MICROENCAPSULATION: A PROMISING TECHNIQUE TO REDUCE THE ENVIRONMENTAL CONTAMINATION BY ANTIBIOTICS

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SPECIALIZATION IN BIOLOGICAL ENGINEERING

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“Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito. Condições de palácio tem qualquer terra larga, mas onde estará o palácio se o não fizerem ali?”

In Livro do Desassossego, Fernando Pessoa
ACKNOWLEDGMENTS

I would like to express my very great appreciation to all those who supported me in any way during this work.

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I deeply thank my parents, Manela and Rui, and my close friends and family, for the patience, love and encouragement, and for always believing in me.

I want to wholeheartedly thank my best friend, Paulo, the true friendship, love, unconditional support and encouragement. Thank you for always being there.

To all those I did not mentioned personally, but that somehow helped me to conclude this work, I also express my gratitude.

“What if today, we were just grateful for everything?”

Charles Schulz

I dedicate this thesis to my parents.
ABSTRACT

The massive and irrational consumption of antibiotics for several decades, combined with their high rates of excretion as parental compounds, is becoming a serious problem once it leads to an increasing concentration of antibiotics in active form in the environment. This is becoming progressively more alarming due to development and spread of antibiotic resistance, that leads to the lack of functions of these drugs that once saved millions of lives. Thus, microencapsulation of antibiotics may be a promising solution to this problem. With this technique the amount of the administered antibiotic required to cure the infections is reduced, since it allows less drug doses and a small amount per dose.

In this work, metronidazole (MNZ) and sulfamethoxazole (SMX) loaded ethylcellulose microparticles were prepared by double emulsion solvent evaporation technique (w/o/w). UV-Vis spectrophotometry methods for MNZ and SMX determination were developed and validated. The methods presented good linearity with correlation coefficients greater than 0.995, and good precision results with coefficients of variation values lower than 5%. In case of MNZ, the limits of detection were 1.29 mg/L for PBS pH 2 and 0.92 mg/L for PBS pH 7, while the limits of quantification were 4.30 mg/L and 3.06 mg/L, respectively. For SMX, the limits of detection were 1.19 mg/L for PBS pH 2 and 0.37 mg/L for PBS pH 7, while the limits of quantification were 3.97 mg/L and 0.92 mg/L, respectively. It was concluded that is possible to encapsulate MNZ and SMX using ethylcellulose through a double emulsion process. The product yield was 71.4% for MNZ and 95.7% for SMX. Drug loadings of 1.1% for MNZ and 7.8% for SMX were obtained and encapsulation efficiency was 4.6% in case of MNZ and 32.1% in case of SMX. SEM analysis revealed round microparticles with smooth and porous surface and mean particle sizes of 0.50 μm (SMX) and 1.26 μm (MNZ) were obtained. In vitro release studies in PBS buffer at pH 2 and pH 7 showed a first initial burst release, a constant sustained release of the remaining antibiotic, and a constant concentration after complete release of antibiotic. MNZ microparticles exhibited, for both pH values, a fast initial release (≈ 80% after 3 h) and a sustained behavior until complete MNZ release at around 20 h. In case of SMX loaded ethylcellulose microparticles, different release profiles were observed for the two studied pH values. At pH 2, SMX microparticles showed a slower release profile, reaching low % of release (≈ 40% after 2 h). It means that these microparticles are a good approach for the delivery of SMX in the gastrointestinal tract, once the antibiotic is not significantly released in the stomach. Regarding pH 7, SMX microparticles exhibited a fast initial release (≈ 30% after 15 min) and it was observed 80% of release after 5 h. Complete SMX release occurred at around 16 h. In the future, the development of safe and efficient microencapsulation techniques for antibiotics will require in-depth investigations of both the biological and technological fields.

Key words: antibiotics, environment, microencapsulation, MNZ, SMX, controlled drug delivery, UV-Visible Spectrophotometric Analysis
RESUMO

O uso massivo e abusivo dos antibióticos por várias décadas, aliado às elevadas taxas de excreção destes fármacos, principalmente na forma de composto parental, provocou um aumento da sua concentração na sua forma ativa no meio ambiente. Este problema está a tornar-se cada vez mais alarmante uma vez que potencia o desenvolvimento e propagação de resistência a antibióticos e consequentemente perda da eficácia destes fármacos. Posto tudo isto, a microencapsulação surge como uma técnica promissora para minimizar este problema. Com esta tecnologia, a quantidade de antibiótico administrada é reduzida, sem comprometer a terapêutica, isto porque permite ao paciente realizar menos tomas e em menor quantidade.

Neste trabalho, os antibióticos metronidazol (MNZ) e sulfametoxazol (SMX) foram microencapsulados com etilcelulose como agente encapsulante pela técnica de dupla emulsão. Foi validado o método de quantificação dos antibióticos em estudo usando a espectroscopia UV-Vis. Este método apresentou boa linearidade, com coeficientes de correlação superiores a 0,995, e boa precisão, com coeficientes de variação inferiores a 5%. No caso do MNZ, os limites de detecção foram 1,29 mg/L para a análise em tampão fosfato salino (PBS - phosphate buffered saline) pH 2 e 0,92 mg/L em PBS pH 7, enquanto os limites de quantificação foram 4,30 mg/L e 3,06 mg/L, respectivamente. Para o SMX, os limites de detecção foram 1,19 mg/L para a análise em PBS pH 2 e 0,37 mg/L em PBS pH 7, enquanto os limites de quantificação foram 3,97 mg/L e 0,92 mg/L, respectivamente. Foi possível encapsular MNZ e SMX usando etilcelulose por dupla emulsão. O rendimento do processo foi de 71,4% para as micropartículas de MNZ e de 95,7% para as micropartículas de SMX. A carga de antibiótico foi de 1,1% para o MNZ e 7,8% para o SMX. Foram obtidas eficiências de encapsulação de 4,6% (MNZ) e 32,1% (SMX). As partículas analisadas mostraram ser redondas, com uma superfície suave e porosa, com tamanhos médios de 0,50 µm (SMX) e 1,26 µm (MNZ). Os estudos de liberação controlada em tampão PBS a pH 2 e pH 7 mostraram uma fase inicial de liberação rápida, seguida de uma fase de liberação constante até se atingir a liberação completa. As micropartículas de MNZ revelaram, para ambos os valores de pH do meio, uma liberação inicial rápida (≈ 80% em 3 h) seguida de uma liberação controlada até à completa liberação do MNZ após 20 h. No caso do SMX, a liberação foi diferente para os dois valores de pH estudados. A pH 2, as micropartículas revelaram uma liberação mais lenta (≈ 40% em 2 h). Isto poderá significar que as micropartículas podem ser aplicadas para a liberação controlada de SMX no trato gastrointestinal, uma vez que não há uma liberação significativa do antibiótico no estômago. A pH 7, a liberação inicial foi mais rápida (≈ 30% em 15 min) e atingiu-se 80% de libertação em 5 h. A liberação total ocorreu passado aproximadamente 16 h.

No futuro, o desenvolvimento de técnicas de microencapsulamento eficientes para antibióticos necessitará de mais investigações tanto no campo biológico como tecnológico.

Palavras-chave: antibióticos, ambiente, microencapsulação, MNZ, SMX, libertação controlada de fármacos, espectrofotometria UV-Visível.
DECLARATION

The author declares, under oath, that this work is original and that all non-original contributions were properly referenced.

[Signature]
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NOTATION AND GLOSSARY

Roman symbols

\( a \) Slope of calibration curve \( \text{mAU} \)
\( b \) Intercept of calibration curve \( \text{(mAU.L)/} \mu \text{g} \)
\( K_{\text{ow}} \) Octanol-water partition coefficient
\( pK_a \) Acidic dissociation constant
\( S/N \) Signal-to-noise ratio
\( s_a \) Standard deviation of slope \( \text{(mAU.L)/} \mu \text{g} \)
\( s_b \) Standard deviation of intercept \( \text{mAU} \)
\( T \) Temperature \( \text{K} \)
\( t_{s_a} \) Confidence interval for slope \( \text{(mAU.L)/} \mu \text{g} \)
\( t_{s_b} \) Confidence interval for intercept \( \text{mAU} \)

Abbreviations and acronyms

5-FU 5-Fluorouacil
\( \text{APA} \) Agência Portuguesa do Ambiente
\( \text{ARB} \) Antibiotic resistance bacteria
\( \text{CEMUP} \) Centro de Materiais da Universidade do Porto
\( \text{CIPRO} \) Ciprofloxacin
\( \text{CV} \) Coefficient of variation
\( \text{DCM} \) Dichloromethane
\( \text{DOX} \) Doxycycline
\( \text{EcoFEUP} \) Sistema de Gestão Ambiental da FEUP
\( \text{FDA} \) Food and Drug Administration
\( \text{FTI} \) Fourier Transform Infrared
\( \text{HIV} \) Human Immunodeficiency Virus
\( \text{HPLC} \) High Performance Liquid Chromatography
\( \text{IgY} \) Immunoglobulin Y
\( \text{LOD} \) Limit of detection
\( \text{LOQ} \) Limit of quantification
\( \text{MIC} \) Minimal Inhibitory Concentration
\( \text{MNZ} \) Metronidazole
\( \text{MRSA} \) Methicillin-resistant Staphylococcus aureus
\( \text{Mw} \) Molecular weight
\( \text{o/o} \) Oil in oil
\( \text{o/w} \) Oil in water
\( \text{PABA} \) para-aminobenzoic acid
\( \text{PBP} \) Penicillin binding protein
Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics

PBS Phosphate Buffered Saline
PCL Polycaprolactone
PHB Polyhydroxybutyrate
PLA Poly(lactic acid)
PLGA Poly(lactic-co-glycolic acid)
PLHMGA Poly(lactic-co-hydroxymethyl-glycolic acid)
PVA Poly(vinyl alcohol)
R Correlation coefficient
SEM Scanning Electron Microscopy
SMX Sulfamethoxazole
TAU Taurocholate
TFE Trifluoroethanol
TMP Trimethoprim
UV Ultraviolet
VA Vancomycin
Vis Visible
w/o Water in oil
w/o/w Water in oil in water
WHO World Health Organization
WWTP Waste Water Treatment Plant
1. BACKGROUND AND AIMS

Antibiotics are frequently used today for the therapy of infectious diseases both in human and veterinary medicine. In human medicine, antibiotics pose the third biggest group among all pharmaceuticals and in veterinary medicine, more than 70% of all consumed pharmaceuticals are antibiotic agents. So, antibiotics are designed to act very effectively even at low doses and, after a short time of residence, 50% to 95% of administered antibiotics are excreted from the body. Consequently, these substances are released to the environment and residual concentrations of pharmaceutical antibiotics are already found in the environment. Antibiotics became a part of our lives, both in terms of health as in terms of their invisible presence in supermarket product shelves (Thiele-Bruhn 2003).

The massive and irrational consumption of antibiotics, combined with their high rates of excretion in active form, is becoming a serious problem due the increasing concentration of antibiotics in the environment. This is becoming progressively more alarming due to development and spread of antibiotic resistance, that leads to the lack of functions of these drugs that once saved millions of lives. Antibiotic resistance became a matter of public news and executive orders of governments. Undoubtedly, the misuse and overuse of antibiotics are major contributors to development and spread of resistance (Zgurskaya 2015a).

Worldwide consumption of antibiotics increased by 30% between 2000 and 2010, and more than 50% of sold antibiotics are administered to animals for human consumption. Estimates indicate that by 2030 the consumption of antibiotics in livestock can grow about 67%, from 63 to 106 thousand tons (Union 2012; Philpott 2013; Reardon 2015; Van Boeckel et al. 2015). Knowing that microparticles can improve the effectiveness of various medical treatments, the microencapsulation of antibiotics may be an effective approach to reduce the amount of the administered antibiotic required to cure the infections. Consequently, this technique can be an interesting and promising option to reduce the amount of antibiotics discharged into the environment. Nevertheless, the implementation of this strategy to minimize this environmental and human health concern has not yet been explored (Singh et al. 2010).

It is known that controlled drug delivery system offers potential advantages over conventional drug delivery systems. Microcapsules are established as unique carrier systems for many pharmaceuticals and can be tailored to adhere to targeted tissue systems. Hence, microcapsules can be used not only for controlled release but also for targeted delivery of drugs to a specific site in the body. Although significant advances have been made in the field of microencapsulation, there are still many challenges ahead in this field (Singh et al. 2010). To the antibiotics in particular, the challenge is to microencapsulate them with success. Therefore, the development of safe and efficient particular systems will require, in the future, in-depth investigations of both the biological and technological aspects of these systems (Singh et al. 2010).
2. INTRODUCTION

2.1. ANTIBIOTICS

2.1.1. Classification and physicochemical and pharmacological properties

Antibiotics are classically defined as chemical compounds, synthetized through the secondary metabolism of living organisms, which inhibit or abolish the growth of bacteria by specific interactions with the microorganism targets without having toxic effects on the host. However, this definition has changed over the years and now the term antibiotics includes a wider diversity of bioactive, or bio-functional compounds, including semi-synthetic and synthetic drugs (Thiele-Bruhn 2003; Sergio Sánchez and Arnold L. Demain 2015). The empirical search for natural products in new and diverse places yielded most of the antibiotics that are currently used in medical and non-medical applications. This approach continues to expand the chemical diversity and biological activities of antibiotics (Zgurskaya 2015b). Antibiotics are small molecules with a molecular weight of less than 1000 Da (Kümmerer 2009a).

Although several criteria are used to classify antibiotics, the most useful and important is based on their chemical structure since antibiotics within a structural class will generally have similar pharmacological properties (spectrum of activity, effect on bacteria, and mode of action). Their diverse physicochemical properties results from the presence of different side-chains (Thiele-Bruhn 2003; Kümmerer 2009b). In Table 1 is presented an overview of the most important classes of antibiotics as well as their chemical structure and some physicochemical and pharmacological properties.
Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics

Table 1. Important classes and groups of antibiotic compounds and their physicochemical properties. Adapted from Booth & McDonald (1988), Thiele-Bruhn (2003).

<table>
<thead>
<tr>
<th>Compound class</th>
<th>Chemical structure</th>
<th>Water solubility (mg/L)</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt; / pK&lt;sub&gt;a&lt;/sub&gt; / pK&lt;sub&gt;a&lt;/sub&gt;</th>
<th>log K&lt;sub&gt;ow&lt;/sub&gt;</th>
<th>Pharmacological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>Aminoglycosides consist of two or more amino sugars bounded to a hexose (2-deoxystreptamine) centrally placed ring by a glycosidic linkage. Particularly, streptomycins have two amino sugars linked to a hexose (streptidine)</td>
<td>1x10&lt;sup&gt;4&lt;/sup&gt;-5x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.9-8.5</td>
<td>-8.1-(-0.8)</td>
<td>Spectrum of activity: activity against Gram-negative aerobes and some anaerobic bacilli</td>
</tr>
<tr>
<td></td>
<td>Effect on bacteria: bactericidal</td>
<td></td>
<td></td>
<td></td>
<td><strong>Effect on bacteria:</strong> bactericidal</td>
</tr>
<tr>
<td></td>
<td>Mode of action: inhibition of protein synthesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lactams</td>
<td>Penicillins</td>
<td>22.10100</td>
<td>2.7</td>
<td>0.9-2.9</td>
<td>Spectrum of activity: mainly active against Gram-positive bacteria, yet the development of broad-spectrum β-lactams against various Gram-negative organisms</td>
</tr>
<tr>
<td></td>
<td>Effect on bacteria: bacteriostatic and bactericidal</td>
<td></td>
<td></td>
<td></td>
<td><strong>Effect on bacteria:</strong> bacteriostatic and bactericidal</td>
</tr>
<tr>
<td></td>
<td>Mode of action: inhibition of the synthesis of cell walls by inhibiting peptidoglycan synthesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorquinolones</td>
<td>Particularly, fluoroquinolones have a C-6 fluorine atom attached to ring (R&lt;sub&gt;4&lt;/sub&gt; substituent)</td>
<td>3.2-17790</td>
<td>8.6</td>
<td>-1.0-1.6</td>
<td>Spectrum of activity: broad spectrum</td>
</tr>
<tr>
<td></td>
<td>Effect on bacteria: bactericidal</td>
<td></td>
<td></td>
<td></td>
<td><strong>Effect on bacteria:</strong> bactericidal</td>
</tr>
<tr>
<td></td>
<td>Mode of action: binds DNA coiling enzymes, inducing double-stranded DNA breaks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Basic structure of glycopeptide antibacterials are glycosylated cyclic or polycyclic nonribosomal peptide</td>
<td>&gt;1000</td>
<td>5.0</td>
<td>not soluble in octanol</td>
<td>Spectrum of activity: narrow spectrum</td>
</tr>
<tr>
<td></td>
<td>Effect on bacteria: bacteriostatic and bactericidal only against the enterococci</td>
<td></td>
<td></td>
<td></td>
<td><strong>Effect on bacteria:</strong> bacteriostatic and bactericidal only against the enterococci</td>
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<tr>
<td></td>
<td>Mode of action: inhibition of the synthesis of cell walls by inhibiting peptidoglycan synthesis</td>
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</tbody>
</table>

Introduction
**Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics**

<table>
<thead>
<tr>
<th>Compound class</th>
<th>Chemical structure</th>
<th>Water solubility (mg/L)</th>
<th>(pK_a^1/ pK_a^2/ pK_a^3)</th>
<th>(\log K_{ow})</th>
<th>Pharmacological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Imidazoles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fenbendazole</td>
<td></td>
<td>6.3-407</td>
<td>2.4</td>
<td>-0.02-3.9</td>
<td>Spectrum of activity: broad spectrum</td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Effect on bacteria: bactericidal</td>
</tr>
<tr>
<td>Oxendazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mode of action: inhibition of the biosynthesis of ergosterol</td>
</tr>
<tr>
<td><strong>Macrolides</strong></td>
<td>Macrolides are characterized by a large macrocyclic lactone, generally with 12, 14, or 16 members, attached to one or more sugars</td>
<td>0.45-15</td>
<td>7.7-8.9</td>
<td>1.6-3.1</td>
<td>Spectrum of activity: broad spectrum against Gram-positive and limited Gram-negative bacteria</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Effect on bacteria: bactericidal</td>
</tr>
<tr>
<td>Oleandomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mode of action: inhibition of protein synthesis</td>
</tr>
<tr>
<td>Tylosin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Polyethers</strong></td>
<td>Polyethers are characterized by multiple tetrahydrofuran and tetrahydropyran rings connected by aliphatic bridges, direct C-C linkages or spiro linkages</td>
<td>2.2x10^{-6}-3.1x10^{-3}</td>
<td>6.4</td>
<td>5.4-8.5</td>
<td>Spectrum of activity: broad spectrum</td>
</tr>
<tr>
<td>Monensin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Effect on bacteria: bactericidal</td>
</tr>
<tr>
<td>Salinomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mode of action: selectivity kill of cancer stem cells and multidrug-resistant cancer cells</td>
</tr>
<tr>
<td><strong>Polypeptides</strong></td>
<td>Polypeptides are a chemical diverse class of anti-infective and antitumor antibiotics containing no-protein polypeptide chains</td>
<td>not soluble in water</td>
<td>-1.0-3.2</td>
<td>1.0-3.2</td>
<td>Spectrum of activity: broad spectrum</td>
</tr>
<tr>
<td>Avermectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Effect on bacteria: bactericidal</td>
</tr>
<tr>
<td>Bactitracin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mode of action: bind to membranes</td>
</tr>
<tr>
<td>Ivermectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virginiamycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Quinoxaline-derivates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olaquindox</td>
<td></td>
<td>1x10^{6}</td>
<td>10.0</td>
<td>-2.2</td>
<td>Spectrum of activity: highly active against Gram-positive bacteria</td>
</tr>
<tr>
<td><strong>Sulfonamides</strong></td>
<td>Sulfadiazine</td>
<td>7.5-1500</td>
<td>2 · 3 / 4.5-10.6</td>
<td>-0.1-1.7</td>
<td>Spectrum of activity: broad spectrum</td>
</tr>
<tr>
<td></td>
<td>Sulfadimethoxine</td>
<td></td>
<td></td>
<td></td>
<td>Effect on bacteria: bacteriostatic and bactericidal</td>
</tr>
<tr>
<td></td>
<td>Sulfadimidine</td>
<td></td>
<td></td>
<td></td>
<td>Mode of action: inhibition of the synthesis of cell walls by inhibiting peptidoglycan synthesis and also exhibit antitumoral effect</td>
</tr>
<tr>
<td></td>
<td>Sulfamethoxazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulfanilamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulfapyridine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Introduction**
Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics

<table>
<thead>
<tr>
<th>Compound class</th>
<th>Chemical structure</th>
<th>Water solubility (mg/L)</th>
<th>pKₐ₁/ pKₐ₂/ pKₐ₃</th>
<th>log Kᵦw</th>
<th>Pharmacological properties</th>
</tr>
</thead>
</table>
| Tetracyclines  | ![Chemical structure](image) | 230-52000 | 3.3/ 7.7/ 9.3 | -1.3-0.05 | Spectrum of activity: broad spectrum  
Effect on bacteria: bacteriostatic  
Mode of action: inhibition of protein synthesis |

pKₐ: acidic dissociation constant  
Kᵦw: octanol-water partition coefficient
As presented in Table 1, most antibiotics tend to ionize accordingly to the pH of the medium and their acidic dissociation constants (pK<sub>a</sub>) values, which are associated to the different functional groups of antibiotics. The different ionic species that are presented in aqueous solutions at different pH values often have different properties, such as water solubility, ultraviolet (UV) absorption, and volatility (Qiang and Adams 2004). Moreover, octanol-water partition coefficient (K<sub>ow</sub>) values can be considered to have interest, since they represent the tendency of the chemical to partition itself between an organic phase, such as a soil, and an aqueous phase, such as groundwater.

There is a relationship between the chemical structure of antibiotics and their physicochemical, microbiological, and pharmacological properties. Consequently, the compound class and the consumption of these drugs are interrelated. Moreover, the abovementioned physicochemical and pharmacological properties of antibiotics are an important background for their microencapsulation since they allow to infer the most suitable mechanism and agent of microencapsulation for each antibiotic compound. They are also important characteristics for the determination of the occurrence of antibiotics into the environment, which is presented in the section 2.1.3.1.

2.1.2. Market

Between 1940 and 1962, more than 20 new classes of antibiotics were marketed. Since then, only two new classes have reached the market. Numerous analogues of existing classes have reached the market in this time period (Coates et al. 2011).

The antibiotics sales by class in 2009 in US$ billions are presented in Figure 1.

Figure 1. Antibiotic sales by class in 2009. Adapted from (Hamad 2010).

Antibiotics are widely commercialized, mostly cephalosporins, penicillins, fluoroquinolones and macrolides, according to Figure 1. The antibiotic compound classes primarily administered in veterinary medicine are tetracyclines, sulfanomides,
aminoglycosides, β-lactams and macrolides. In human medicine β-lactams, tetracyclines and macrolides are mostly prescribed (Thiele-Bruhn 2003).

2.1.3. The environmental problem

2.1.3.1. Occurrence of antibiotics into the environment

Antibiotics are widely commercialized for use in human and animals to treat diseases. In animals, they are also used as growth promoters and to improve feed efficiency (Sarmah et al. 2006). Nowadays, antibiotics play a major role in modern agriculture and livestock industries and their use has been on the rise in many developed nations (Barbosa and Levy 2000). However, these pharmaceuticals are poorly adsorbed in the gut of the animal, resulting in as much as 30–90% of the parent compound being excreted (Halling-Sørensen et al. 1998; Alcock et al. 1999). In addition, antibiotic metabolites can also be bioactive and can be transformed back to the parent compound after excretion. Because of that, the intra-corporal administration of antibiotics inevitably leads to residual concentration in excrements. The excretion rates for different antibiotic compounds have also been described by Kümmnerer (2009) and Thiele-Bruhn (2003). In humans, about 50% to 90% of antibiotics administered are excreted after a short time of residence. Like other drugs, these compounds are designed and optimized in their pharmacokinetics to not accumulate in the organism. After administration, antibiotics for human use or their metabolites are excreted into the effluent and reach the sewage treatment plant. As already described by Kümmnerer (2009) and Thiele-Bruhn (2003) antibiotics are not totally eliminated in sewage treatment plants. As a consequence, if they are not eliminated during the purification process, they may end up in the environment, mainly in water compartment. Besides, residual amounts can reach surface waters, groundwater, drinking water, soils or sediments. The different sources and pathways of antibiotics on the environment are presented in Figure 2.
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Agricultural waste storage structures. Other pathways for dissemination are via land application of human and agricultural waste, surface runoff and unsaturated zone transport (Sarmah et al. 2006). WWTP: Waste Water Treatment Plant.

The sources of antibiotics in the environment, such as natural background, production and manufacturing, human and veterinary medicine, plant agriculture, feeding animals or aquaculture result in detectable residual concentrations in diverse environmental compartments. The concentration range of the antibiotic compounds in aquatic environments and in soils is presented in tables 2 and 3, respectively.

Table 2. Examples of measured concentrations of antibiotics in the aquatic environment.

<table>
<thead>
<tr>
<th>Antibiotics Classes</th>
<th>Active agents</th>
<th>WWTP effluent</th>
<th>Surface water</th>
<th>Ground water</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrolides</td>
<td>Azithromycin</td>
<td>3</td>
<td>65</td>
<td>49</td>
<td>(Christian et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Clarithromycin</td>
<td>240</td>
<td>260</td>
<td></td>
<td>(Giger et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>287</td>
<td>1700</td>
<td></td>
<td>(Hirsch et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>Macrolide</td>
<td>700</td>
<td>20</td>
<td>2</td>
<td>(Christian et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Roxithromycin</td>
<td>1000</td>
<td>560</td>
<td>26</td>
<td>(Hirsch et al. 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td></td>
<td></td>
<td>(Sacher et al. 2001)</td>
</tr>
<tr>
<td>Penicillins</td>
<td>Fluoxacillin</td>
<td>7</td>
<td></td>
<td></td>
<td>(Christian et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>200</td>
<td>3</td>
<td></td>
<td>(Christian et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Piperacillin</td>
<td>48</td>
<td></td>
<td></td>
<td>(Christian et al. 2003)</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Ciprofloxacin</td>
<td>30</td>
<td>26</td>
<td></td>
<td>(Kolpin et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Fluorquinolone</td>
<td>100</td>
<td>5</td>
<td></td>
<td>(Calamari et al. 2003)</td>
</tr>
<tr>
<td>Sulfanomides</td>
<td>Sulfadimidin</td>
<td>7</td>
<td></td>
<td></td>
<td>(Christian et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Sulfamethazin</td>
<td>160</td>
<td></td>
<td></td>
<td>(Hirsch et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>Sulfamethoxazole</td>
<td>370</td>
<td>163</td>
<td>410</td>
<td>(Alexy et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>Sulfanomide</td>
<td>2000</td>
<td>480</td>
<td>470</td>
<td>(Sacher et al. 2001)</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Oxytetracycline</td>
<td>1000</td>
<td>40</td>
<td>20</td>
<td>(Hirsch et al. 1999)</td>
</tr>
<tr>
<td>Others</td>
<td>Chloramphenicol</td>
<td>560</td>
<td>60</td>
<td></td>
<td>(Christian et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>110</td>
<td></td>
<td></td>
<td>(Hirsch et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>Spiramycin</td>
<td>74</td>
<td></td>
<td></td>
<td>(Calamari et al. 2003)</td>
</tr>
</tbody>
</table>

These studies have shown that antibiotic compounds are transported into surface water and groundwater from urban and agricultural sources. It should be noted that the concentration of antibiotics decreases from effluents to surface water, and it reaches minimal values in drinking water. Nevertheless, there are still significant concentrations of these compounds in tap water, which represents a risk.

Table 3. Examples of measured concentrations of antibiotics in soils, sediments and slurry.

<table>
<thead>
<tr>
<th>Antibiotics Classes</th>
<th>Active agents</th>
<th>Concentration (µg/g)</th>
<th>Samples</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazoles</td>
<td>Metronidazole</td>
<td>100–2000 mg/g</td>
<td>pig manure</td>
<td>(Rabelle and Spliid 2000)</td>
</tr>
<tr>
<td>Lipoglycosides</td>
<td>Efrotomycin</td>
<td>1.0–135</td>
<td>sandy loam (pH 7.5)</td>
<td>(Yeagert and Halley 1990)</td>
</tr>
</tbody>
</table>
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The presence of residues of pharmaceutical antibiotics in soils and aquatic environments may be related to emergence of antibiotic resistance (Sarmah et al. 2006). This phenomenon is discussed in the next section.

#### 2.1.3.2. Antibiotics resistance

Antibiotics have been overused and misused. Even though they were developed originally to treat human infectious diseases, their properties in veterinary, animal and plant agriculture and aquaculture were applied soon thereafter (Barbosa and Levy 2000). Once in the environment, if antibiotics are not efficiently degraded, it is possible that these residues may assist in maintaining or developing antibiotic resistant microbial populations (Sarmah et al. 2006). Antibiotic resistance can occur by three main ways: by natural resistance in certain types of bacteria, by genetic mutation, or by one species acquiring resistance from another. Acquired resistance is the result of contamination by antibiotics (Bebell and Muiru 2014). Even when used appropriately, antibiotics and bacteria resistant to antibiotics seep into the local drinking water sources after human, agricultural, and animal use and wastewater treatment (Bebell and Muiru 2014). An increase in antibiotic resistance is observed and consequently a growing number of infections and patients who are dying from infectious diseases. Additionally, the number of reports on drug-resistant strains of bacteria is rising, while industrial efforts in antibiotic discovery and development have been declining (C. Walsh., Von Döhren 2003).

Due to the evolution of antibiotic resistance, the enormous success of using antibiotics to save lives is being challenged. This resistance has led to predictions that we will soon be returning to the pre-antibiotic era whereby even the most minor infection has the potential to have severe consequences on the infected person. Fortunately, there has been an increase in the efforts to combat this rise in antibiotic resistance (Schofield 2015). Available quantitative evidence on excess harm caused to patients through drug resistance comes mainly from experiences with malaria, tuberculosis, and to some extent, human immunodeficiency virus

<table>
<thead>
<tr>
<th>Antibiotics Classes</th>
<th>Active agents</th>
<th>Concentration (µg/g)</th>
<th>Samples</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrolides</td>
<td>Tylosin</td>
<td>50000</td>
<td>kaolinite, illite, montmorillonite, and bentonite</td>
<td>(Bewick 1979)</td>
</tr>
<tr>
<td>Polypeptides</td>
<td>Avermectin</td>
<td>0.006 - 2.17</td>
<td>sand (pH 7.5)</td>
<td>(Gruber et al. 1990)</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Ciprofloxacin</td>
<td>2 - 200</td>
<td>loamy sand (pH 5.3)</td>
<td>(Halling-Sørensen 2000)</td>
</tr>
<tr>
<td></td>
<td>Ofloxacinc</td>
<td>2 - 200</td>
<td>loamy sand (pH 5.3)</td>
<td>(Nowara et al. 1997)</td>
</tr>
<tr>
<td>Quinoxaline</td>
<td>Olaquindox</td>
<td>100 - 2000 mg/g</td>
<td>pig manure</td>
<td>(Halling-sørensen 2016)</td>
</tr>
<tr>
<td>derivatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfanomides</td>
<td>Sulfadimidine</td>
<td>1.25 - 25</td>
<td>sand (pH 5.6)</td>
<td>(Rabølle and Spliid 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 - 25</td>
<td>sand (pH 5.2)</td>
<td>(Burkhardt et al. 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>loamy sand (pH 5.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>clay silt (pH 6.9)</td>
<td></td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Sulfapyridine</td>
<td>0.1 - 500</td>
<td>sit loam (pH 7.0)</td>
<td>(Thiele 2000)</td>
</tr>
<tr>
<td></td>
<td>Oxytetracycline</td>
<td>2.5 - 50</td>
<td>sand (pH 5.6)</td>
<td>(Rabølle and Spliid 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>285</td>
<td>organic marine sediment</td>
<td>(Smith and Samuelsen 1996)</td>
</tr>
</tbody>
</table>
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(HIV) (WHO 2012). In Table 4, it is presented the estimated annual burden due to selected antibiotic-resistance bacteria in European Union Member States, Iceland and Norway, in 2007.

<table>
<thead>
<tr>
<th>Antibiotic-resistance bacteria</th>
<th>No. of cases of infection*</th>
<th>No. extra deaths</th>
<th>No. extra hospital days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimicrobial resistant Gram-positive bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin-resistant <em>Staphylococcus aureus</em> (MRSA)</td>
<td>171200 (12%)</td>
<td>5400 (37%)</td>
<td>1050000 (16%)</td>
</tr>
<tr>
<td>Vancomycin-resistant <em>Enterococcus faecium</em></td>
<td>18100 (9%)</td>
<td>1500 (28%)</td>
<td>111000 (22%)</td>
</tr>
<tr>
<td><strong>Antimicrobial resistant Gram-negative bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd generation cephalosporin-resistant <em>Escherichia coli</em></td>
<td>32500 (27%)</td>
<td>5100 (52%)</td>
<td>358000 (27%)</td>
</tr>
<tr>
<td>3rd generation cephalosporin-resistant <em>Klebsiella pneumoniae</em></td>
<td>18900 (27%)</td>
<td>2900 (52%)</td>
<td>208000 (27%)</td>
</tr>
<tr>
<td>Carbapenem-resistant <em>Pseudomonas aeruginosa</em></td>
<td>141900 (3%)</td>
<td>10200 (7%)</td>
<td>809000 (3%)</td>
</tr>
</tbody>
</table>

* Bloodstream infections, lower respiratory tract infections, skin and soft tissue infections, and urinary tract infections. Numbers in parentheses indicate percentage bloodstream infections.

According to WHO (2012), there were an increase on resistance among bacteria causing pneumonia, which kills about 1.8 million children annually. Besides, in 2013 there were about 480 000 new cases of multidrug-resistant tuberculosis. Extensively drug-resistant tuberculosis has been identified in 100 countries. Another consequence of antimicrobial resistance in healthcare facilities and community-associated infections is the need to change prescribing practices to newer, more costly medicines, some of which are also associated with higher rates of adverse reactions (WHO 2012).

A well-known case of bacteria resistance to antibiotics is methicillin-resistant *Staphylococcus aureus* (MRSA). *S. aureus* is a gram-positive bacterium that colonizes a variety of animal species and humans (Smith 2015). In 1941, virtually all strains of *S. aureus* worldwide were susceptible to penicillin G, but by 1944 *S. aureus* was capable to hydrolyze penicillin by penicillinase, which today is called a β-lactamase. Methicillin, a semisynthetic penicillin, has then commercialized to overcome the problem. Resistance to methicillin was noted in hospitals in several countries and in the 1980s MRSA became a problematic, particularly in long-term care facilities and among narcotics abusers. MRSA is resistant to all β-lactams, penicillins, cephalosporins, carbapenems, and penems because of a mecA gene that produces a new penicillin binding protein (PBP) that has a low affinity for β-lactam antibiotics. Approximately 25% of patients who carry MRSA will have an episode of infection, compared to 4% of those who are colonized with susceptible *S. aureus*, which is alarming. As a result of transposition and site-specific integration in many MRSA, the chromosome mediates resistance not only to β-lactam antibiotics but to other antibiotics such as erythromycin, fusidic acid, tetracycline, minocycline, streptomycin, spectinomycin, and sulfonamides and to disinfectants and toxic metals such as cadmium and mercury. Besides, the emergence of MRSA has resulted in an increased use of vancomycin (VA), the only agent that effectively treats these bacteria, but this increased use of VA has created vancomycin resistance in other species such as enterococci.
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MRSA is a classic example of the downfall of a new antimicrobial agent with respect to one specie (Neu 1992). The possible ways for the spread of MRSA are presented in Figure 3.

![Figure 3. Antibiotic-resistant Staphylococcus aureus, a growing public health concern.](image)

It is concluded that the massive and irrational use of antibiotics is a major force associated with the high numbers of antibiotic resistant pathogenic bacteria worldwide. Both the volume and the mode of antibiotic administration contributes to the selection of resistant strains (Barbosa and Levy 2000). In this context, preventing the development and rapid expansion of antibiotic resistance by reduction of the consumption of these drugs is a future priority which requires significant research advances (Kümmerer 2009b). In this work, microencapsulation appears as a promising solution to reduce this environmental contamination by antibiotics, as presented in the next sub-chapters.

### 2.2. Microencapsulation

#### 2.2.1. Definition

Microencapsulation had its origin in the late 1930s as a cleaner substitute for carbon paper and carbon ribbons as sought by the business machines industry. This is a process by which solids, liquids or even gases may be enclosed in microscopic particles formation of thin coatings of wall material around the substances. Microencapsulation provides the conversion of liquids to solids, the alteration of colloidal and surface properties, the environmental protection, and the control release of the coated materials. Several of these properties can be attained by macro packaging techniques. However, the uniqueness of microencapsulation is the smallness of the coated particles and their subsequent use and adaptation to a wide variety of dosage forms (Wang et al. 2006; Agnihotri et al. 2012).
The term “microparticle” refers to a particle with a diameter ranging in size from one micron to several millimeters (Agnihotri et al. 2012). Within the broad category of microparticles, microspheres specifically refer to spherical microparticles. In turn, microcapsules refer to microparticles composed by a core surrounded by a distinctively different encapsulating material (Singh et al. 2010). Some variations on microparticle structures are given in Figure 4.

Microencapsulation can alter some characteristics or availability of coated materials. Several of these properties can be attained by macro packaging techniques. However, the uniqueness of microencapsulation is the smallness of the coated particles and their subsequent use and adaptation to a wide variety of dosage forms (Agnihotri et al. 2012). Microencapsulation technologies are used in food and beverage, pharmaceutical, agriculture and detergent industries and they are expected to play a significant role in driving market growth (Grand View Research 2015). The microencapsulation market is presented in the next section.

2.2.2. Market

Global microencapsulation market is expected to reach USD 8.73 billion by 2020, according to a new study by Grand View Research (2015). Pharmaceutical was the largest application, accounting approximately 70% of market revenue share in 2013. Growing demand for microencapsulation for controlled release of active ingredients and targeted drug delivery is expected to have a positive impact on the market. Pharmaceutical growth in emerging economies of India, China, and Brazil is expected to augment microencapsulation market over the forecast period. Emergence of nanotechnology and microtechnology in pharmaceutical industry is expected to challenge market growth over the next six years (Grand View Research 2015). Microcapsule drug carrier systems possess high potential for various applications in therapeutic and pharmaceutical fields, such as anti-inflammation, antibiotics, anti-tumor,
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proteins, and vitamins (Lam and Gambari 2014a). In fact, microencapsulation offers various advantages for pharmaceutical applications, the sector that leads the market (Figure 5).

![Figure 5. Statistical distribution of microencapsulation over different fields of application (Martins et al. 2014).](image)

In pharmaceutical application, microencapsulation is actually used to modify and retard drug release. Microparticles offer various significant advantages as drug delivery systems, including: (i) an effective protection of the encapsulated active agent against degradation, (ii) the possibility to accurately control the release rate of the incorporated drug over periods of hours to months, (iii) an easy administration (compared to alternative parenteral controlled release dosage forms), (iv) desired, pre-programmed drug release profiles can be provided which match the therapeutic needs of the patient and (v) masking drug flavors (Chemtob et al. 1986; Singh et al. 2010). Many different coating materials and processes or techniques can be used for drug microencapsulation, as presented in the next sections.

### 2.2.3. Techniques

The various microencapsulation processes for drugs can be divided into chemical, physicochemical, and electrostatic and mechanical. Some examples are given in table 5.

<table>
<thead>
<tr>
<th>Microencapsulation process category</th>
<th>Example</th>
</tr>
</thead>
</table>
| Chemical                           | • Interfacial polymerization  
|                                    | • In-situ polymerization   |
| Physicochemical                    | • Coacervation phase separation  
|                                    | • Complex emulsion         
|                                    | • Meltable dispersion      
|                                    | • Power bed methods        |
| Electrostatic and mechanic         | • Air suspension method    
|                                    | • Pan coating              
|                                    | • Spray drying             
|                                    | • Spray congealing         
|                                    | • Micro-orifice system     
|                                    | • Rotary fluidization bed granulator method |

A brief description of reported methods used for microencapsulation of drugs is presented in table 6, as well as their advantages, disadvantages and applications.
**Table 6. List of reported methods used for the microencapsulation of drugs, as well as their advantages, disadvantages and applications (Nidhi et al., 2014).**

<table>
<thead>
<tr>
<th>Method</th>
<th>Brief description</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray drying</td>
<td>• Dispersion of the core material in a liquefied coating substance&lt;br&gt; • Spraying of the core coating mixture under specific environmental conditions&lt;br&gt; • Coating solidification is affected by rapid evaporation of a solvent in which the coating material is dissolved&lt;br&gt; • Obtaining polymer blended microspheres, which size range from 1 to 100 µm</td>
<td>• Rapid process&lt;br&gt; • Formation of porous microparticles&lt;br&gt; • Complete evaporation of organic solvent&lt;br&gt; • Operation feasible under aseptic conditions (ideal for sterile product manufacturing)&lt;br&gt; • Suitable for both hydrophobic and hydrophilic polymer</td>
<td>• High operating temperature&lt;br&gt; • Very low yield due the sticking of microparticles to the drying chamber&lt;br&gt; • Alteration of the polymorphism of spray dried drugs&lt;br&gt; • Cost of equipment</td>
<td>Production of PLGA microparticles loaded with thyrotropin releasing hormone</td>
</tr>
<tr>
<td>Single emulsion</td>
<td>• Dissolution of drug in an aqueous medium (dispersed phase), and addition of an oil phase, (continuous phase)&lt;br&gt; • Stabilization by addition of either crosslinker or by heating</td>
<td>• Simple method&lt;br&gt; • In expensive</td>
<td>• Toxicity of chemical crosslinker&lt;br&gt; • Lengthy process</td>
<td>Preparation of microspheres containing protein and peptide drugs</td>
</tr>
<tr>
<td>Double emulsification</td>
<td>• Formation of two emulsions, one is primary and other is secondary&lt;br&gt; • For primary emulsion: dispersion of drug in an aqueous phase in an oil phase containing the coating material&lt;br&gt; • Homogenization of primary phase and dropping into an aqueous solution of an emulsifier, which acts as secondary phase</td>
<td>• Controlled release&lt;br&gt; • Used for hydrophilic drugs, proteins, vaccines</td>
<td>• Stability problem&lt;br&gt; • Coalescence</td>
<td>Microencapsulation of leuprolide acetate in the PLGA (75/25) polymer</td>
</tr>
<tr>
<td>Solvent evaporation</td>
<td>• Preparation of microcapsules either by o/w or w/o or o/o type of emulsion&lt;br&gt; • Dispersion/dissolution of the core material in a polymer solution&lt;br&gt; • Addition to a continuous phase, in which either water or oil is used as</td>
<td>• Suitable for microencapsulation of lipophilic drugs like peptide for sustained delivery</td>
<td>• Removal of oil from final product in w/o type of emulsions&lt;br&gt; • Cost effective&lt;br&gt; • Use of organic solvent which is toxic, and need its</td>
<td>Preparation of drug containing Poly (D-L-lactide) microparticles</td>
</tr>
</tbody>
</table>
Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics

<table>
<thead>
<tr>
<th>Interfacial polymerization</th>
<th>Preparation of doxycycline microcapsules</th>
</tr>
</thead>
</table>
| • Dissolution of one monomer and dispersion of another separately in two immiscible liquids.  
  • Reaction at the interface between two solutions, creating a thin interfacial polymer film | • Fast, rapid  
  • Much controlled approach  
  • Efficient  
  • Complete removal from final product  
  • Denaturation of proteins and formation of aggregates  
  • Fragile and difficult to handle microcapsules  
  • Inactivation of enzymes or proteins due to large w/o interface  
  • Difficult control of the polymerization reaction  
  • Large number of washing steps for the complete removal of monomers and other by-products |

Vehicle to obtain microcapsules (heating is necessary for the complete removal of organic solvent)
In the 21st century, microencapsulation technology has been extensively applied to drug deliveries. Biopharmaceuticals can be simply produced by an oil-in-water (o/w) system or a water-in-oil (w/o) system. Generally, an o/w system refers to the processing emulsion in which the oil is used as a dispersed phase while the water-soluble/hydrophilic material is employed as a dispersed medium during the microcapsule formation process. However, a w/o system refers to the processing emulsion which contains the water-soluble/hydrophilic material as the dispersed phase and the oil as the dispersed medium during the formation process (Lam and Gambari 2014b). Double emulsion technique or water-in-oil-in-water (w/o/w) is also widely used in pharmaceutical industry, in particular in antibiotics microencapsulation (Liu et al. 2004; Mundargi et al. 2007; Patel et al. 2008; XU et al. 2008; Chaisri et al. 2009; Chaisri et al. 2011; Raval et al. 2011; Rodriguez-Contreras et al. 2013; Aina et al. 2015). Spray drying, due its advantages, namely for being a quickly and simple process, is also used for antibiotic microencapsulation by Gupta, Johnson, & Alllexon (1993), Gavini et al. (2004), Geno, Demirel, & Yazan (2006), Orhan (2006) and Pradip Patel, Raghavendra C. Mundargi, V. Ramesh Babu, Dharmendra Jain, Vidhya Rangaswamy (2008). Chaisri, Hennink, & Okonogi, (2009) evaluated the effects of emulsion type on the properties of PLGA microspheres and concluded that double emulsion technique was the most suitable method for the production of the microparticles.

Concluding, several methods and techniques are potentially useful for the preparation of polymeric microparticles in the broad field of microencapsulation. The preparation method determines the type and the size of microparticles and influence the ability of the interaction among the components used in microparticle formulations (Singh et al. 2010). The coating material used in the different processes is also a very important parameter. Its characteristics and examples of use in the pharmaceutical industry are presented in the next section.

2.2.4. Coating materials for drugs

The selection of the appropriate coating material needs consideration of some criteria, such as: (i) what are the specific dosage forms or product requirements (e.g. stabilization, reduced volatility, released characteristics, and environmental conditions); (ii) what coating material will satisfy the product objective and requirements; (iii) what microencapsulation method is best suited to accomplish the coated product objectives. This selection decides the physical and chemical properties of the resultant microparticles (Singh et al. 2010).

In recent years, much research has been focused on the usage of biodegradable polymeric microspheres as drug delivery systems. Using biodegradable and biocompatible polymeric matrices in microspheres has many benefits for developing successful drug delivery systems. Among the different classes of biodegradable polymers, the thermoplastic aliphatic esters as poly(lactic acid) (PLA) and its glycolic copolymer poly(lactic-co-glycolic acid) (PLGA) are most commonly used as drug carriers due to their excellent biocompatibility, biodegradability, and mechanical strength. They can degrade by non-enzymatic hydrolysis of the ester backbone in
body fluids yielding metabolic compounds. PLA was extensively studied in medical implants, sutures, and drug delivery systems. A large number of drugs have been delivered using this polymer. However, a wide variety of coating materials has already been used. Some polymeric material used for microencapsulation of drugs are presented in table 7.

Table 7. Some polymeric materials used for microencapsulation of drugs.

<table>
<thead>
<tr>
<th>Polymeric materials</th>
<th>Entrapped drugs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural or semi-natural</td>
<td>Alginate</td>
<td>Insulin</td>
</tr>
<tr>
<td>Alginate-chitosan</td>
<td>Isoniazid</td>
<td>(Lucinda-Silva and Evangelista 2003)</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Curcumin</td>
<td>(Das et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Celecoxib</td>
<td>(Thakkar et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Hydrocortisone</td>
<td>(Filipović-Grčić et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>5-FU</td>
<td>(Zhu et al. 2009)</td>
</tr>
<tr>
<td>Chitosan-alginate</td>
<td>Insulin</td>
<td>(Sarmento et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>IgY</td>
<td>(Canada et al. 2007)</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Ibuprofen</td>
<td>(Li et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Vitamine A palmitate</td>
<td>(Junyaprasert et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>(Lam et al. 2014)</td>
</tr>
<tr>
<td>Gelatin-agar</td>
<td>Metronidazole</td>
<td>(Chemtob et al. 1986)</td>
</tr>
<tr>
<td>Ethylcellulose</td>
<td>Bovine serum albumin</td>
<td>(Zhang et al. 2006)</td>
</tr>
<tr>
<td>PCL</td>
<td>Insulin</td>
<td>(Ibrahim et al. 2005)</td>
</tr>
<tr>
<td>PLA</td>
<td>Insulin</td>
<td>(Ibrahim et al. 2005)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Aciclovir</td>
<td>(Martínez-Sancho et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Plasmid DNA</td>
<td>(Ando et al. 1999)</td>
</tr>
</tbody>
</table>

The coating material used in the microencapsulation process should be capable of forming a film that is cohesive with the core material. It also should be chemically compatible, non-reactive with the core material and provide the desired coating properties such as strength, flexibility, impermeability, optical properties and stability (Singh et al. 2010). Generally hydrophilic polymers, hydrophobic polymers or a combination of both are used. Biodegradable polymeric microcapsules have received great attention as potential drug delivery vehicles in consideration of their applications in targeted drug delivery (Lam and Gambari 2014b).

It should be pointed out that there are a few published articles on the antibiotics microencapsulation with different techniques and microencapsulating agents. These references on antibiotic microencapsulation had positive results in terms of antibiotic performance, however the values are still low. Therefore, it is necessary to carry out further tests to prove the effectiveness and efficiency of the methods. In fact, there are some important considerations when microencapsulating antibiotics, such as the type of drug release, pharmaceutical considerations, limitation and challenges, as presented in the next section.
2.2.5. A solution for antibiotic resistance

2.2.5.1. Pharmacological considerations

Antibiotics absorption occurs in the intestine, so the coating material should only degrade and release the antibiotic when it reaches that neutral pH in the gastrointestinal tract. This is an important consideration for the controlled release studies of the microcapsules.

Another important consideration is the minimal inhibitory concentration (MIC) of the drug, which is the lowest concentration of drug that inhibits the growth of the organism. A MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. Because a lower MIC value indicates that less of the drug is required in order to inhibit growth of the organism, drugs with lower MIC scores are more effective antimicrobial agents. Currently, there are a few web-based, freely accessible MIC databases.

MIC scores are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. This is also a relevant factor when microencapsulating antibiotics because populations of bacteria exposed to an insufficient concentration of a particular drug or to a broad-spectrum antibiotic (one designed to inhibit many strains of bacteria) can evolve resistance to these drugs. Therefore, the concentration of drug released from the microcapsules should always be higher than the MIC for that population of bacteria (Boundless 2016). It is also important consider that microencapsulation may not modify or inactivate the antibiotic, since antibiotics are unstable compounds and they are susceptible to degradation by multiple processes.

2.2.5.2. Controlled release

Microparticles are of great interest in controlled drug release because of relative ease in design and formulation and partly on the advantages of microparticulate delivery systems. Controlled drug delivery technology represents one of the frontier areas of science, which involves multidisciplinary scientific approach, contributing to human health care (Singh et al. 2010). These delivery systems offer numerous advantages compared to conventional dosage forms, which include improved efficacy, reduced toxicity, and improved patient compliance and convenience. Such systems often use macromolecules as carriers for the drugs (Ravi Kumar 2000).

The release of the active agent from the microparticle can occur from different ways. Three types of mechanisms for drug release from particulate systems are represented in Figure 6.
Drug release from particulate systems depends upon the morphology, size, and density of the particulate system, as well as the physicochemical properties of the drug. In vitro release also depends upon pH, polarity, and presence of enzymes in the dissolution media. In majority of cases, drug release follows more than one type of mechanism. In case of release from the surface, adsorbed drug instantaneously dissolves when it comes in contact with the release medium. Drug entrapped in the surface layer of particles also follows this mechanism. This type of drug release leads to burst effect (Agnihotri et al. 2004b).

Based on the release mechanism, oral controlled release products can be classified as (i) diffusion-controlled products; (ii) dissolution-controlled products; (iii) erosion products; (iv) osmotic pump systems; and (v) ion exchange resins. In diffusion-controlled products there is a water insoluble polymer and diffusion occurs when a drug passes through the polymer that forms the controlled release device. The diffusion can occur through pores in the polymer matrix or by passing between polymer chains. In dissolution controlled products, the rate of dissolution of the drug is controlled by slowly soluble polymers or by microencapsulation. Once the coating is dissolved, the drug becomes available for dissolution. By varying the thicknesses of the coat and its composition, the rate of drug release can be controlled. Some preparations contain a fraction of the total dose as an immediate release component to provide a pulse dose soon after administration, which is important to overcome MIC scores in case of antibiotics. In erosion systems, biodegradable polymers degrade within the body as a result of natural biological processes and drug release occurs at constant rate. Most biodegradable polymers are designed to degrade as a result of hydrolysis of the polymer chains into biologically acceptable and progressively smaller compounds. The release of drug from these products is controlled by the erosion rate of a carrier matrix, so the rate of release is determined by the rate of erosion. In osmotic pump systems, pressure is generated within the device which forces the active agent out of the device via an orifice (of a size designed to minimize solute diffusion, whilst preventing the build-up of a hydrostatic pressure head which has the effect of decreasing the osmotic pressure and changing the dimensions (volume) of the device). The advantage of this type of product is that the constant release is unaltered by the environment of the
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gastrointestinal tract and relies simply on the passage of water into the dosage form. The rate of release can be modified by altering the osmotic agent and the size of the hole. Drug-resin complexes for extended release have also been successfully used commercially. The drug is bound to the resin and released by exchanging with appropriately charged ions in contact with the ion exchange groups. This technique is applicable to certain drugs which have particular characteristics in terms of their relatively affinity for the polymers being used (Anal et al. 2013).

Controlled release drug delivery system is one of the most efficient methods to overcome most of the difficulties associated with other methods of administration. Controlled release drug delivery includes carriers such as polymer-based disks, microparticles, nanoparticles, pellets in which the release of encapsulated drug occurs at controlled rates for relatively long periods of time. Such kind of systems often show several advantages over other methods of administration. First advantage is the drug release rates can be adjusted according to the needs of a specific application (e.g. providing a constant rate of delivery or pulsatile release). Controlled release systems also protect drugs, especially proteins, from degradation that are otherwise rapidly destroyed by the body. By using controlled release systems, frequent (daily) dosing can be replaced by giving once per month injection, which ultimately increases patient comfort and compliance (Figure 7) (Nidhi et al. 2014).

![Figure 7. Immediate release vs controlled drug release scheme.](image)

With traditional drug delivery systems, the drug level in the blood rises after each administration of the drug and then decreases until the next administration. The key point with traditional drug administration is that the blood level of the agent should remain between a maximum value, which may represent a toxic level, and a minimum value, below which the drug is no longer effective. The goal of many of the original controlled-release systems was to achieve a delivery profile that would yield a high blood level of the drug over a long period of time. The release of the active agent may be constant over a long period, it may be cyclic over a long period, or it may be triggered by the environment or other external events. In any case, the purpose behind controlling the drug delivery is to achieve more effective therapies while eliminating the potential for both under and overdosing (Innovation 2012).
While the advantages of these systems can be significant, the potential disadvantages cannot be ignored like the possible toxicity or non-biocompatibility of the materials used, undesirable by-products of degradation, any surgery required to implant or remove the system, the chance of patient discomfort from the delivery device, and the higher cost of controlled-release systems compared with traditional pharmaceutical formulations. The ideal drug delivery system should be inert, biocompatible, mechanically strong, comfortable for the patient, capable of achieving high drug loading, safe from accidental release, simple to administer and remove, and easy to fabricate and sterilize (Innovation 2012).

In the next section, some limitations and challenges on the application of microencapsulation technology for antibiotics are presented.

2.2.5.3. Limitations and challenges

Drug microencapsulation possesses a significant potential for pharmaceutical and therapeutic fields in the future as it provides the sustained and controlled release of pharmaceutical agents for various medical applications (Agnihotri et al. 2012). However, microencapsulation technology still faces the existing obstacles and limitations in drug deliveries. This results in a distance between drug microencapsulation and clinical practice in real life. The challenge involves the scale-up of drug microencapsulation process to achieve the high reproducibility of the microencapsulated drug formulations. Another challenge is to not allow the controlled release in such low doses that could favor antibiotic resistance. Technical, quality, ethical, and economic issues should be also considered when the mass production of pharmaceuticals using microencapsulation technology is implemented (Lam and Gambari 2014b).

Precise characterization of drug loaded microcapsules is fundamental to the development of a pharmaceutical formulation for the disease treatment. For the therapeutic applications, the physical, chemical, and therapeutic properties of microcapsules should be carefully examined and identified. Drug entrapment efficiency of microcapsules is closely related to the drug doses and, therefore, it should be cautiously controlled to avoid an overdose administration of the drug. Particle size of microcapsules could alter the total surface area for the drug release. Besides, the stability of microcapsules should be manipulated in order to produce the microencapsulated pharmaceuticals with a clinically acceptable shelf life. The materials involved in the microcapsule formation should be standardized in terms of chemical compositions, purification and reaction situations so as to achieve the biosafety and efficiency requirements consistently and minimize the product variability in different laboratories (Singh et al. 2010; Lam and Gambari 2014b).

Although the challenges and limitations of drug microencapsulation, there are some successful microencapsulation products which have been commercially available in the market (Lam and Gambari 2014b). The future work is to adapt this proven success to antibiotics to
solve the environmental problems of the massive use of antibiotics and its low level of metabolism by human and animal organisms. The main challenge is to microencapsulate antibiotics effectively and efficiency. It is also important to study the release pattern of the drug, considering that too low doses can favor resistance, as discussed in previous sections.

2.3. STUDY CASES

2.3.1. Antibiotics: metronidazole and sulfamethoxazole

2.3.1.1. Physicochemical characterization

Metronidazole (MNZ) belongs to the group of 5-nitroimidazoles. It is used in veterinary and human medicine for the treatment of infections with protozoa (Trichomas, Treponema, Histomonas) and with obligatory anaerobic bacteria (Bacteroides, Fusobacterium, Campylobacter, Clostridium) (European Medicines Agency 1997). It is also often used to eradicate Helicobacter pylori along with other drugs and to prevent infection in people recovering from surgery (FDA Professional Drug Information).

Sulfamethoxazole (SMX) is a sulfonamide related chemically to sulfisoxazole, with a similar antibacterial spectrum, but a slower rate of absorption from the gastrointestinal tract and urinary excretion. SMX is used for the treatment of bacterial infections, such as urinary tract infections, bronchitis, and prostatitis. This antibiotic is effective against both Gram negative and positive bacteria, for example Listeria monocytogenes and E. coli, and it is often used in combination with trimethoprim (SMX-TMP) (FDA Professional Drug Information). Studies have shown that bacterial resistance develops more slowly with the combination of the two drugs than with either Trimethoprim or Sulfamethoxazole alone (World Health Organization 2001).

The chemical structure and speciation diagram of MNZ and SMX are presented in Figure 8 and Figure 9, respectively.

![Figure 8](image)

Figure 8. (A) Chemical structure and (B) speciation diagram of MNZ. Adapted from Carrales-Alvarado et al. (2014).
Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics

Introduction

The values of $pK_a$ are important when analyzing antibiotics in aqueous samples, once different forms of the antibiotic molecule are present for different pH values. Some biological properties and the mechanism of action of MNZ and SMX are presented in the next section.

Other important physicochemical properties of MNZ and SMX are listed in the table 8.

Table 8. Physicochemical properties of MNZ and SMX.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Molecular weight (g/mol)</th>
<th>Log $k_{o/w}$</th>
<th>Water solubility (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNZ</td>
<td>171.15</td>
<td>-0.02</td>
<td>9500 (25 °C)</td>
</tr>
<tr>
<td>SMX</td>
<td>253.28</td>
<td>0.89</td>
<td>610 (37 °C)</td>
</tr>
</tbody>
</table>

References: (1) (FDA Professional Drug Information); (2) (Myrdal et al. 1992).

The partition coefficient of sulfamethoxazole is 0.89, higher than the partition coefficient of metronidazole (-0.02). The greater lipophilicity allows easier penetration of biological membranes, thus facilitating penetration into the body tissues. Hydrophilicity influences the circulation of molecules in aqueous spaces (Saivin and Houin 1988). SMX is slightly soluble in water, benzene, chloroform, diethyl ether and isopropanol. It is soluble in ethanol and methanol (World Health Organization 2001). MNZ, besides water, is also soluble in ethanol (5000 mg/L), methanol, chloroform (<500 mg/L), and DMSO (34000 mg/L) at 25 °C (FDA Professional Drug Information).

2.3.1.2. Biological properties

Metronidazole inhibits nucleic acid synthesis by disrupting the DNA of microbial cells. However, this only occurs when metronidazole is partially reduced, and reduction usually happens only in anaerobic cells. Consequently, it has relatively little effect upon human cells or aerobic bacteria.

Sulfamethoxazole inhibits bacterial synthesis of dihydrofolate acid by competing with para-aminobenzoic acid (PABA) for binding to dihydropteroate synthetase (dihydrofolate...
Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics

Synthetase). Sulfamethoxazole is bacteriostatic in nature. Inhibition of dihydrofolic acid synthesis decreases the synthesis of bacterial nucleotides and DNA.

Some biological properties of these two antibiotics are summarized in the next table.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Bioavailability (%)</th>
<th>$T_{1/2}$ after administration (h)</th>
<th>Protein binding (%)</th>
<th>Metabolism</th>
<th>Excretion rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNZ</td>
<td>95 (1)</td>
<td>8.3 (1)</td>
<td>20 (2)</td>
<td>Hepatic (1)</td>
<td>60-80 renal (4)</td>
</tr>
<tr>
<td>SMX</td>
<td>-</td>
<td>10 (2)</td>
<td>70 (2)</td>
<td>Hepatic (3)</td>
<td>20 parent compound (3)</td>
</tr>
</tbody>
</table>

References: (1) (European Medicines Agency 1997); (2) (FDA Professional Drug Information); (3) (World Health Organization 2001).

Individual sulfonamides differ markedly in their absorption, distribution, and elimination. With the exception of sulfapyrimidine and sulfasalazine, which are only slightly absorbed, sulfonamides are generally well absorbed from the gastrointestinal tract. According to World Health Organization (2001), 70-90% of an oral dose of the absorbable sulfonamides is absorbed from the small intestine. Besides, small amounts may also be absorbed from the stomach. In urine, approximately 20% of the sulfamethoxazole present is unchanged drug, 50-70% is the acetylated derivative, and 15-20% is the glucuronide conjugate.

Metronidazole has higher excretion rates (60-80%) when compared to SMX. The excretion rates are important factors once these compounds and their metabolites may be present in the environment, which has the associated risks, as previously discussed.

2.3.1.3. Consumption and applications

Sulfamethoxazole has been used since the 1960s in the treatment of various systemic infections in humans and other species. The main use has been in the treatment of acute urinary tract infections. Despite its relatively unfavorable pattern of tissue distribution, it is the sulfonamide most commonly used around the world in combination with trimethoprim or pyrimethamine for the treatment of various systemic infections. The combination with trimethoprim is used mainly for the treatment of urinary tract infections.

Metronidazole has been used in human medicine since about 30 years. This antibiotic is on the World Health Organization’s List of Essential Medicines, a list of the most important medication needed in a basic health system (FDA Professional Drug Information). MNZ is not labeled for animal use, but is widely used to treat infections of *Giardia* in dogs, cats, and other companion animals, although it does not reliably clear infection with this organism and is being supplanted by fenbendazole for this purpose in dogs and cats. The U.S. Food and Drug Administration suggests it only be used when necessary because it has been shown to be carcinogenic in mice and rats.
2.3.2. **Microencapsulation**

### 2.3.2.1. Technique: double emulsion (w/o/w)

In pharmaceutical industry, double emulsification technique is mostly used for controlled release. In this technique the drug is dissolved in water (aqueous phase) and the polymer is dissolved in an organic solvent (oil phase). The aqueous phase is added to the oil phase and sonicated with the formation of the first emulsion (w/o). The resulting emulsion is added to a second aqueous phase containing an emulsifying agent (e.g. poly(vinyl alcohol) (PVA)) under magnetic stirring. Then, microparticles are collected by filtration, washed with water and desiccated in vacuum at room temperature, as schematically presented in Figure 10 (Kundap et al. 2013).

![Double emulsion (w/o/w) technique](image)

**Figure 10.** Double emulsion (w/o/w) technique: a) Emulsification/Sonication (primary w/o emulsion); b) Emulsification (final w/o/w emulsion).

### 2.3.2.2. Coating material: ethylcellulose

Ethylcellulose (Figure 11) is a derivative of cellulose in which some of the hydroxyl groups on the repeating glucose units are converted into ethyl ether groups. The number of ethyl groups can vary depending on the manufacturer (Zijlstra et al. 2010).

![Chemical structure of ethylcellulose](image)

**Figure 11.** Chemical structure of ethylcellulose. R = H or CH₂CH₃.

Ethylcellulose is a free-flowing, white to light tan powder used in the pharmaceutical and food manufacturing industries. It is prepared from wood pulp or cotton by treatment with alkali and ethylation of the alkali cellulose with ethyl chloride. This polymer is used in pharmaceutical industry as a coating agent, flavoring fixative, tablet binder and filler, film-
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former, and as a viscosity-increasing agent. It is also used in the food industry as an emulsifier. It is biocompatible and approved for use in regulated markets such as food and pharmaceuticals (DOW). Ethylcellulose is used for controlled release for longer period of time (Kundap et al. 2013).

### 2.3.2.3. Microparticle characterization

Once the microcapsules are prepared, their characterization is important, for example in terms of size and number. Drug loading, encapsulation efficiency and product yield are the main parameters to evaluate microencapsulation. The product yield is the quantity of powder recovered in relation to the amount of raw materials used. Drug loading is defined as the mass fraction of a particle that is composed of drug and encapsulation efficiency is the fraction of drug incorporated into microparticles compared with the total amount of drug that was added during microparticle synthesis. These parameters are calculated as presented in the equations 1 to 3 (Patel et al. 2008)

\[
\% \text{ Drug loading} = \left( \frac{\text{Weight of drug in microspheres}}{\text{Weight of microspheres}} \right) \times 100
\]

\[
\% \text{ Encapsulation efficiency} = \left( \frac{\% \text{Drug loading}}{\% \text{Theoretical loading}} \right) \times 100
\]

\[
\% \text{ Product yield} = \left( \frac{\text{Weight of microspheres}}{\text{Weight of polymer} + \text{weight of drug}} \right) \times 100
\]

To determine the antibiotic content of microspheres (weight of drug in microspheres), 20 mg of microspheres were added to 5 mL of a PBS solution and kept under stirring for 8 days, followed by UV-Visible (UV-Vis) spectrophotometric analysis at 320 nm (MNZ) or 275 nm (SMX). Theoretical loading is the weight of drug in relation to the weight of drug and polymer in a sample of microspheres, considering the homogeneity of the sample.

Particle morphology can be evaluated by Scanning Electron Microscopy (SEM) and particle size distribution can be measured by laser granulometry.

### 2.3.3. Analytical methodology: Spectrophotometry UV-Vis

#### 2.3.3.1. Spectrophotometric methods

Spectrophotometric methods can be used for the determination of antibiotics in aqueous samples. Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a sample as a function of wavelength. It uses spectrophotometers (double or single
beam) that can measure a light beam’s intensity as a function of its wavelength. A scheme of a double beam spectrophotometer is presented in Figure 12.

![Double beam spectrophotometer scheme.](image)

In microencapsulation release studies, spectrophotometry is used instead of other analytical methods, for example high performance liquid chromatography (HPLC). Due to the different sizes of microparticles, they could pass through the filter during filtration process and plug HPLC equipment. However, if the evaluated microparticles size after microencapsulation technique is uniform and big enough to not pass through membrane filter, HPLC could be used as the analytical methodology for the quantification of microencapsulated antibiotics.
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In this section, published works on the antibiotics microencapsulation with different microencapsulating agents will be presented and discussed. Table A1 (Annexes A1) summarizes the microencapsulating agents and techniques reported for antibiotics, as well as some obtained results.

About 50% to 90% of antibiotics administered to humans and animals are excreted after a short time of residence (Thiele-Bruhn 2003; Kümmerer 2009b). The massive and irrational consumption of antibiotics for several decades, combined with these high rates of excretion as parental compounds, is becoming a serious problem once it leads to an increasing concentration of antibiotics in active form in the environment. Knowing that microparticles can improve the effectiveness of various medical treatments, the microencapsulation of antibiotics may be an effective approach to reduce the amount of the administered antibiotic required to cure the infections. Microencapsulation is used to modify and retard drug release (Chemtob et al. 1986). Consequently, this technique can be an interesting and promising option to reduce the amount of antibiotics discharged into the environment. Nevertheless, the implementation of this strategy to minimize this environmental and human health concern has not yet been explored (Singh et al. 2010).

Metronidazole (MNZ) and sulfamethoxazole (SMX) has already been encapsulated with different agents and techniques.

Chemtob et al. (1986) prepared microcapsules of MNZ with ethylcellulose type N (ethoxy content 47.5-49%), viscosity 95 mPa (5% w/w solution in toluene-ethanol 80:20) by phase separation technique. Prolonged dosage forms were obtained and 70-80% of metronidazole was released after 3 h. It was proven that lower percentages of larger microcapsules and higher percentages of smaller microcapsules were obtained at a higher stirring rate. The use of polyisobutylene (7%) (Mw 400000) increased the percentages of small microcapsules much more than the modification of stirring rate, but they were aggregated.

Labhasetwar and Dorle (1990) used albumin (human, fraction V powder of 96-99% purity) and gelatin (type I from swine skin) as agents for the microencapsulation of primaquine and metronidazole by denaturation at high temperature (albumin) and combined emulsification and congealing procedure (gelatin). Particle size ranging between 500 and 1200 nm and a drug entrapment of 8.7% for primaquine and 8.9% for MNZ were obtained with albumin as the encapsulating agent. When using gelatin, particle size ranging between 100 and 800 nm and a drug entrapment of 0.5% for primaquine and 0.5% for MNZ were obtained.

Egbaria and Friedman (1990) also prepared albumin microspheres for the sustained release of MNZ by heat denaturation. Nevertheless, it was used three different albumin types: bovine albumin powder (clinical reagent grade), egg albumin (grade 2), and human albumin (fraction V powder). The percentage of metronidazole released from microspheres of bovine, egg and human albumin was in the order bovine > egg ≥ human albumin systems. Drug payloads
were 19.25% for egg albumin microspheres, 15.5% for human albumin microspheres, and 12.75% for bovine albumin microspheres. The amount of drug released in the initial phase (burst effect) up to 10 h was similar for the three systems. This suggested that overall release of metronidazole from the microspheres may be a function of the diffusivity of the drug in the albumin matrix.

Saravanan and Rao (2009) studied the microencapsulation of MNZ using pectin and alginate by complex coacervation with gelatin. Pectin produced coacervation with gelatin with all acidifiers but alginate produced coacervation only with acetic acid. Addition of sodium carboxymethyl cellulose reduced aggregation between the microparticles. MNZ was poorly encapsulated (4-7% w/w) by this technique. The percentage of encapsulation was 2.92% and 6.25% and an average particle size of 45.14 µm and 41.34 µm were obtained for pectin-gelatin and alginate-gelatin microspheres, respectively. The microcapsules showed low drug release in gastric fluid and sustained release in intestinal fluid. Alginate was found to be better than pectin for coacervation with gelatin in terms of less aggregation, smaller particle size and easy dispersion.

Sohail et al. (2012) used alginate as microencapsulating agent for the production of MNZ microcapsules by impinging aerosols method, with an encapsulation efficiency of 21%. Around 75 and 96 % of the MNZ content was released from hydrated and dried microspheres respectively in simulated gastric fluid within 20 min.

PLGA was also used as microencapsulating agent for the microencapsulation of MNZ by Aina et al. (2015) and Prabhakaran et al. (2015). They used water-in-oil-in-water (w/o/w) double emulsion technique and electrospraying, respectively. By w/o/w double emulsion technique metronidazole it was shown that MNZ can be encapsulated into PLGA scaffolds even at low concentration as permitted by aqueous solubility. By electrospraying technique it was used PLGA 90:10, (Mw 10000) and it was studied the microencapsulation of MNZ with two different solvents: dichlorometane (DCM) and trifluoroethanol (TFE). The polymeric solution in DCM produced particles of bigger sizes than using TFE. Particles with spherical morphology, with sizes of (3946±407) nm and (1774±167) nm, respectively for PLGA-metronidazole(DCM) and PLGA-metronidazole(TFE) were obtained. It was demonstrated a sustained drug release for at least 41 days.

Nivasu and Tammisetti (2006) encapsulated SMX into polyester polyol acrylate polymers by a method that consisted in a diacid-diol condensation and dispersion in a solvent. Polymers were prepared and they were used to microencapsulate SMX as a model drug. The in vitro delivery of the drug followed the expected trend depending on the polymer hydrophobicity. All microspheres were analyzed by Scanning Electron Microscopy (SEM) and they were found to be very spherical, entirely free of any defects, nonporous, and most of them were in the range of 10-20 µm in diameter. Microcapsule yield between of 78% to 79% and microencapsulation efficiency in the range of 83-91% were obtained. All the tested formulations had a burst release up to about two days probably due to release of SMX near the periphery of the microspheres.
Although MNZ and SMX have been microencapsulated by the abovementioned techniques, one of the most referenced techniques for the antibiotics microencapsulation is double emulsion solvent evaporation (w/o/w). This technique is used in this work to encapsulate MNZ and SMX and had been reported by several authors. Doxycycline (DOX), the most administered antibiotic in veterinary medicine in 2013, is an example of an antibiotic that had been encapsulated by this method.

Mundargi et al. (2007) developed a technique for the production of biodegradable microspheres containing DOX prepared by water-in-oil-in-water (w/o/w) double emulsion technique using blends of poly(D,L-lactide-co-glycolide) (PLGA) and poly(ε-caprolactone) (PCL) in different ratios. This method was tested for the controlled drug delivery of doxycycline in the treatment of human periodontal pocket. It was used PLGA 50:50 Resomer® RG 504 (Mw=60,000) and PCL (Mw=32 kDa). DOX encapsulation of up to 24% was achieved within the polymeric microspheres. Mean particle size of the microspheres as measured by dynamic laser light scattering method ranged between 90 and 200 µm. In vitro release studies performed in 7.4 pH media indicated the release of DOX from 7 to 11 days, depending upon the blend ratio of the matrix. Up to 11 days, DOX concentrations in the gingival crevicular fluid were higher than the minimum inhibitory concentration (MIC) of DOX against most of the periodontal pathogens.

Patel et al. (2008) tested the use of PLGA polymers with three different molecular weights (91342; 75857; 46257) to microencapsulate DOX. Microparticles were produced by spray drying and water-in-oil-in-water (w/o/w) double emulsion techniques to encapsulate 86% of DOX for the use of periodontitis. By spray drying technique, particle size ranged from 10 to 25 µm for different formulations, and encapsulation efficiency ranged from 40 to 60%. Using w/o/w technique, mean particle size was 26 µm and it was obtained an encapsulation efficiency of 19%. Antimicrobial studies performed on one formulation and placebo microspheres suggested that drug concentrations during in vitro release were above the minimum inhibitory concentration (MIC) for Staphylococcus aureus growth.

Raval et al. (2011) produced doxycycline-loaded PCL (Mw 70000-90000 by GPC) microspheres prepared by water-in-oil-in-water (w/o/w) double emulsion solvent evaporation technique with different formulation variables such as concentrations of drug and polymer. A yield of production between 82.90% and 93.55% and encapsulation efficiencies between 20.09% and 81.38% were obtained. It was found that drug/polymer ratio does not affect the production yield but it affects the encapsulation efficiencies. Observed MICs for K. pneumoniae and P. aeruginosa were 5 µg/mL and 25 µg/mL, respectively. S. aureus exhibited susceptibility at lower concentration levels (1 µg/mL) while E. coli shows susceptibility at 2.5 µg/mL.

Poly(3-hydroxybutyrate) (PHB) (from microbial fermentation with Cupriavidus necator and glycerol as carbon source (Mw 313 KDa and polydispersity index of 4.15) was also used for the microencapsulation of DOX. PHB can be produced by bacteria and is remarkable for this application due to its excellent biocompatibility and biodegradability. Rodríguez-Contreras et
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al. (2013) used PHB to prepare micro and nanoparticles of DOX by polymer precipitation via dialysis, simple emulsion (o/w) or multiple emulsion (w/o/w) applying solvent evaporation in the last two cases. Pluronic-F127, poly(vinyl alcohol) (PVA) and sodium taurocholate (TAU) were used as surfactants both for simple and multiple emulsion techniques. The highest drug loading was obtained by single emulsion with high speed stirring (4.83%). In the case of multiple emulsion, the combination of ultrasound with high speed stirring resulted in the most elevate encapsulation efficiency (16.18%).

Recently, Kassab et al. (2014) presented a comparative study of doxycycline and tetracycline polymeric microspheres. Solvent evaporation technique was used for the preparation of PLA (average Mw 75000-120000) microspheres loaded with the two antibiotics, introducing different masses for each drug. Moderate drug encapsulation values of 38% and 24% were observed for DOX and tetracycline, respectively. Microspheres of both drugs showed homogeneous size distribution varying between 80 and 110 µm. In vitro release studies showed that tetracycline release was slightly faster (98% in less than 5 hours), in comparison with DOX which attained the same percentage after 7 hours.

Besides doxycycline, there are several other antibiotics that have already been microencapsulated with proven therapeutic effects into different coating materials. Clarithromycin is a macrolide antibiotic recommended for the treatment of upper respiratory tract infections. Gupta et al. (1993) tested the encapsulation of this antibiotic with a biodegradable polymer, PLA (Mw 50000) by solvent evaporation technique with recovery by filtration or spray-drying. Microspheres with a diameter varying between 5 and 40 µm were obtained, which is acceptable for administration. Drug loadings, with batches prepared by spray-drying as well as by solvent-extraction technique, were within 10-15% of the theoretical values, suggesting no major loss of drug with either formulation processing. Other biodegradable polymers, such as Eudragit L30D, Eudragit RL 100, Eudragit RS 100, CAP, gum arabic and maltodextrine were shown to be possible agents for the microencapsulation of clarithromycin by different methods (Zgoulli et al. 1999; Geno et al. 2006).

Ciprofloxacin (CIPRO) was microencapsulated with PLGA by Martinez et al. (1997), Ramchandani and Robinson (1998), and JEONG et al. (2009). Martinez et al. (1997) used PLGA 50:50 and 75:25 and two different techniques: solvent evaporation and evaporation-extraction (o/w emulsion). PLGA 50:50 formulation had shown a faster release profile than PLGA 75:25. Evaporation-extraction method produced higher values of ciprofloxacin content of PLGA microspheres, both for PLGA 50:50 and 75:25. Ramchandani and Robinson (1998) tested nonsolvent-induced phase separation technique with PLGA 50:50. A CIPRO recovery greater than 90% at all drug loading levels were obtained. Sustained drug levels, greater than the minimum inhibitory concentration (MIC) of CIPRO, up to 70 mm from the site of implantation, were detected for a period of six weeks. JEONG et al. (2009) used PLGA 50:50 to prepare CIPRPO microspheres by solvent evaporation (o/o) technique. Particle sizes ranged from 10 to
50 µm and an in vitro drug release study showed that CIPRO was continuously released over 3 weeks from the microspheres, and a burst effect was observed for the first 3 days.

Yu et al. (1998) tested phase separation technique for the production of CIPRO microspheres with PLA (different molecular weights). The encapsulation was in the range of 97.9-99.0%, as expected with CIPRO hydrophilic property.

Pectin and chitosan were also used by Orhan (2006) for the microencapsulation of CIPRO by spray-drying (Model 191, Büchi Labortechnik AG, Flawil, Switzerland). Several polymer:drug ratios were tested. For all formulations, the production yield was from 46% to 48%, the particle diameters were less than 5 µm and the encapsulation efficiencies were above 98%. PLGA were used for the preparation of microcapsules of other antibiotics, such as vancomycin (Gavini et al. 2004), rifampicin (Ito and Makino 2004), tetracycllin (Liu et al. 2004; Kim et al. 2005), amoxicillin (XU et al. 2008), cephalexin (Chaisri et al. 2009), and gentamicin (Chaisri et al. 2011). Erythromycin was microencapsulated by Zgoulli et al. (1999) using biodegradable polymers (Eudragit L30D, CAP, gum arabic, maltodextrine) by spray drying technique (Niro Minor (Denmark) spray-drying apparatus). Microencapsulation of 80% was obtained. With this study, it was proven that microencapsulation of macrolides using a spray-drying technique is feasible.

In conclusion, microcapsules have been used as drug delivery systems in the pharmaceutical field for sustained or controlled release of drugs, and for artificial cells and organs. Biodegradable polymers have been widely used in this field, particularly PLGA. In past two decades PLGA has been among the most attractive polymeric candidates used to fabricate devices for drug delivery and tissue engineering applications (Makadia and Siegel 2011). The main factors involved in the preparation of PLGA microspheres with high encapsulation efficiency are the PLGA concentration in the organic phase, PLGA molecular weight and composition, the water miscibility of the organic solvent and/or cosolvents that have been utilized to dissolve the polymer or the drug, the type and concentration of emulsifier and the pH of internal/external water phases in case of ionizable drugs. (Gert et al. 2016) Understanding the release mechanisms, as well as which factors that affect drug release, is important in order to be able to modify drug release (Fredenberg et al. 2011).

A representation of the percentage of reported techniques for microencapsulation of antibiotics, as well as the most used encapsulating agents are presented in Figure 13. The most common microencapsulation methods are based on emulsification procedures, in which emulsified droplets of polymer and drug solidify into microspheres when the solvent is extracted from the polymeric phase (Gert et al. 2016).
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Figure 13. Representation of the percentage of reported techniques for microencapsulation of antibiotics, as well as the most used encapsulating agents.

These aforementioned microencapsulation techniques for antibiotics were tested for the sustained release of drugs and hence a more patient-friendly dosing regimen. Drug loaded microspheres have also been administered with the intention to overcome poor local distribution of drugs (Gert et al. 2016). Nevertheless, no studies were found in which the aim for microencapsulation was the environmental contamination by antibiotics. For that reason, this application is a challenge.
4. MATERIALS AND METHODS

4.1. MATERIALS

4.1.1. Standards and reagents

Metronidazole and sulfamethoxazole (purity, 98%) were supplied from Sigma-Aldrich (Steinheim, Germany). Analytical reagent grade samples of poly(vinyl alcohol) (PVA) (Mw~130000 and 86.7%-88.7% hydrolyzed) and dichloromethane (DCM) were purchased from Sigma-Aldrich (Steinheim, Germany), and methanol from VWR (Fontenay-sous-Bois, France). Ethylcellulose (viscosity 46 cP) was procured from Sigma-Aldrich (Steinheim, Germany). Filtration membrane (nylon membrane 0.45 µm x 47 mm) was purchased from Sigma-Aldrich (Steinheim, Germany). Phosphate-buffered saline (PBS) was prepared with 8.0 g/L NaCl, 0.2 g/L KCl, 1.42 g/L Na₂HPO₄ and 1.8 g/L KH₂PO₄. Ultrapure water was used throughout the work, and it was prepared with an Elix Essential Water Purification System (3 L/h) from Merck Millipore (Beeston, United Kingdom). All the chemicals were used without further purification.

4.1.2. Equipment

All weight measurements were performed using a Mettler Toldedo AG245 analytical balance (Columbus, OH, USA). All spectrophotometric analysis were performed using a double-beam Jasco V-530 UV-Vis Spectrophotometer (Easton, USA). pH measurements were performed using a 900 Multiparameter Water Quality Meter (A & E Lab; Guangzhou, China). Vacuum drying chamber Buchi GKR-51 was used for microparticles drying. Particle morphology was evaluated by Scanning Electron Microscopy, SEM (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M) and particle size distribution was measured by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyser (Miami, FL, USA).

4.2. METHODS

4.2.1. Microencapsulation of antibiotics

4.2.1.1. Preparation of microcapsules

Microparticles containing MNZ and SMX were prepared by double emulsion solvent evaporation (w/o/w) technique. For each essay, 100 mg of antibiotic was dissolved in 2 mL of ultrapure water and the drug solution was added to an oil phase (10 mL of DCM:MeOH (1:1)
containing 300 mg of ethylcellulose) and emulsified by an ultrasonic bath for 2 min to yield a w/o emulsion.

The w/o primary emulsion was slowly added to 100 mL of a 1% PVA aqueous solution and further emulsified with mechanical stirring at 400 rpm for 2 h to yield a w/o/w emulsion. The obtained microparticles were collected by filtration (gouch filter) under vacuum and washed twice with water. Then, the microparticles were dried overnight in a vacuum chamber at room temperature. The microparticles storage occurred at -20 °C.

4.2.1.2. Characterization of microcapsules

To determine the antibiotic content of microspheres, 20 mg of microspheres were added to 5 mL of a PBS solution and kept under stirring for 8 days, followed by UV-Vis spectrophotometric analysis at 320 nm (MNZ) or 275 nm (SMX). Drug loading, encapsulation efficiency and product yield are the main parameters to evaluate microencapsulation and they were calculated as mentioned in section 2.3.2.3.

Particle morphology was evaluated by Scanning Electron Microscopy, SEM (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M). Samples of microparticles were gold coated under vacuum in a Jeol JFC 100 apparatus at Centro de Materiais da Universidade do Porto (CEMUP) and SEM analysis was performed at 15 kV for surface structure observation.

Particle size distribution was measured by laser granulometry using a Coulter-LS 230 Particle Size Analyser. A small powder sample was suspended in water. The particles were characterized by volume and number distribution using Laser Diffraction and Polarization Intensity Differential Scattering. The results were obtained as an average of two 60 s runs.

4.2.1.3. Controlled release study

The in vitro drug release of MNZ and SMX from the microcapsules was performed in PBS buffer at different pH values (pH 2 and pH 7 at room temperature) to simulate stomach and intestinal tract conditions, respectively. PBS was used as the release medium once its osmolarity and ion concentration match those of the human body (isotonic). Twenty milligrams of microcapsules were put in 5 mL of PBS in amber glass flasks. The release tests were carried out in a mechanical shaker with a constant shaking speed of 40 rpm at room temperature. The antibiotic concentration in PBS solution was determined directly by measuring UV-Vis absorption at 320 nm for MNZ and 275 nm for SMX. A different sample was prepared for each time of analysis.
4.2.2. Analytical method for antibiotics quantification

4.2.2.1. Procedure

In order to determine the best detection wavelength for the analysis of each antibiotic, in which the maximum absorption occurs, MNZ and SMX (15.0 mg/L in PBS pH 2 and pH 7) was scanned between wavelengths 190 to 700 nm and the absorption spectra was obtained. The UV-Vis absorption spectrum of each antibiotic was obtained for the determination of the maximum absorption wavelength ($\lambda_{\text{max}}$) of the compound. It was necessary to know the $\lambda_{\text{max}}$ since it allows the quantification with a higher sensitivity in analysis, and thereby the detection of low concentrations in samples. Stock solutions (250 mg/L) of metronidazole and sulfamethoxazole were prepared in 50% of methanol and 50% of water and stored at -20 °C. For the validation of the method, standard solutions at different concentrations were daily prepared by appropriate dilution of the stock solution of each antibiotic with PBS (used as release medium in controlled release tests), at pH 2 and pH 7 at room temperature. The standards stabilization occurred at 2 °C for 2 hours.

4.2.2.2. Validation

The UV-Vis spectrometric method was validated, in order to demonstrate that this method is suitable for quantitative determination of MNZ and SMX and ensure the reliability of the results. Validation performance parameters: quantification parameters (linearity, sensitivity and limits of detection and quantification) and reliability parameters (repeatability and intermediate precision) were determined.

The linearity is the ability of a method to demonstrate that the results obtained are directly proportional to the concentration of the analyte in the sample within a specific range. The results of the standard solutions analysed (in duplicate) were processed statistically to determine the calibration curve equation (least squares method) and the correlation coefficient (R) using the Microsoft Excel 2013 software. The calibration standards should be prepared in the same matrix as the matrix of the intended study samples by spiking the blank matrix with known concentrations of the analyte (EMA 2012). The calibration curves were constructed plotting the values of absorption in the vertical axis and antibiotic concentrations in the horizontal axis. The linearity of MNZ and SMX was investigated at pH 2 and pH 7 by an eleven points calibration curve from 0.5 to 15.0 mg/L. For the validation of the calibration curve, one typically considers five criteria: (i) minimum of 5 concentration levels; (ii) concentration range above 10; (iii) relative standard deviation of the slope ($\frac{s_a}{a} \times 100$) less than 5%; (iv) intercept contains the origin ($b - s_b < 0 < b + s_b$); (v) correlation coefficient (R) greater than 0.995.

The limit of detection (LOD) is the lowest analyte concentration that can be detected and identified with a given degree of certainty (UNODC 2009). The limit of quantification (LOQ)
is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision (EMA 2012).

The precision describes the closeness of the results obtained in a series of measurements of the same sample. Precision is further subdivided into intra-day precision or repeatability, which assesses precision within a short period of time with the same analyst and instrumentation, and inter-day precision or intermediate precision, which measures the precision with time and may involve different analysts, equipment, and reagents. Precision was assessed by testing the repeatability of three different standard solutions (low, medium and high concentration) six times, and by intermediate precision, analyzing the same three standard solutions three times on different days. Precision was expressed as coefficient of variation (CV%), and these values should be less than 5%.

4.2.3. Quality assurance and control

Antibiotics are sensitive compounds, namely light sensitive. Methanolic stock solutions of MNZ and SMX were preserved in the freezer (-20 °C) and protected from light to avoid degradation. Aqueous intermediate solutions and individual standards were prepared and analysed freshly, and were not stored longer than 24 h. The obtained microparticles were wrapped in aluminium foil to protect from light and stored at -20 °C.

4.2.4. Storage, fate and treatment of wastes

Liquid wastes were generated containing water, methanol, DCM, PVA, PBS and traces amounts of MNZ, SMX and ethylcellulose. These wastes were collected in closed containers properly labeled and stored in protected locations of light and ignition sources for further treatment by Sistema de Gestão Ambiental da FEUP (EcoFEUP).
5. RESULTS AND DISCUSSION

5.1. ANALYTICAL METHOD VALIDATION

Considering the need to evaluate the percentage of MNZ and SMX released from the microparticles during the controlled release studies, it was necessary to develop an analytical methodology for the quantitative determination of antibiotics in aqueous samples. As discussed in section 2.3.3.1, spectrophotometric methods were used.

Firstly, the absorption spectra for both tested antibiotics were obtained between wavelengths 190 to 700 nm, for both controlled release mediums (PBS pH 2 and PBS pH 7) in order to determine the wavelength of maximum absorption in each medium, as presented in Figure 14.

![Absorption spectra of a) MNZ (15.0 mg/L) and b) SMX (15.0 mg/L) between 190 and 700 nm for PBS pH 2 and pH 7.](image)

In both cases, for MNZ and SMX, the results showed a difference in the maximum wavelength of absorption between pH 2 and pH 7. However, this difference is most significant in case of MNZ and it was considered irrelevant in case of SMX. Since previous release tests had been made using the same wavelength for both pH values, that wavelength was used throughout the work to allow comparison of results. The detection was performed at 320 nm for MNZ and at 265 nm for SMX. Those values were also reported in the literature (Carrales-Alvarado et al. 2014; Qi et al. 2014).

The UV-Vis Spectrophotometry analytical methods were also validated, in order to demonstrate that they are suitable for quantitative determination of MNZ and SMX in aqueous samples and ensure the reliability of the results. For MNZ, seven standards in a range of concentrations between 1.5 and 15.0 mg/L were analyzed in duplicate for both pH values. In case of SMX, eight standards were analyzed in a range of concentrations between 1.4 and 15.0 mg/L for pH 2 and nine standards between 0.5 and 15.0 mg/L were analyzed for pH 7. The results allowed the construction of the calibration curves, which are presented in Figure 15.
Results and discussion

Quantification parameters (linearity, sensitivity and limits of detection and quantification) and reliability parameters (repeatability and intermediate precision) were determined.

The results of the quantification parameters of the UV-Vis spectrophotometry method for MNZ and SMX quantification in PBS are presented in the next table.

Table 10. Quantification parameters of the UV-Vis spectrophotometry for MNZ and SMX quantification in PBS.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MNZ</th>
<th>SMX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS pH 2</td>
<td>PBS pH 7</td>
</tr>
<tr>
<td>Linearity range (mg/L)</td>
<td>1.5-15.0</td>
<td>1.5-15.0</td>
</tr>
<tr>
<td>Regression slope/Sensitivity ((L.AU)/mg)</td>
<td>0.0219</td>
<td>0.0596</td>
</tr>
<tr>
<td>Regression intercept (AU)</td>
<td>-0.0076</td>
<td>-0.0006</td>
</tr>
<tr>
<td>Correlation coefficient (R)</td>
<td>0.9992</td>
<td>0.9996</td>
</tr>
<tr>
<td>LOD (mg/L)</td>
<td>1.29</td>
<td>0.92</td>
</tr>
<tr>
<td>LOQ (mg/L)</td>
<td>4.30</td>
<td>3.06</td>
</tr>
</tbody>
</table>

In studied concentration ranges, all regression curves were linear. LOD and LOQ values were satisfactory low, unless they were a little high in some cases. Nevertheless, LOD values were less than the lowest concentration of the calibration curve and LOQ values were lower than most of the concentration analyzed in the controlled release studies.

The linearity conditions for the validation of the calibration curves are presented in Table 11.
Table 11. Linearity conditions for the validation of the UV-Vis spectrophotometry calibration curves.

<table>
<thead>
<tr>
<th></th>
<th>MNZ</th>
<th>SMX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS pH 2</td>
<td>PBS pH 7</td>
</tr>
<tr>
<td>Number of standards ≥ 5</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Linearity factor range ≥ 10</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Correlation coefficient (R) ≥ 0.995</td>
<td>0.9992</td>
<td>0.9996</td>
</tr>
<tr>
<td>Slope error ( \left( \frac{\text{Slope}}{100} \right) \leq 5%</td>
<td>1.80%</td>
<td>1.28%</td>
</tr>
<tr>
<td>Intercept confidence interval ( b - s_b &lt; 0 &lt; b + s_b )</td>
<td>-0.011&lt;0&lt;0.004</td>
<td>-0.008&lt;0&lt;0.007</td>
</tr>
</tbody>
</table>

The results obtained were considered satisfactory since the linearity requirements were all met, unless the intercept confidence interval for some calibration curves. Nevertheless, the values were close to zero, so the linearity conditions were considered achieved.

Reliability results for MZ and SMX are presented in Tables 12 and 13, respectively. Precision, expressed as the coefficient of variation (CV%), was assessed by testing the repeatability (intra-day precision) and the intermediate precision (inter-day precision) at different concentrations within the working range: low, medium, and high standard concentrations.

Table 12. Reliability parameters of the UV-Vis spectrophotometry for MNZ quantification in PBS pH 2 and pH 7.

<table>
<thead>
<tr>
<th>MNZ standard concentration (mg/L)</th>
<th>Repeatability</th>
<th>Intermediate precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 2(^{(a)})</td>
<td>pH 2(^{(b)})</td>
</tr>
<tr>
<td>5.4(^{(a)})/3.2(^{(b)})</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>10.3(^{(a)})/8.0(^{(b)})</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>15.0(^{(a)})/8.0(^{(b)})</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 13. Reliability parameters of the UV-Vis spectrophotometry for SMX quantification in PBS pH 2 and pH 7.

<table>
<thead>
<tr>
<th>SMX standard concentration (mg/L)</th>
<th>Repeatability</th>
<th>Intermediate precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 2(^{(a)})</td>
<td>pH 2(^{(b)})</td>
</tr>
<tr>
<td>4.1(^{(a)})/1.4(^{(b)})</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>8.2(^{(a)})/8.0(^{(b)})</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>15.0(^{(a)})/8.0(^{(b)})</td>
<td>0.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The acceptable precision at lower concentrations is 20%, and, at higher concentrations it is expected a CV under 5%. Both intermediate precision and repeatability are in line with these criteria. From the obtained results it can be concluded that there were no significant response variations and that the precision was very satisfactory. Thus, the UV-Vis spectrophotometric method was developed and validated to detect and quantify MNZ and SMX in PBS solutions, in the studied range of concentrations.
5.2. MICROENCAPSULATION AND CHARACTERIZATION OF MICROPARTICLES

5.2.1. Product yield, drug loading and encapsulation efficiency

Two different antibiotics, MNZ and SMX, were encapsulated into ethylcellulose by a double emulsion (w/o/w) solvent evaporation process. The product yield (quantity of powder recovered in relation to the amount of raw materials used) was (71.4 ± 8.1)% for MNZ and (95.7 ± 2.7)% for SMX. The obtained results were satisfactory for the technique and the scale that were used and showed that there were no significant losses in the encapsulating process. It should be noted that microparticles could still contain remains of emulsifier or solvents if they were not properly washed and dried, resulting in higher product yield values. Double emulsion method has successfully been used by other authors for the encapsulation of antibiotics with similar product yields ranging from 84.2% to 95.5% (Gavini et al. 2004).

Drug loading (mass fraction of a particle that is composed of drug) and encapsulation efficiency (fraction of drug incorporated into microparticles compared with the total amount of drug that was added during microparticle synthesis) were also evaluated. Drug loadings of 1.1% for MNZ and 7.8% for SMX were obtained. Encapsulation efficiency was 4.6% in case of MNZ and 32.1% in case of SMX. Chaisri et al. (2011) obtained similar drug loadings (around 4-10%) when encapsulating gentamicin sulfate by double emulsion technique to develop a suitable formulation for the sustained release of the drug. Encapsulation efficiencies ranging from 11.5% to 17.9% (Liu et al. 2004) and 18.1% to 30.1% (Sendil et al. 1999) were also obtained for the encapsulation of tetracycline loaded PLGA by w/o/w technique.

Product yield, drug loading and encapsulation efficiency could be affected by several factors in a w/o/w process. Chaisri et al. (2011) concluded that the polymer concentration of the DCM solution is an important parameter to achieve a high loading. On the other hand, it was found that emulsifiers in the continuous medium for the microencapsulation of the drug were influential on the encapsulation efficiency in microparticles (Sendil et al. 1999). The agitation during microencapsulation process can also affect encapsulation efficiency. Therefore, it is necessary, in the future, to study and optimize all these parameters in order to obtain a more suitable and efficient method for the encapsulation of MNZ and SMX by w/o/w technique.

5.2.2. Particle morphology and particle size distribution

The prepared microparticles were analysed by scanning electron microscopy (SEM) for particle morphology analysis and laser granulometry to determine particle size distribution. Figure 16 shows SEM images of MNZ and SMX loaded ethylcellulose microparticles. The microstructural analysis confirmed a population of smooth, regular, porous and spherical particles for both encapsulated antibiotics. Once ethylcellulose is water insoluble and
microparticles were found to be porous, a diffusion controlled release is predictable. Diffusion occurs when a drug passes through the polymer that forms the controlled release device. The diffusion can occur through pores in the polymer matrix or by passing between polymer chains (Anal et al. 2013).

Figure 16. SEM micrographs of MNZ (a,b) and SMX (c,d) loaded ethylcellulose microparticles. Amplified 100 (a, c), 500 (b) and 1000 (d) times, beam intensity (HV) 15 kV, distance between the sample and the lens (WD) around 10 mm.

Particle size and size distribution are important microsphere properties, as they can influence the biopharmaceutical properties of the particle preparations (Martínez-Sancho et al. 2004). They influence active ingredient loading, the release profile and the stability of the active compound inside the microparticles. Particle size and size distribution can also influence
in vivo distribution, biological fate, toxicity and the targeting ability of microparticle systems. Particle size distribution results are presented in Figures 17 and 18 and in Table 14.

Figure 17. Size distribution in number of (a) MNZ and (b) SMX microparticles.

Figure 18. Size distribution in volume of (a) MNZ and (b) SMX microparticles.

Table 14. Particle mean diameter results by laser granulometry analysis.

<table>
<thead>
<tr>
<th>Microparticles</th>
<th>Mean diameter (µm)</th>
<th>Differential number</th>
<th>Differential volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNZ</td>
<td>1.26</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>SMX</td>
<td>0.50</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n/a: not applicable

Particle size was analyzed from 0.3 µm to 1822 µm. Mean diameter of microparticles (obtained through normal distribution graphics) were similar for both microencapsulated antibiotics. Number distributions were close to normal, whereas diameter values do not follow an expected distribution in terms of volume. Because of that, it was only possible to estimate the mean diameter of microparticles by the analysis of size distribution in number. The volume distribution may be explained by aggregation factors. Big particles or agglomerates were present and at the same time a high number of small particles were found. In case of number distribution of microparticles, the results indicated that the 90% of the particles is below 2.66 µm for MNZ and 1.26 µm for SMX. Volume distribution of 90% of the particles is below 1660 µm for MNZ and 1512 µm for SMX. Average particle sizes in the range of 3 to 5 µm and similar
particle size distributions were also reported in cephalixin, gentamicin and doxycycline encapsulation into PLGA and PLHMGA by a w/o/w process (Patel et al. 2008; Chaisri et al. 2009; Chaisri et al. 2011).

5.3. CONTROLLED-release FROM THE MICROPARTICLES

The in vitro drug release of MNZ and SMX from the microcapsules was performed in PBS buffer at different pH values (pH 2 and pH 7 at room temperature) to simulate stomach and intestinal tract conditions, respectively. Once antibiotics absorption only occurs in the intestine, the antibiotic release from the microparticles should only occur when they reach the neutral pH in the gastrointestinal tract. This was an important consideration for the use of two different pH values in release studies. MNZ (a) and SMX (b) release profiles from PBS at pH 2 and pH 7 are presented in Figure 19. Figure 20 compares MNZ and SMX release profiles in PBS at pH 2 (a) and pH 7 (b).

Generally, the in vitro release profiles obtained for each antibiotic showed three phases: (1) a first initial burst release, possibly due to desorption of antibiotic initially associated with microparticles on their surfaces by weak linkages to ethylcellulose, (2) a constant sustained release of the remaining antibiotic, resulting possibly from the diffusion through the polymer wall, and (3) a constant concentration after complete release. The initial burst effect is a very important factor when encapsulating antibiotics, once the MIC should be rapidly reached in order to prevent antibiotic resistance. After that initial release, the antibiotic concentration in the medium should be gradually higher to achieved the desired controlled release from the microparticles.

MNZ loaded ethylcellulose microparticles exhibited a fast initial release (= 80% after 3 h) and a sustained behavior until complete MNZ release at around 20 h. This profile was observed for both pH values, with no significant differences (Figure 19a). In case of SMX loaded ethylcellulose microparticles (Figure 19b), different release profiles were observed for the two studied pH values. At pH 2, SMX microparticles showed a fast initial profile, reaching low % of release (= 40% after 2 h). For the next hours, the were no significant release. It means that these microparticles are a good approach for the delivery of SMX in the gastrointestinal tract, once the antibiotic is not significantly released in the stomach. Regarding pH 7, SMX microparticles exhibited a fast initial release (= 30% after 15 min) and then a constant phase until 2 h of release. After that, the antibiotic release was again observed (= 80% after 5 h) and a sustained behavior occurred until complete SMX release at around 16 h.
Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics

Results and discussion

Figure 19. Comparison between the MNZ (a) and SMX (b) release profiles from the microparticles in PBS at pH 2 and pH 7, for 24 h.

Figure 20. Comparison between the MNZ and SMX release profiles from the microparticles in PBS at pH 2 (a) and pH 7 (b), for 24 h.
When microparticles were prepared by w/o/w method, the water soluble drugs showed a significant tendency to migrate to aqueous dissolution medium, thereby concentrating at the surface of microparticles with the burst effect. Once ethylcellulose is water insoluble, drug release may be due to diffusion, but not due to polymer degradation. In addition, porous structure of the microspheres as seen from SEM images also confirms the idea of diffusion controlled-release from the microparticles (Mundargi et al. 2007).

In general, a fast release profile at an initial phase was observed during the in vitro release assay, which was in accordance with previous data for the w/o/w encapsulating technique. Gavini et al. (2004) encapsulated vancomycin into PLGA and obtained 80% of drug release after 3 h, in USP phosphate buffer at pH 7. Liu et al. (2004) also studied the release of alginate coated tetracycline loaded PLGA in PBS at pH 7.4 and 70% of the drug was release after 6 h. However, slower release profiles were observed in other studies. Chaisri et al. (2011) studied gentamicin release from PLGA and PLHMGA microcapsules at pH 7. The particles showed a burst release of the drug depending on their porosity, followed by a phase of 35 days where hardly any release occurred. The drug was then slowly released for around 25 days likely due to degradation of the microspheres.

These preliminary release tests in PBS at pH 2 and pH 7 indicated that ethylcellulose could be used as encapsulating agent for a more controlled release of MNZ and SMX, in microparticles produced by a double emulsion technique. However, further studies should be done to obtain microparticles that control the release of antibiotics more effectively, since a slower release is intended after reaching MIC score. The profile of compound release from microparticles can be affected by the method of encapsulation, the release medium, the pH and by the interactions between the drug, encapsulating agent and auxiliary ingredients added, and these factors should be optimized to obtain an optimum sustained release system.
6. CONCLUSIONS

Two different antibiotics, metronidazole (MNZ) and sulfamethoxazole (SMX), were encapsulated into ethylcellulose by a double emulsion solvent evaporation (w/o/w) technique. The results demonstrated that it is possible to encapsulate MNZ and SMX by this method, however this is probably a better approach for the encapsulation of SMX, since the obtained results were more satisfactory in that case. The product yield was 71.4% for MNZ and 95.7% for SMX. Drug loadings of 1.1% for MNZ and 7.8% for SMX were obtained and encapsulation efficiency was 4.6% in case of MNZ and 32.1% in case of SMX.

UV-Vis spectrophotometry methods for MNZ and SMX determination were developed and validated. The methods presented good linearity with correlation coefficients greater than 0.995, and good precision results with coefficients of variation values lower than 5%. In case of MNZ, the limits of detection were 1.29 mg/L for PBS pH 2 and 0.92 mg/L for PBS pH 7, while the limits of quantification were 4.30 mg/L and 3.06 mg/L, respectively. For SMX, the limits of detection were 1.19 mg/L for PBS pH 2 and 0.37 mg/L for PBS pH 7, while the limits of quantification were 3.97 mg/L and 0.92 mg/L, respectively. The proposed method was used to predict the drug loading and encapsulation efficiency and the release profile of MNZ ans SMX from ethylcellulose particles.

SEM analysis revealed round microparticles with smooth and porous surface and mean particle sizes of 0.50 µm (SMX) and 1.26 µm (MNZ) were obtained by laser granulometry. Porous structure of the microspheres as seen from SEM images also confirms the fast initial release rates, thus contributing to the rapid release of drug by diffusion. In vitro release studies showed a first initial burst release, a constant sustained release of the remaining antibiotic, and a constant concentration after complete release of antibiotic. The first initial burst release is essential in antibiotics application since the MIC has to be exceeded rapidly in order not to cause resistance to antibiotics. It was investigated the controlled release profile of MNZ and SMX in PBS under simulated stomach (pH 2) and gastrointestinal tract conditions (pH 7). MNZ microparticles exhibited, for both pH values, a fast initial release (= 80% after 3 h) and a sustained behavior until complete MNZ release at around 20 h. In case of SMX loaded ethylcellulose microparticles, different release profiles were observed for the two studied pH values. At pH 2, SMX microparticles showed a slower release profile, reaching low % of release (= 40% after 2 h). It means that these microparticles are a good approach for the delivery of SMX in the gastrointestinal tract, once the antibiotic is not significantly released in the stomach. Regarding pH 7, SMX microparticles exhibited a fast initial release (= 30% after 15 min) followed by a constant phase and then the antibiotic release was again observed (= 80% after 5 h) and a sustained behavior occurred until complete SMX release at around 16 h.

These preliminary tests revealed the potential of encapsulating antibiotics in ethylcellulose by a w/o/w technique. However, the method needs further optimizations for in depth studies and its application at large scale.
7. LIMITATIONS AND FUTURE WORK

Several drawbacks emerged during the development of this work, which limited the obtained results. Nevertheless, proposed laboratorial work for this project was successfully completed. The main limitations were time, microparticles filtration and available instrumentation, coating materials and antibiotics. Due to high occupancy rate of spray-dryer, it was also not possible to use it and compare the obtained results by two different processes.

As preliminary tests, the results in this study are significant and prove the success of MNZ and SMX microencapsulation with ethylcellulose by double emulsion solvent evaporation technique, suggesting the need to explore new delivery systems for antibiotics. In fact, positive results on microencapsulation of antibiotics are reported in literature, using different techniques and coating materials, mainly for targeted delivery of drugs to a specific site in the body. However, to the author’s knowledge, it has not been scientifically demonstrated that microencapsulation of antibiotics is an effective solution to the environmental problem that was presented. In addition to antibiotics, other drugs have also been successfully microencapsulated and its pharmacokinetic/pharmacodynamic activity has been improved. The future work is to adapt this proven success to decrease the environmental problems of massive and irrational use of antibiotics and its low level of metabolism by human and animal organisms.

It is also known that the release profile from microparticles can be affected by the microencapsulation technique, the pH of release medium and by the interactions between the compound, encapsulating agent and auxiliary ingredients. In future work in this area, these parameters should be investigated and optimized to obtain a system with the intended properties. Different encapsulating agents should be tested, as well as other techniques, and the addiction of cross-linking agents. Considering the low product yields obtained, a variety of scale-up was performed and experimented throughout this work. Nevertheless, there were several limitations in filtration process, namely the size of the glass gooch filter and the pores that were constantly blocking. For this reason, the scale-up have not been correctly performed and the obtained microparticles were not used on further tests. As the obtained mass is a limiting factor, it would be interesting to invest in an efficient scale-up to get a more detailed release profile from the microparticles.

The interactions of MNZ and SMX with ethylcellulose during the process of encapsulation could also be investigated using Fourier Transform Infrared (FTI) spectroscopy analysis and thermal properties of the particles could also be evaluated.

Microencapsulation can be a promising solution to decrease the increasing problem of antibiotic resistance. Despite this, it must be borne in mind that the amount of released antibiotic from the microcapsule should not be too low, since low antibiotic concentration could enhance resistance. The development of safe and efficient microencapsulation techniques will require, in the future, in-depth investigations of both the biological and technological aspects of these systems.
REFERENCES


Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics

10.1021/es020158e.


References
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A. ANNEXS

A1. SUPPLEMENTARY CONTENT TO THE STATE OF THE ART

Table 15. Microencapsulating agents and techniques reported for antibiotics, as well as some obtained results.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Microencapsulation agent</th>
<th>Microencapsulation technique</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Metronidazole | Ethylcellulose | Phase separation | - Microcapsules irregularly shaped  
- Lower percentages of larger microcapsules and higher percentages of smaller microcapsules at a higher stirring rate  
- Use of polyisobutylene (7%) increases the percentages of small microcapsules more than the modification of stirring rate, but they are aggregated  
- Total drug content is essentially the same for the different processes, and the free drug content does not really depend on the core:wall ratio, but on the process followed during the microcapsule preparation | (Chemtob et al. 1986) |
| Primaquine | Albumin | Denaturation at high temperature | - Particle size: 500-1200 nm  
- Drug entrapment: 8.7% for primaquine and 8.9% for metronidazole | (Labhasetwar and Dorle 1990) |
| Metronidazole | Gelatin | Combined emulsification and coagulating procedure | - Particle size: 100-800 nm  
- Drug entrapment: 0.5% for primaquine and 0.5% for metronidazole | (Labhasetwar and Dorle 1990) |
| Metronidazole | Albumin | Heat denaturation | - Drug payload (wt.%) = 12.75  
- Molar extinction coefficient = 6.84x10^3  
- Detection limit = 1.39x10^-6  
- Maximal drug release at pH 3  
- % of metronidazole released from microspheres of bovine, egg and human albumin prepared using heat denaturation was in the order bovine > egg ≥ human albumin systems  
- Drug loads for the systems were: egg (19.25%) > human (15.5%) > bovine albumin microspheres (12.75%) | (Elgaria and Friedman 1990) |
| Clarithromycin | PLA | Solvent evaporation (recovering by filtration or spray-drying) | - Retention time for clarithromycin: 6.0 min  
- Microspheres were spherical  
- Microspheres recovered by spray-drying demonstrated surface concavities typical of this technique  
- SEM photomicrographs: microspheres with 5-40 µm in diameter, which is acceptable for administration  
- Drug loadings, were within 10-15% of the theoretical values, suggesting no major loss of drug with either formulation processing | (Gupta et al. 1993) |
| Ciprofloxacin | PLGA | Solvent evaporation  
Evaporation-extraction (o/w emulsion) | Ciprofloxacin content of PLGA microspheres:  
- Solvent evaporation method: 15.25±0.99 µg CIPRO/mg microsphere (50:50) and 7.31±1.16 µg CIPRO/mg microsphere (75:25)  
- Evaporation extraction method: 39.19±4.11 µg CIPRO/mg microsphere (50:50) and 23.26±3.83 µg CIPRO/mg microsphere (75:25)  
- PLGA 50:50 formulation has shown a faster release profile than PLGA 75:25  
- PLGA microspheres were round with smooth surface (independent of the preparation methods and the polymeric composition) | (Martinez et al. 1997) |
| Ciprofloxacin | PLA | Phase separation | - Recovery of CIPRO in the microcapsules was 97.6-98.7%  
- Encapsulation in the range of 97.9-99.0%  
- In the first 8h, microparticle released almost 95% of its total drug. The addition of PLA(D,L) decreased the CIPRO release rate, so that 90% of the drug is released in about 96 h | (Yu et al. 1998) |
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Microencapsulation agent</th>
<th>Microencapsulation technique</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Ciprofloxacin hydrochloride | PLGA 50:50               | Nonsolvent-induced phase separation                                                          | - Recovery greater than 90% at all drug loading levels from microcapsules prepared by both polar and nonpolar procedures  
- Drug release from implants made by the nonpolar method was more rapid as compared to implants made by the polar method  
- In vivo studies indicated that PLGA 50:50 implants were almost completely resorbed within five to six weeks  
- Sustained drug levels, greater than the minimum inhibitory concentration (MIC) of ciprofloxacin, up to 70 mm from the site of implantation, were detected for a period of six weeks | (Ramchandani and Robinson 1998) |
| Erythromycin                | Biodegradable polymers (Eudragit L30D, CAP, gum arabic, maltodexrine)                        | Spray-drying                                                                                   | - Microencapsulation of 80% of each drug as determined by HPLC  
- Particle size ranged from 2 to 300 µm  
- Yield of encapsulation was poor (about 33%)  
- Decreasing the size of the antibiotic crystals by additional grinding and sieving or by vigorous mixing did improve YE significantly  
- Microencapsulation of macrolides using a spray-drying technique is feasible | (Zgoulli et al. 1999)             |
| Clarythromycin              |                          | Emulsification/spray-drying                                                                   | - Drug encapsulation efficiencies close to the theoretical values (84.2-99.5%)  
- Average particle size was about 11 µm  
- Increasing the viscosity of the microsphere suspension by addition of a suspending-viscosizing agent (hydroxypropylcellulose) did not produce an increase of the ocular bioavailability  
- PLGA microspheres can be proposed as a system for ocular delivery of peptide drugs | (Gavini et al. 2004)             |
| Vancomycin                  | PLGA                     | Solvent evaporation (membrane emulsification technique using Shirasu porous glass (SPG))     | - Average diameters of RFP/PLGA microspheres: 1.3, 2.2, 5.2, and 9.0 µm  
- Microspheres relatively monodisperse and the values of the coefficient of variation (CV) for the size distributions of the microspheres were in the range between 7.0 and 16.0%  
- Loading efficiency of RFP was in the range between 50.3 and 67.4% independent of the microsphere size  
- Microspheres with average diameters of 1.3 and 2.2 µm: almost 60% of RFP loaded in the microspheres was released in the initial day and the release was terminated almost within 10 days  
- Microspheres with average diameters of 5.2, and 9.0 µm: release of RFP was observed even 20 days after the release started | (Ito and Makino 2004)            |
| Rifampicin                  | PLGA                     | w/o/w double emulsion/solvent evaporation                                                     | - Average diameters of RFP/PLGA microspheres showed smoother surface but enlarged their particle sizes compared with those of uncoated ones  
- Alginate coated microspheres showed enhanced Tc encapsulation efficiency (EE) from 11.5±0.5% of uncoated ones to 17.9±0.5%  
- Coated PLGA microspheres could prolong Tc release from 9-12 days with 50% or higher in cumulative release of Tc compared with those of uncoated ones | (Liu et al. 2004)                |
| Tetracycline hydrochloride  | PLGA and alginate coated PLGA | Reverse micelle-based encapsulation process                                                   | - Free-flowing, spherical microspheres with a size mode of 88 µm, suitable for delivering bioactive agents via intramuscular or subcutaneous injections  
- Maximal loading efficiency of 63.19±0.64%  
- Successful microencapsulation of tetracycline hydrochloride into PLGA75:25 microspheres  
- Loading efficiency of TH ranged only from 1.83±0.10 to 3.45±0.92%  
- Microparticles surfaces had numerous pores, while their internal architecture was honey-combed  
- This method yielded very poor microencapsulation efficiencies | (Kim et al. 2005)                |
| Tetracycline hydrochloride  | PLGA                     | Methylene chloride-based double emulsion process                                               | - Loading efficiency of TH ranged only from 1.83±0.10 to 3.45±0.92%  
- Microparticles surfaces had numerous pores, while their internal architecture was honey-combed  
- This method yielded very poor microencapsulation efficiencies | (Kim et al. 2005)                |
### Antibiotic: Clarithromycin
- **Microencapsulation technique:** Eudragit RL 100 and RS 100
- **Results:**
  - Total CL loaded into the products: 32–47% in the dried formulations.
  - Preparation yields: 22–35%.
  - Mean particle size: 6–11 µm.
  - Clarithromycin content: 32–47%.
  - Microparticles prepared by the spray-drying method had a slower release compared to the casting-drying method. The spray-drying method seems to yield microparticles for prolonged release of CL.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Microencapsulation technique</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>Eudragit RL 100 and RS 100</td>
<td>Spray-drying</td>
<td>(Geno et al. 2006)</td>
</tr>
</tbody>
</table>

### Antibiotic: Sulfamethoxazole
- **Microencapsulation technique:** Polyester polyol acrylate polymer dispersed in silicone oil
- **Results:**
  - Microspheres were found to be very spherical, entirely free of any defects, nonporous, and most of them were in the range of 10–20 µm in diameter.
  - Microcapsule yield: 78–79%.
  - Microencapsulation efficiency: 83–91%.
  - All the formulations have a burst release up to about two days probably due to release of sulfamethoxazole near the periphery of the microspheres.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Microencapsulation technique</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethoxazole</td>
<td>Polyester polyol acrylate polymer dispersed in silicone oil</td>
<td>Spray-drying</td>
<td>(Nivasu and Tammisetti 2006)</td>
</tr>
</tbody>
</table>

### Antibiotic: Ciprofloxacin hydrochloride
- **Microencapsulation technique:** Pectin and chitosan
- **Results:**
  - Production yield was from 46% to 48%, the particle diameters were less than 5 µm and the encapsulation efficiencies were above 98%.
  - All microsphere formulations were spherical.
  - The full amount of ciprofloxacin hydrochloride was released from PMS1-4 microspheres in 48 hours but only 35.4% was released from CMS microspheres in 120 hours.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Microencapsulation technique</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin hydrochloride</td>
<td>Pectin and chitosan</td>
<td>Spray-drying</td>
<td>(Orhan 2006)</td>
</tr>
</tbody>
</table>

### Antibiotic: Doxycycline
- **Microencapsulation technique:** PLGA and PCL
- **Results:**
  - FT-IR: no interaction between drug and polymers.
  - DSC: confirmed the polymorphism of doxycycline and indicated a molecular level dispersion of doxycycline in the microspheres.
  - SEM: confirmed the spherical nature and smooth surfaces of the microspheres produced.
  - Mean particle size of the microspheres: 90 to 200 µm.
  - In vitro release studies indicated the release of doxycycline from 7 to 11 days, depending upon the blend ratio of the matrix. Up to 11 days, doxycycline concentrations in the gingival crevicular fluid were higher than the minimum inhibitory concentration of doxycycline against most of the periodontal pathogens.
  - Significant results were obtained with respect to both microbiological and clinical parameters up to 3 months even as compared to commercial doxycycline gel in the in vivo studies.
  - Results of in vitro and in vivo testing suggest that the novel blend microparticles prepared from PLGA and PCL are effective in controlled delivery of doxycycline to periodontal pockets.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Microencapsulation technique</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline</td>
<td>PLGA and PCL</td>
<td>Water-in-oil-water (W/O/W) emulsion technique</td>
<td>(Mundargi et al. 2007)</td>
</tr>
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</table>

### Annexes

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Microencapsulation technique</th>
<th>Results</th>
<th>Reference</th>
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<td>Doxycycline</td>
<td>PLGA</td>
<td>Spray-drying</td>
<td>(Pradip Patel, Raghavendra C. Mundargi, V. Ramesh Babu, Dharmendra Jain, Vidhya Rangaswamy 2008)</td>
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<th>Microencapsulation technique</th>
<th>Results</th>
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<tr>
<td>Doxycycline</td>
<td>PLGA</td>
<td>Spray-drying</td>
<td>(Pradip Patel, Raghavendra C. Mundargi, V. Ramesh Babu, Dharmendra Jain, Vidhya Rangaswamy 2008)</td>
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### Reference

- Geno et al. (2006)
- Nivasu and Tammisetti (2006)
- Orhan (2006)
- Mundargi et al. (2007)
- Pradip Patel, Raghavendra C. Mundargi, V. Ramesh Babu, Dharmendra Jain, Vidhya Rangaswamy (2008)
**Microencapsulation: A promising technique to reduce the environmental contamination by antibiotics**

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</table>
| Doxycycline           | PLGA                         | Water-in-oil-in-water double emulsion solvent evaporation  
|                       |                              | - Particle size: 26 µm  
|                       |                              | - Doxycycline loaded: 10%  
|                       |                              | - Encapsulation efficiency: 19%  
|                       |                              | - Particle surfaces are smooth with regularly shaped morphologies  
|                       |                              | - FT-IR indicated no chemical interactions between doxycycline and PLGA  
|                       |                              | - XR D suggested molecular level dispersion of doxycycline in PLGA  
|                       |                              | - In vitro release data of the spray-dried formulations and of the solvent evaporated formulations indicated quick release of about 40-50% of doxycycline in about 5-7 h  
|                       |                              | - The release of doxycycline was continued for longer times up to 72 and 48 h, respectively, for spray-dried and solvent evaporated formulations  
|                       |                              | - Antimicrobial studies performed on one formulation and placebo microspheres suggested that drug concentrations during in vitro release are above the minimum inhibitory concentration (MIC) for Staphylococcus aureus growth  
|                       |                              | (Pradip Patel, Raghavendra C. Mundargi, V. Ramesh Babu, Dharmendra Jain, Vidhya Rangaswamy 2008)  
| Amoxicillin           | PLGA                         | Solvent extraction/evaporation methods (w/o/w and s/o/w)  
|                       |                              | - w/o/w method: the PVA surfactant produced statistically larger microspheres (51.9 µm) than SDS (40.2 µm) or DSS (46.6 µm) at the same concentration of 1%  
|                       |                              | - s/o/w method: statistically significant reduction in particle size was observed compared with the w/o/w method  
|                       |                              | - The anionic surfactants SDS and DSS produced smaller microspheres than PVA  
|                       |                              | - The s/o/w method achieved higher EE of 40% in PLGA microspheres using surfactant SDS compared with the conventional water-in-oil-in-water (w/o/w) method (about 2%)  
|                       |                              | - Triphasic release profiles were observed for all PLGA microspheres (s/o/w) with slight drug burst, a slow diffusion-controlled release within the period of about 7 days and followed by the degradation-controlled sustained release for further 30 days  
|                       |                              | (XU et al. 2008)  
| Cephalexin            | PLGA                         | w/o/w double emulsion and o/w single emulsion  
|                       |                              | - CPX-loaded PLGA microspheres prepared using a w/o/w double emulsion technology were slightly larger (~3-5 µm) but much higher in drug content (~18% w/w) than those obtained using o/w single emulsion preparation technology (average size was 2 µm, encapsulation efficiency was less than 2%)  
|                       |                              | - Stirring during emulsification and a change in both the internal and external phase of the emulsion, affected the size and the drug entrapment efficiency of the microspheres obtained  
|                       |                              | (Chaisri et al. 2009)  
| Ciprofloxacin hydrochloride | PLGA                        | Solvent evaporation (o/o method)  
|                       |                              | - CIPRO-encapsulated PLGA microspheres showed spherical shapes under a SEM and their particle sizes ranged from 10 to 50 µm  
|                       |                              | - In an in vitro drug release study, CIPRO was continuously released over 3 weeks from the microspheres, and a burst effect was observed for the first 3 days  
|                       |                              | - Encapsulated CIPRO keeps its antibacterial activity after microencapsulation  
|                       |                              | (JEONG et al. 2009)  
| Metronidazole hydrochloride | Pectin-gelatin and alginate-gelatin | Complex coacervation  
|                       |                              | Pectin-gelatin  
|                       |                              | - Yield: (0.55±0.14) g  
|                       |                              | - % of loading: (5.32±0.31)  
|                       |                              | - % of encapsulation: 2.92  
|                       |                              | - Average particle size: 45.14 µm  
|                       |                              | - Specific surface area: 0.2301 m²/g  
|                       |                              | Alginate-gelatin  
|                       |                              | - Yield: (0.96±0.37) g  
|                       |                              | - % of loading: (6.51±0.43)  
|                       |                              | - % of encapsulation: 6.25  
|                       |                              | - Average particle size: 41.34 µm  
|                       |                              | - Specific surface area: 0.2827 m²/g  
|                       |                              | (Saravanan and Rao 2009)  

**Annexes**
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| Gentamicin       | Albumin                  | Supercritical Assisted Atomization (SAA) | - All the microspheres produced by SAA exhibited large drug content and very high encapsulation efficiency  
- In all cases, spherical and noncoalescing particles were successfully produced with a mean particle size of 2 µm and with a standard deviation of about 1 µm  
- The release of GS from the microspheres was found to be diffusion controlled and correlated with BSA concentration | (Chaisri et al. 2011)                                                                                     |
| Gentamicin       | PLGA and PLHMGA          | Double emulsion evaporation technique | - Neither ethanol nor surfactants had beneficial effects on the drug loading efficiency (around 4-10%)  
- An increase in buffer concentration (and thus osmotic pressure) of the external phase resulted in a substantial increase of GS-loading (from 10 to 28%)  
- The best formulation identified in this study had a loading efficiency of around 70% resulting in PLGA microspheres with a 6% (w/w) loading  
- The particles showed a burst release of the drug depending on their porosity, followed by a phase of 35 days where hardly any release occurred  
- The drug loading efficiency of GS in PLHMGA was not significantly different from PLGA microspheres (64%) | (Raval et al. 2011)                                                                                     |
| Doxycycline      | PCL                      | Water-in-oil-in-water double emulsion solvent evaporation | - Yield of production: 82.90% to 93.55%  
- Encapsulation efficiencies of doxycycline-loaded PCL microspheres: 20.09% to 81.38%  
- Drug/polymer ratio does not affect the production yield but it affects the encapsulation efficiencies  
- SEM: microspheres are almost spherical with smooth surface and no drug crystals were found on the microsphere surface  
- Observed MICs for K. pneumoniae and P. aeruginosa: 5 µg/mL and 25 µg/mL, respectively  
- S. aureus exhibited susceptibility at lower concentration levels (1 µg/mL) while E. coli shows susceptibility at 2.5 µg/mL | (Chaisri et al. 2011)                                                                                     |
| Metronidazole    | Alginate                 | Impinging aerosols method | - Maximum loadings: 7%  
- Encapsulation efficiency: 21%  
- Around 75 and 96% of the metronidazole content was released from hydrated and dried microspheres respectively in simulated gastric fluid within 20 min | (Sohail et al. 2012)                                                                                     |
| Doxycycline      | PLA                      | Solvent evaporation technique (o/w) | - Maximum drug encapsulation: 38%  
- Drug loading: 0.15% to 0.25%  
- Average particle size: 87-91 µm  
- Size distribution, varying between 80 and 110 µm, with a spherical profile and porous surface  
- FT-IR study hasn't revealed any drug-polymer interaction  
- In vitro release study of PLA microspheres loaded with doxycycline reveals a rather fast release which requires only few hours to go to completion | (Kassab et al. 2013)                                                                                     |
| Doxycycline      | PHB                      | Polymer precipitation via dialysis | - The highest drug loading was obtained by single emulsion with high speed stirring. In the case of multiple emulsion, the combination of ultrasound with high speed stirring resulted in the most elevate process yield and drug loading capability. | (Rodriguez-Contreras et al. 2013)                                                                  |
| Doxycycline      | Tetracycline             | o/w solvent evaporation | - Moderate drug encapsulation values: 38% and 24% for doxycycline and tetracycline, respectively  
- Microspheres of both drugs show homogenous size distribution: between 80 and 110 µm  
- In vitro release studies: tetracycline release is slightly faster and reaches 98% in less than 5 hours, in comparison with doxycycline which attains the same percentage after 7 hours | (Rima Kassab, Paolo Yammine, Dima Moussa 2014)                                                       |
| Metronidazole    | PLGA                     | Water in oil in water emulsion technique | - Metronidazole can be encapsulated into PLGA scaffolds even at low concentration as permitted by aqueous solubility | (Aina et al. 2015)                                                                                     |

**Annexes**
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<td>Metronidazole</td>
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<td>Electrospraying</td>
<td>- XRPD has proven to be a veritable tool for monitoring the observed encapsulation&lt;br&gt;- Particles with spherical morphology, with sizes of (3946±407) nm and (1774±167) nm, respectively for PLGA-metronidazole(DCM) and PLGA-metronidazole(TFE)&lt;br&gt;- Sustained drug release for at least 41 days</td>
<td>(Prabhakaran et al. 2015)</td>
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