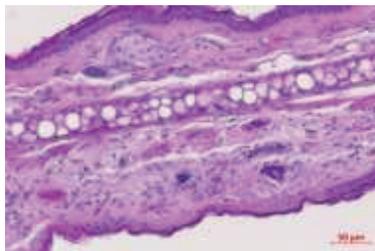
(b). Xylene induced mice ear edema treated with distilled water (negative control) showing normal cell histology structure.



(c). Xylene induced mice ear edema treated with nanoparticles loaded with indomethacin.



(d). Xylene induced mice ear edema treated with nanoparticles loaded with indomethacin and *Nigella Sativa L.* Essential oil, showing remarkable amelioration of the lesions, epidermal-dermal separation, and inflammatory cells.



(e). Mice ear treated with indomethacin gel (positive control).

Figure 9:-Histology images of mice ear with a magnification of 20X, Scale bars represent for (a), (c), (d) 100 μm and for, (b) and, (e) 50 μm . It shows that epidermal-dermal layers separation and infiltration of granulocyte into the skin reduced by nanoparticles loaded with NSSEO and indomethacin (d) in comparison with other formulations (a), (b), (c) and (d) is prominent thanks to NSSEO presence.

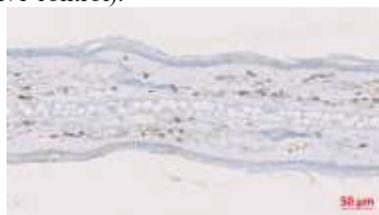
The homogeneous eosinophilic substance accumulation was observed at the epidermal-dermal separation areas implying skin damage and/or inflammation associated with xylene application. Our findings in this research are in accordance with Gunasekar et al, in 2003 (Gunasekar et al., 2003). In addition, inflammatory lesions were more evident than was previously reported (Sadeghi et al., 2014). The topical application of designed formulations including indomethacin gel (1 %), nanoparticles loaded with indomethacin alone and nanoparticles loaded with indomethacin and NSSEO, could turned down the previously histological changes in different ratio. However, mice ears treated with distilled water (negative control) and mice ears treated with blank nanoparticles (indomethacin free) did not show any changes in the above mentioned histological changes. Treated mice ears nanoparticles loaded with indomethacin and NSSEO shown extravagant lesions, epidermal-dermal separation, and inflammatory cells (lymphocytes and neutrophils) infiltration reduction effect as showed in Figure 9(d).



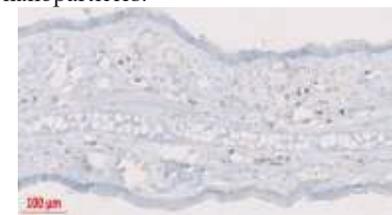
(a). Xylene induced mice ear edema treated with distilled water (negative control).



(b). Xylene induced mice edema ear treated with blank nanoparticles.



(c). Xylene induced mice ear edema treated with nanoparticle loaded with indomethacin.



(d). Xylene induced mice ear edema treated with nanoparticles loaded with indomethacin and *Nigella Sativa* L. essential oil.



(e). Xylene induced mice ear edema treated with indomethacin (1 %) gel (positive control).

Figure 10:-Microscopic evaluation of inflammatory cell infiltration differential profile, epidermis integrity, dermis thickness, and edema in mice ears, all samples were assessed in representative areas with increased 20x.

Immunohistochemical evaluation of CD45⁺ cells in the inflamed and non-inflamed ears tissues of mice. Samples were taken after 9 h xylene application. The mice ear sections were stained with Hematoxylin-Phloxine and all representative tissue section slides were observed with increased. The images show the thickness of the dermis, sebaceous glands, blood vessels and leukocyte infiltration as the criteria for evaluation of anti-inflammatory activity. These criteria are most strongly decreased in (d).

As can be seen in images (Figure 10), the blue color shows the normal cells while the brown color indicates the CD45 that is directly correlated with the inflammation. The number of normal and inflamed cell nucleus were counted and provided the percentage of inflammatory inhibition. Tissue inflammation quantity was determined by the division of CD45 number on the normal cell nucleus number. The anti-inflammatory inhibition percentage of designed formulations such as blank nanoparticles, indomethacin gel (1 %), nanoparticles loaded with indomethacin, and nanoparticles loaded with indomethacin and *Nigella Sativa L.* seeds essential oil were respectively 9 %, 47 %, 79 % and 90 %.

The obtained data from this research support the concept that NSSEO would exert its anti-inflammatory activity and boost the efficacy of indomethacin, and decrease its dosage and consequent side effects.

Conclusions:-

This study firstly reports that PCL based nanoparticles loaded with NSSEO can significantly improve cutaneous penetration of indomethacin as a noninvasive approach. In another words there would not be required to irritate, or disturb skin functions for enhancing drug delivery to skin and overcoming SC barrier properties. Furthermore, this study reinforces the anti-inflammatory activity enhancement of indomethacin by NSSEO within the polymeric nanoparticles. Consequently, for providing the same efficacy by taken dose of indomethacin can be decreased due to NSSEO existence with indomethacin in the formulation in order to reduce its side effects throughout the digestive system. Indeed NSSEO anti-inflammatory is associated with presence of thymoquinone. Further researches are necessary to investigate NSSEO different amounts effects on its anti-inflammatory activity and to support NSSEO clinical applications.

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PartIV

General discussion and conclusion

General discussion and conclusion

The goal of our project was to extract the *Nigella Sativa* L. Seeds Essential Oil and its encapsulation together with indomethacin within polymer based nanoparticles to decrease taken amount and to enhance indomethacin cutaneous penetration, and anti-inflammatory activity. Inflammation is taken from latin inflammare word that indicates to set on fire. Inflammation could be indicated in health problems such as arthritis, cancer, stroke, neurodegenerative to name a few in a response of harmful agents including irritants, infection, or damaged cells and other injuries. The common signs of an inflammation are raised blood flow, amplified cellular metabolism, cellular influx, soluble mediators release, vasodilation, fluids extravasation, on site heat, redness, swelling and pain. Non-steroidal Anti-Inflammatory Drugs (NSAIDs) are broadly employed heterogeneous group of drugs that have not the steroids structure within their chemical. The benefit/risk profiles of NSAIDs are different from each other such as their chemical formula. On the daily basis NSAIDs by approximately 30 million people take NSAIDs around the world. Generally NSAIDs are exerting anti-inflammatory activity by inhibition of cyclooxygenase (COX) enzymes. Indeed, COX-1 is a constitutive enzyme whereas COX-2 is an inducible enzyme. Indomethacin has selected as active to be encapsulated because it is a gold standard NSAID that has a sufficient anti-inflammatory activity for the external application, indomethacin is a potent NSAID. Since indomethacin as other NSAIDs to provide its pharmacological activity would inhibit COX enzymes and consequently could cause or intensify gastro-intestinal side effects (ulcers or bleeding). In addition, direct contact of indomethacin as an acid can boost the pharmacological mechanism based side effect. Therefore, to avoid indomethacin direct contact with digestive mucus and its first pass effect consequently to reduce indomethacin side effects, skin may use as indomethacin route of administration. However, skin because of its drug low permeability (Stratum Corneum) play the role of a barrier towards drugs development for skin application. *Nigella Sativa* L. Seeds Essential Oil (NSSEO) biological properties (anti-inflammatory, analgesic, immunomodulatory, spasmolytic, and anti-oxidant) have been well studied. Thymoquinone is the most abundant part of *Nigella Sativa* L. that is mostly responsible for NSSEO therapeutic properties. There is a direct proportion between indomethacin taken dose and its side effects severity at the digestive system. Thus, it is important to decrease the employed indomethacin dosage and consequently its side effects for providing the indomethacin anti-inflammatory activity. To overcome this challenge NSSEO could be used together with indomethacin in order to decrease indomethacin taken dosage while for handling with skin barrier properties nanotechnology would be the best approach among the skin penetration enhancement approaches. All these reasons and facts are motivating to encapsulate indomethacin with NSSEO within poly (ϵ -caprolactone) polymer for the skin application. According to the made literature review, we have decided to orient our works to polymer-based nanoparticles and use nanoprecipitation for the preparation of nanoparticles. The first challenge in our research to handle with was the using of

nanoprecipitation as a method of nanoparticles preparation for design of nanoparticles with suitable properties for skin penetration. In fact, safety, simplicity, ease of scalability, low energy input, obtaining of submicron particle sizes with narrow size distribution and good reproducibility are from the advantages of nanoprecipitation method. Acetone has selected as solvent due to its low boiling point (56.3 °C), immiscibility with water and its ability to dissolve polycaprolactone properly. To have a suitable colloidal dispersion stability polyvinyl alcohol (PVA) and polysorbate 80 (Tween[®] 80) were used as stabilizers. The objective of this work was to prepare the PCL based nanoparticles loaded with indomethacin and *Nigella Sativa* Seeds Essential Oil (NSSEO). Accordingly, a series of experiments were carried out:

In first part, a systematic study has done to optimize the condition (formulation and process related parameters) for obtaining nanoparticles with optimal wanted properties (size, surface charge etc.) to the skin administration, namely a monodisperse population of nanoparticles with diameter near to 100 nm and negative zeta potential. Our findings were divided into two parts of formulation related parameters and operating conditions related parameters. The usage of PCL as biodegradable polymer and acetone as solvent with low boiling point made this part original. In this part of study, it was found out that in formulation related parameters polymer concentration, and aqueous phase volume had a paramount impact on colloidal particles properties. Indeed, we have observed that when 4 mg/ml PCL concentration was used, nanoparticles with size of 152 ± 1.15 nm were obtained while 24 mg/ml PCL concentration has used as highest concentration, nanoparticles with size of 258 ± 2.50 nm were obtained. In addition, aqueous phase volume increasing from 25 ml to 100 ml lead nanoparticles size from 345 ± 3.95 nm to 181 ± 1.11 nm. In operating conditions, agitation speed organic phase and injection rate are the key factors to be taken into consideration. Stirring speed alteration from 75 rpm to 600 rpm decreased nanoparticles size from 211 ± 1.27 nm to 172 ± 2.25 nm. The change of organic phase injection rate from 1 ml/min to 9 ml/min caused nanoparticles size enlargement from 195 ± 1.34 nm to 189 ± 1.37 nm. However, upon organic phase injection speed, increasing to 13 ml/min this rule was not applicable anymore and the nanoparticles size was 197 ± 3.62 nm. Simplicity and low variability of nanoparticles preparation process are the other two important factors that should take into account beside the characteristics of produced nanoparticles within a systematic study. Prepared nanoparticles zeta potential were stable for all parameters except PVA concentration, while PVA concentration was getting increased nanoparticles zeta potential was decreased. The findings of this study proved that systematic study is essential before investigation of any drug encapsulation in order to design the nanoparticles with proper characteristics for *in-vivo* and *in-vitro* applications.

After optimization of nanoparticles preparation condition that was too crucial for figuring out the effective parameters, in second part, PCL based nanoparticles containing alone indomethacin were prepared. This part of study carried out to see the indomethacin presence effect and to prepare nanoparticles for comparison in terms of skin penetration and *in-vivo* anti-inflammatory activity with

the nanoparticles loaded with indomethacin and NSSEO together, which have prepared in the following experiment. Consecutively these nanoparticles were characterized in terms of size, surface charge, morphology, and pre-stability study. Along this study PCL based particles containing IND have been successfully prepared and characterized. The results obtained from DSC and FTIR studies of NPs indicated that no chemical interaction between drug and polymer in the formulation was occurred. The designed nanoparticles were pointed out stable for one month under storage temperatures of 4 °C, RT, and 40 °C. The pH of prepared colloidal dispersion was ranged between 4 and 6. The results showed that NPs hydrodynamic size was between 220 to 245 ± 79.75 nm and the zeta potential value ranges from -19 to -13 ± 4.61 mV at pH 5 and 1 mM NaCl. The encapsulation efficiency of designed nanoparticles upon preparation and after one-month storage in three above-mentioned temperatures was around 70 % and the drug loading was about 14 to 17 %. SEM and TEM images confirmed that the obtained nanoparticles were spherical and properly isolated with smooth surface. In addition, CLSM images proved that obtained NPs can penetrate the skin as well. The prepared nanoparticles are submicron in nature, with good colloidal stability and penetrate the stratum corneum layer of the skin. This formulation potentiates IND skin penetration and as a promising strategy would be able to decline the side effects of IND. This study supports indomethacin loaded nanoparticles penetration potential as a modern topical formulation that would decline indomethacin frequency of administration, side effects and consecutively patients' compliance.

In third part, indomethacin and NSSEO together were encapsulated within PCL by nanoprecipitation method. Successively, the characterization of prepared nanoparticle in terms of size, zeta potential, morphology, fluorescent microscopy, TEM, FTIR, DSC and stability was performed. These techniques all together confirm the encapsulation of indomethacin and NSSEO. Furthermore, skin penetration of designed nanoparticles was studied as well. The size and zeta potential of obtained nanoparticles were respectively (230 nm - 260 nm) and (-20 mV and -30 mV). Images provided by TEM analysis shown that prepared nanoparticles have a spherical and regular form. Encapsulation efficiency of nanoparticles for indomethacin and NSSEO were correspondingly 70 % and 84 %. The size of nanoparticles was not changed significantly within 30 days of pre-stability investigation. The zeta potential of nanoparticles throughout the period of pre-stability study was altered from -22 mV up to -28 mV that was non-significant as well. Nanoparticles had a size in nano scale with round shape and a proper stability. Indomethacin and NSSEO were successfully encapsulated that would boost the anti-inflammatory and analgesic effects of indomethacin.

In fourth part, *ex vivo* skin penetration and *in vivo* anti-inflammatory activity of Poly (ϵ -caprolactone) based nanoparticles loaded with indomethacin and NSSEO that were previously prepared, has been assessed. Our findings shown that since the application of designed formulations up to one hour no

significant differences were observed in mice ears thickness. Nevertheless, in 1st hour, mice ear thickness was reduced significantly by indomethacin gel whereas the thickness was stable for three other formulations. Furthermore, in 2nd hour the thickness reduction by nanoparticles containing indomethacin and NSSEO in comparison with the blank nanoparticles was significant that was less than indomethacin gel. From the other hand in 3rd hour, thickness depression by nanoparticles loaded with indomethacin and NSSEO was highly significant in comparison with indomethacin gel. Furthermore, in 6th and 8th hours the declined thickness by nanoparticles loaded with indomethacin and NSSEO together, nanoparticles loaded with indomethacin alone and indomethacin gel was gradually significant. This inflammatory inhibition of nanoparticles containing indomethacin and *Nigella Sativa* L. Seeds Essential Oil has reached its peak 8 h after xylene application. According to the found results the anti-inflammatory activity was time dependent. Histology and immunohistochemistry analysis figured out that the anti-inflammatory inhibition percentage of blank nanoparticles, indomethacin (1 %) gel, nanoparticles loaded with indomethacin alone and nanoparticles loaded with indomethacin and NSSEO are respectively 9 %, 47 %, 79 %, and 90 %. The *ex vivo* and *in vivo* results of this work confirmed that NSSEO possesses a significant anti-inflammatory activity and can be used as an enhancer for indomethacin topical application within the nanoparticles. Nanoparticles containing indomethacin and NSSEO had skin better penetration than nanoparticles loaded with indomethacin alone that was confirmed with CLSM. Based on our hypothesis, designed nanoparticles having negative charge can penetrate into the skin by appendage route (hair follicle) that can be time and size dependent. However, it is difficult to draw a conclusion regarding nanoparticles penetration precise mechanism because of skin heterogeneous structure and composition. Hair follicle can play a role as a reservoir for topically applied nanoparticles. In conclusion, poly (ϵ -caprolactone) based nanoparticles were elaborated that could be loaded with indomethacin and NSSEO. Prepared particles as noninvasive penetration enhancement technique have upgraded the skin penetration of indomethacin, and reduce its systemic concentration and side effects.

Part V
PERSPECTIVES

Perspectives

The highly encouraging results of this PhD thesis open new ingenious avenue to enhance inflammation inhibitory properties of indomethacin. It could be hoped that the encapsulation of indomethacin together with *Nigella Sativa* L. Seeds Essential Oil within poly- ϵ -caprolactone enhance the anti-inflammatory activity, the efficacy of indomethacin treatment and decline its side effects. Furthermore, another way, which is opened by this PhD thesis, is to search the possibility of using anti-inflammatory and penetration enhancer from natural source. In the light of this research, different drugs will be encapsulated together with *Nigella Sativa* L. Seeds Oil for the treatment of various diseases such as cancer, tuberculosis and so on.

For better confirmation concerning encapsulation of indomethacin and *Nigella Sativa* L. Essential Oils, it is required to performed several other investigations. In addition, nanoparticles penetration and drug release in each layer of the skin, long-term stability study, indomethacin release kinetic and clinical application of nanoparticles loading indomethacin and *Nigella Sativa* L. Essential Oil would be other steps towards previously carried research.

Part VI

LIST OF PUBLICATIONS INCLUDED IN MANUSCRIPT

- 1 Encapsulation of NSAIDs for inflammation management: Overview, progress, challenges and prospects. Waisudin Badri, Karim Miladi, Qand Agha Nazari, Helene Greige-Gerges, Hatem Fessi, Abdelhamid Elaissari (2016).
- 2 Plant extracts: from encapsulation to application. Brenda Armendáriz Barragán, Nadiah Zafar, Waisudin Badri, Sergio Arturo Galindo Rodríguez, Kabbaj D, Hatem Fessi, Abdelhamid Elaissari (2016).
- 3 Nanoprecipitation process: From encapsulation to drug delivery. Claudia Janeth Martínez Rivas, Mohamad Tarhini, Waisudin Badri, Karim Miladi, Helene Greige-Gerges, Qand Agha Nazari, Sergio Arturo Galindo Rodríguez, Rocío Álvarez Román, Hatem Fessi, Abdelhamid Elaissari (2017).
- 4 Effect of process and formulation parameters on polycaprolactone nanoparticles prepared by solvent displacement. Waisudin Badri, Karim Miladi, Qand Agha Nazari, Hatem Fessi, Abdelhamid Elaissari (2016).
- 5 Polycaprolactone Based Nanoparticles Loaded with Indomethacin for Anti-Inflammatory Therapy: From Preparation to *Ex Vivo* Study. Waisudin Badri, Karim Miladi, Sophie Robin, Céline Viennet, Qand Agha Nazari, Géraldine Agusti, Hatem Fessi, Abdelhamid Elaissari. (2017).
- 6 Poly (ϵ -caprolactone) nanoparticles loaded with indomethacin and *Nigella Sativa* L. essential oil for the topical treatment of inflammation, Inflammation topical enhanced treatment. Waisudin Badri, A. El Asbahani, Karim Miladi, Abdellatif Baraket, Géraldine Agusti, Qand Agha Nazari, Abdelhamid Errachid, Hatem Fessi, Abdelhamid Elaissari (2018).
- 7 Topical co-delivery of indomethacin and *Nigella Sativa* L. Essential oil in poly- ϵ -caprolactone nanoparticles: *In Vivo* study of anti-inflammatory activity. Waisudin Badri, Farahidah Mohamed, Mohd Affendi, A. El Asbahani, Karim Miladi, Qand Agha Nazari, Céline Viennet, Sophie Robin, Hatem Fessi, Abdelhamid Elaissari (2018).