SUMMARY

Antibiotic-loaded nanoparticles for combating the antimicrobial resistance in veterinary medicine

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Introduction

During the course of a century, antibiotics have made an impressive leap from being considered one of the greatest and most important discoveries in modern medicine to being one of the greatest threats to public health. According to government reports from the United Kingdom (Hutchings et al., 2019; Shankar, 2016), it is estimated that 10 million people die each year due to antibiotic-resistant bacteria, a phenomenon known as antimicrobial resistance (AMR). Although the causes are multiple, varied, and widely debated in the specialized literature, an important causal factor in accelerating this process is the misuse of antimicrobial substances in veterinary medicine, as first reported in 2015 by the European Centre for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA), and by the European Medicines Agency (EMA) (Prestinaci et al., 2015a). This phenomenon has led to the rapid emergence of animal-origin bacteria that are resistant to a wide range of antimicrobial substances (Economou & Gousia, 2015).

Considering that the discovery and development of new antimicrobial substances is a relatively slow process (Miethke et al., 2021), nanotechnology has opened up new possibilities for improving the effectiveness of antibiotics already used in human and veterinary therapeutics (Cerbu et al., 2021; Dupuis et al., 2022; Patra et al., 2018). Defined as the study of materials within the dimensions of 1 to 1000 nm, nanotechnology makes it possible to significantly reduce the dosage of antimicrobial substances used by improving their bioavailability. Consequently, it manages to contribute to combating one of the main causes of AMR, namely overdosing. At the same time, it also contributes to improving the pharmacokinetics of antibiotics, thus countering certain limitations in their clinical use.

Florfenicol (d-treo)-1-(methylsulfonylphenyl)2-dichloroacetamide-2-fluoro-1-propanol) (FFC) is a broad-spectrum antibiotic with bacteriostatic action, used exclusively in veterinary medicine (Park et al., 2008). FFC is a derivative analog of chloramphenicol and thiamphenicol (Mechesso et al., 2018), it is non-volatile, with good tissue penetration, and known to be effective against both Gram-positive and Gram-negative bacteria (Shuang, Yu, Weixiao, & Dacheng, 2011). Through its excessive use in veterinary therapy and aquaculture, as well as a feed additive for prophylactic purposes, several resistance genes have been identified against FFC initially in fish in 1990 (Kim & Aoki, 1996) and subsequently in various other animal species such as cattle, poultry, and pigs (Arcangioli et al., 1999; Bolton et al., 1999; Fang et al., 2020). Additionally, the presence of an FFC-resistant *E.coli* isolate has been reported in humans (Fernández-Alarcón et al., 2011), highlighting the causal relationship between AMR in human and veterinary medicine, as FFC is used exclusively in veterinary medicine.

Structuring the thesis

The doctoral thesis entitled ***"Polymeric Nanoparticles Loaded with Antibiotics for Combating Antibiotic Resistance in Veterinary Medicine"*** consists of 139 pages and includes 49 figures and 13 tables. The thesis follows the formatting guidelines of the IOSUD Cluj Napoca, and is structured into two main parts: the current state of knowledge and the personal contribution.

Current State of Knowledge

The first part of the thesis, the current state of knowledge, comprises 28 pages and is organized into three chapters.

Chapter I: ***"Antimicrobial Resistance in Veterinary Medicine"*** **-** This chapter, divided into four subchapters, explores the concept of antimicrobial resistance and its implications in veterinary medicine. It discusses the mechanisms of antimicrobial resistance development, both intrinsic and extrinsic, along with strategies for combating it.

Chapter II: ***"Florfenicol: Uses in Veterinary Medicine and Improvement Methods"*** **-** Providing an overview of florfenicol, this chapter discusses its general aspects, including structure and biochemical properties, as well as its various applications in veterinary medicine. A subchapter titled "Antimicrobial Resistance to Florfenicol and Other Limitations of its Use" addresses the issue of antimicrobial resistance and other constraints associated with the use of florfenicol in veterinary therapeutics.

Chapter III: ***"Nanotechnology in Veterinary Medicine"*** **-** Highlighting the application of nanotechnology in veterinary medicine, this chapter begins with the definition of nanotechnology and explores different types of nanostructures. It further delves into nanostructures with florfenicol, emphasizing their potential in veterinary medicine.

Personal Contribution

The second part of the thesis, the personal contribution in the field of the doctoral thesis, encompasses 80 pages and is organized into eight chapters. It provides detailed information about the tested hypothesis, the research methodology (materials and methods), as well as the synthesis of florfenicol-loaded nanostructures, their characterization, and testing regarding antimicrobial potential, anti-biofilm potential, and cytotoxic effects.

Hypothesis of the Study

The hypothesis of the study posited that the effectiveness of florfenicol can be enhanced by loading it into a nanostructure system, which allows the use of minimal

amount of active substance through controlled and prolonged release. Thus, it is assumed that this technology could improve the antibiotic both in terms of efficacy, by using smaller doses, and in terms of pharmacokinetics, by facilitating the formulation of an aqueous solution and reducing the frequency of administrations.

Study objectives and research methodology

Following a thorough literature review, which presented the current state of knowledge, the importance of identifying factors actively contributing to the perpetuation of antibiotic resistance was highlighted. One of these factors is the improper use of antimicrobial substances, which are often overdosed to achieve the desired therapeutic effect, without considering the pharmacokinetics and bioavailability of the active agent involved. Attention was thus focused on florfenicol, a broad-spectrum antibiotic widely used in veterinary medicine, whose improvement would have a significant impact in veterinary therapy, both clinically and economically. Several steps were followed to conduct the experiments presented in this doctoral thesis, involving the formulation of clear objectives and well-defined working stages. The study objectives can be presented as follows:

1. Synthesis of nanostructures loaded with florfenicol (polymer selection, solvent selection, synthesis method based on previously identified limitations).
2. Characterization of the obtained nanostructures (size, shape, arrangement mode, zeta potential, polydispersity index, release profile of the active substance from nanostructures).
3. Testing the antimicrobial potential and inhibition of bacterial biofilm.
4. Testing the cytotoxic effect to verify the biocompatibility of the obtained nanostructures.

Chapter VI, entitled ***"Synthesis of nanostructures loaded with florfenicol,"*** comprises two subchapters, each addressing aspects related to the synthesis of nanostructures based on PLGA-FFC and PLGA-Lignin-FFC. PLGA-FFC synthesis: It was performed using the solvent evaporation emulsification method. The principle of the method is based on the formation of a mixture between two types of substances known to be heterogeneous and immiscible with each other, using a suitable surfactant compound capable of reducing the interfacial tension between them, thus enabling mixing within a stable homogeneous solution. The method involves emulsifying the organic polymer solution and the active substance to be encapsulated (representing the organic phase) in an aqueous phase, followed by the evaporation of the organic solution (Mallakpour & Behranvand, 2016). The method used initially involved the formation of a water-in-oil (W/O) type mixture, using the active substance and the polymer in a ratio of 1/10, representing the organic phase. Thus, 440 mg of PLGA and 44 mg of FFC dissolved in an organic solvent were added, represented by 10 ml of ethyl acetate. The solvent was chosen based on the existing data in the literature, as it favored the production of homogeneous nanoparticles with smaller sizes compared to other organic solvents such as dichloromethane or chloroform (Karp et al., 2019). The aqueous phase

was obtained by dissolving 2 g of polyvinyl alcohol (PVA) in 100 ml of deionized water. The organic phase was poured into 80 ml of the aqueous phase and homogenized for 15 minutes at room temperature using a magnetic stirrer. This was followed by a high-pressure homogenization step using equipment (high-pressure homogenizer) from Microfluidics Corp, Westwood, MA. The homogenization was carried out at a pressure of 30,000 psi, with four passes at 4°C. Subsequently, the solvent was evaporated using a rotary evaporator R-300 (Buchi Corporation, Switzerland) under vacuum at 33°C for 100 minutes. Purification was performed by ultracentrifugation at 30,000 rpm (150,000 relative centrifugal force) using a Beckman Coulter Optima XPN 80 ultracentrifuge (Danaher Corporation, Brea, CA). The obtained deposit after removing the supernatant was resuspended in 60 ml of ionized water, and 950 ml of trehalose was added. The resulting sample was divided into 10 vials and stored at a temperature of -80°C for 24 hours.

Synthesis of PLGA-Lignin-FFC: The working method used to obtain these types of polymeric nanostructures is the one mentioned by Carlos et al. in a study conducted in 2020 (Astete et al., 2020), and it involves three working steps.

The first step involved adding 4 g of PLGA to a reaction vessel with a magnetic stirrer. Subsequently, the organic solvent represented by 100 ml of dichloromethane (DCM) was added with extra caution (the syringe was introduced under pressure to ensure gradual release of DCM). The mixture was homogenized at room temperature for 5-10 minutes at 500 rpm. A bubbler was connected with the outlet portion directed towards the reaction vessel and the inlet portion towards the argon dosing device. Later, 110 µl of oxalyl chloride (kept at refrigeration temperature) was added. Then, 4 ml of dimethylformamide (DMF) was added as the reaction catalyst. After 30 minutes, the argon flow was stopped, and the reaction was left at room temperature for 5 hours. With the rotavapor cooler turned on one hour earlier, the argon flow from the reaction vessel was increased for 1 minute to remove all gases accumulated inside due to chemical reactions. The obtained polymer was then centrifuged in the rotavapor in a round-bottom flask at a pressure of 300 mbar and 32°C until half of the initial polymer volume remained in the vessel. At this point in the reaction, a deposition could be observed on the vessel walls, and this deposition was quantified by weighing the vessel used in parallel with the weighing of an identical vessel and measuring the difference between them. The obtained polymer was added to another organic solvent, namely ethyl ether, using a 10 ml pipette, and this washing process was repeated 4-5 times. It was then incubated for 24 hours at 30°C under vacuum.

The second step involved drying lignin at 60°C for 24-72 hours. The obtained PLGA polymer was weighed in an incubator (the obtained mass was 4128.3 mg). Then, 2.1 g of lignin was weighed, solubilized in water, and kept in a desiccator. A glass syringe was filled with argon and injected into dimethyl sulfoxide (DMSO) (sealed and under controlled conditions, without humidity). The syringe was thus filled with DMSO due to the pressure difference between the two, with 25 ml. DMSO was injected into the reaction vessel, and in another reaction vessel (a vessel with 3 access ports), lignin and 30 ml of DMSO were placed. Both vessels were placed on a magnetic stirrer for 1 hour. PLGA was added to the vessel containing lignin using a pipette. The bubbler and argon

flow were connected to the reaction vessel. Then, 4 ml of DMF was added, and the reaction took place for 24 hours. The argon flow was stopped after 30 minutes.

The third step involved adding 200-300 ml of ethyl ether to a reaction vessel. The obtained polymer was poured over it and allowed to react for 10 minutes, followed by several successive washes (4-5 washes every 15 minutes) with this organic solvent to remove all traces of DMSO. The polymer was then poured with DCM into the decantation vessel, and water was added to homogenize and clean the vessel walls. Subsequently, the supernatant was collected, and the water on the surface was removed, a process repeated 3 times. The obtained supernatant was centrifuged in the rotavapor at a pressure of 350-400 mbar at 32°C until its viscosity increased (a process that took about an hour). In a thick-walled vessel previously weighed, the resulting polymer was added, carefully recovered from the vessel walls, resulting in a polymer mass of 1287.99 mg. It was placed in the refrigerator slightly tilted to avoid breaking the vessel and frozen by drying.

Loading of PLGA-lignin nanoparticles with florfenicol represents the final stage in obtaining PLGA-lignin-based nanoparticles loaded with florfenicol (PLGA-lignin-FFC NPs). These were synthesized using 900 mg of PLGA-lignin polymer and 90 mg of FFC dissolved in 14 ml of ethyl ether at room temperature by continuous mixing. After dissolution, the organic phase was poured into 150 ml of nanopure water (aqueous phase) pre-saturated with ethyl acetate and mixed for 15 minutes at room temperature, followed by high-pressure homogenization (Microfluidics Corp., Westwood, MA) at 30,000 psi with four passes at 4°C. Subsequently, the ethyl acetate was evaporated using a rotary evaporator R-300 (Buchi Corporation, Switzerland) at 33°C under vacuum for 100 minutes. 1g of trehalose was added for cryoprotection. The obtained sample was equally divided into 14 containers, kept at -80°C for 24 hours, and subsequently lyophilized (Labconco, Corp, Kansas City, MO). Using the same mentioned procedures but without loading with FFC, polymeric nanoparticles based on PLGA and PLGA-lignin were obtained.

Chapter VI, entitled ***"Description and Characterization of Obtained Nanostructures,"*** includes information about the morphology and dimensions of the obtained nanostructures, the evaluation of florfenicol release from the nanostructures, and the description of basic parameters. All synthesized nanoparticles have a spherical shape with irregular size distribution, and the average diameter is 100.2 nm for PLGA-NPs, 100.7 nm for PLGA-FFC-NPs, 84.99 nm for PLGA-lignin, and 86.30 nm for PLGA-Lignin-FFC NPs. The size of the obtained nanoparticles falls within the range necessary for them to withstand the physiological process of endocytosis. The obtained values for the polydispersity index (PDI) (according to the analysis report from Malvern Zetasizer, illustrated in Figures 13, 14, 15, and 16) were 0.061 ± 0.019 for PLGA-FFC NPs, 0.089 ± 0.020 for PLGA-lignin-FFC, and 0.087 ± 0.014 and 0.089 ± 0.020 for PLGA NPs and PLGA-lignin NPs without loaded active substance, respectively. The obtained zeta potential values were -26.80 ± 1.30 and -55.97 ± 3.43 mV for PLGA-FFC NPs and PLGA-lignin-FFC NPs, and -35.20 ± 1.64 and -55.33 ± 2.32 mV for PLGA NPs and PLGA-lignin NPs without loaded active substance. The release profile of florfenicol from the nanostructures

demonstrated complete release within 6 hours for PLGA-based nanostructures, while for PLGA-lignin-based nanostructures, complete release was observed only after 24 hours.

Chapter VII, entitled "***Evaluation of Antimicrobial Activity of Obtained Nanostructures,"*** includes the materials and methods used to evaluate the antimicrobial properties of the nanostructures, as well as the technique for evaluating biofilm inhibition capacity, along with SEM evaluation of the biofilm inhibited by florfenicol-loaded nanoparticles. After culturing the selected bacterial strains (*S. aureus*-ATCC 29213, *E. coli*-ATCC 25922, and *P. aeruginosa*-ATCC), PLGA-based nanoparticles showed a reduction in the minimum inhibitory concentration by ~97.13% for S. aureus, 99.33% for E. coli, and 64.1% for P. aeruginosa compared to free florfenicol. Therefore, by incorporating florfenicol into nanostructure systems, its efficiency is improved by using a significantly lower dose, as indicated by in vitro studies. Tests for evaluating antimicrobial susceptibility, including qualitative or quantitative MIC evaluation, are based on the direct interaction of the isolated pathogen or the standard strain used with the tested active substance. However, this interaction does not take into account other factors that influence the effectiveness of the antimicrobial substance in the animal's body, such as distribution volume, proper functioning of all body systems, or immune system activity, which can significantly affect the final effect of the antibiotic to which the microorganism is susceptible. Clearly, a high degree of susceptibility of the strain and, implicitly, a MIC value below the known minimum limits represented by the Epidemiological Cut-Off Value (ECOFF), increases the chances of achieving microbiological and clinical success. Moreover, it is fundamentally incorrect to assess the dosage of a microbial substance solely based on MIC evaluation since, in addition to the aforementioned aspects, MIC has been shown to be an inconsistent parameter in terms of reproducibility (a 2011 study states that repeated MIC evaluation, even by the same laboratory staff member, can produce MIC values that exhibit statistically significant differences).

Despite these aspects, the significant decrease in the MIC value for the tested bacterial strains is particularly relevant for the applicability of nanostructures as delivery systems for florfenicol in veterinary medicine, as the use of a lower dose could reduce or slow down the development of florfenicol resistance genes, actively contributing to the reduction of antibiotic resistance in veterinary medicine. Achieving therapeutic efficiency with a lower dose is also relevant in applied veterinary medicine since florfenicol could interfere, dose-dependently, with an optimal immune response following the implementation of immunoprophylactic actions in livestock animals (Shuang, Yu, Weixiao, & Dacheng, 2011).

Regarding the activity of florfenicol-loaded nanostructures on the bacterial biofilm produced by S. aureus, E. coli, and P. aeruginosa, the obtained results were favorable, as low concentrations of nanostructures and hence the active substance involved were tested, resulting in partial inhibition of the bacterial biofilm. Furthermore, florfenicol is known as a bacteriostatic antibiotic, acting by inhibiting protein synthesis without affecting the biofilm. Therefore, the lack of complete

inhibition of the biofilm was not an entirely unexpected result. The results obtained from the quantitative evaluation of the biofilm for the three tested strains were confirmed by evaluating it through electron microscopy, illustrating partially inhibited portions of the biofilm compared to the biofilm formed for the positive control.
Regarding the ability of these florfenicol-loaded nanostructures to inhibit the biofilm previously formed by the tested bacterial strains, no effect was observed, as the measured values for the biofilm treated with antibiotics did not show statistically significant differences compared to the positive control. The results are consistent with the data found in the specialized literature (X. Yuan et al., 2020) since florfenicol has been reported previously as ineffective in inhibiting a pre-existing biofilm.

Chapter VIII, titled "***Cytotoxicity Evaluation***," includes information about the generation of primary cell lines used to assess the cytotoxic effect, the MTT method for cytotoxicity evaluation, and the obtained results. Florfenicol-loaded PLGA and PLGA-lignin nanostructures showed very low cytotoxic potential, with differences between cell proliferation observed in the case of the positive control and primary cells treated with the specific antibiotic concentrations being statistically insignificant. Although much higher doses than those indentified in order to evaluate the minimum inhibitory concentration were used, no harmful effects were observed on the proliferation of the primary cells tested. Despite the fact that further studies are considered necessary, until now, through the in vitro results obtained, the polymeric nanostructures loaded with florfenicol can be considered safe in terms of cytotoxic potential at the maximum tested dose of 4 mg/ml.

Chapter IX, titled "***General Conclusions,"*** provides a synthesis of all the information obtained from the conducted research studies. Although florfenicol is a relatively new antibiotic, it is exclusively used in veterinary medicine due to its broad spectrum of activity against a large number of bacterial groups (Wei et al., 2016). Considering all its advantages and extensive use in veterinary medicine, it can be affirmed that it is only a matter of time until the development of bacterial resistance limits its effectiveness (Li et al., 2020). Furthermore, the perpetuation of the phenomenon of bacterial resistance gene development against florfenicol among bacteria with zoonotic potential can have a negative impact on public health. All these aspects reinforce the aforementioned idea that limiting the development of antibiotic resistance has a significant impact in medicine. Therefore, any method to improve an existing antibiotic successfully used in veterinary therapeutics can contribute to reducing the development of antimicrobial resistance.

Loading florfenicol within polymeric nanostructure delivery systems (PLGA and PLGA-lignin) has an increased potential for enhancing this antimicrobial substance. Implicitly, this phenomenon can slow down the antimicrobial resistance process by reducing the dose of FFC and improving its pharmacological properties. Although reducing the active substance dose would seemingly lead to medication underdosing, in vitro tests provide evidence of the efficacy of this antimicrobial at a much lower inhibitory concentration percentage than what is known and identified through testing free florfenicol. Therefore, incorporating florfenicol offers not only the possibility of

using a significantly lower dose but also the advantages of controlled release, which subsequently leads to economical and practical antibiotic use. The reduced MIC for florfenicol, correlated with the lack of cytotoxic effects, indicates that through nanotechnology, FFC can be improved in terms of performance and efficacy, simultaneously providing the desired antimicrobial effects against bacterial pathogens in multiple animal species.

Despite the previously known mechanism of action, florfenicol being a bacteriostatic antibiotic and not having a documented effect on bacterial biofilm, when loaded into polymeric nanostructures, it has the capacity to partially inhibit biofilm formation. However, the efficacy of both the free antibiotic and the one loaded into nanostructures is minimal in situations where a pre-existing biofilm is present.

Originality and Innovative Contributions of the Thesis

The doctoral thesis contains both innovative contributions in the field of veterinary medicine and elements of originality. Although PLGA-based nanostructures loaded with florfenicol have been previously described in the literature, they were synthesized using an adapted and modified protocol, providing additional data regarding their testing that have not been previously reported. The performed tests, such as the evaluation of the antimicrobial effect through MIC determination, the effect on biofilm, and cytotoxicity, represent innovative contributions to veterinary medicine. These favorable results can serve as a starting point for improving florfenicol. Thus, new information about this nanostructure-based delivery system is obtained by highlighting its advantages and limitations.

Moreover, the thesis contains as an element of originality the synthesis of PLGA-lignin-based nanostructures loaded with florfenicol, describing their morphology, structural characteristics, antimicrobial activity, anti-biofilm activity, and potential cytotoxicity. The obtained results constitute new and original information for the synthesis of a novel delivery system that exhibits superior characteristics compared to previously tested ones and could significantly contribute to veterinary therapeutics.

All these laboratory experiments confer an innovative character to the thesis, as they have the potential to contribute to the improvement of florfenicol for its use in veterinary medicine, thereby reducing the development of antibiotic resistance in veterinary medicine and directly impacting

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