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Iron Oxide Nanoparticle Improves the Antibacterial Activity of Erythromycin

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Abstract

Antimicrobial resistance is considered a public health problem around the world. Every day new resistance mechanisms emerge and spread globally. Therefore, it is imperative to improve the treatment schemes that have been developed to cure infections caused by human pathogens for instance, *Streptococcus pneumoniae*. Here, we are proposing a strategy that consists on combining the drug with iron oxide nanoparticles (FeNPs). We synthetized FeNPs by chemical technique and they were functionalized in order to make them soluble and biologically compatible. The antimicrobial agent (erythromycin) was coupled to FeNPs and its antibacterial properties was analysed in bacterial cultures of *S. pneumoniae*. The antibacterial effect was improved when the drug was coupled to FeNPs, also, the bacterial viability was diminished in presence of FeNPs. Additionally, the capsule of the bacterium has an important role in the antibacterial effect, because a mutant without capsule (R6) was more sensible to antibacterial agent than a strain with capsule (TIGR4 or ATCC strain). Maybe the FeNPs help to erythromycin to cross the capsule. In conclusion the presence of FeNPs improve the antibacterial activity of erythromycin.

Keywords: *Streptococcus pneumoniae*; Nanoparticles; Iron; Antibacterial activity; Erythromycin

Abbreviations

PEG: Poliethylen Glicol; MIC: Minimum Inhibitory Concentration (minimum concentration of antibiotic required to inhibit growth of a bacterium); NPs: Nanoparticles; FeNPs: Iron Oxide Nanoparticles (iron particles with a size less than 1 μm); NH_4OH: Ammonium Hydroxide Solution; Uv-Vis spectra: Ultraviolet Spectrum; CLSI: Clinical & Laboratory Standards Institute; ATCC: American Type Culture Collection; CFU: Colony Forming Unit; $FeCl_2.4H_2O$: Iron (II) Chloride; $FeCl_3$: Iron (III) Chloride; Eri: Erythromycin

Introduction

Streptococcus pneumoniae is a human pathogen bacterium, infects principally infants and the elderly, causing often sinusitis, otitis and pneumonia [1]. In some cases the infection is more severe and the bacterium can reach other tissues causing meningitis or septicaemia [2]. Diseases caused by *S. pneumoniae* are treated with antimicrobials such as penicillin (pen), erythromycin (eri), vancomycin (van) and ciprofloxacin (cip). However, the indiscriminate use of these drugs have developed an event known as antimicrobial resistance in which, the patient does not respond to the therapy [3]. For this reason, it is necessary to improve the treatment scheme. A good alternative for this purpose is the nanotechnology [4]. This new discipline is in constant increase and we think that the antimicrobial therapy can be improved considerably using iron oxide nanoparticles (FeNPs) as nanovehicles, because this element is necessary for supporting its cellular growth and viability of this pathogen [5]. In fact it has been reported that bacterium can obtain iron of sources such as haemoglobin haem and Fe^{3+} [5,6]. The principal advantages of FeNPs usage are: they are easy to obtain, they are economical [7], the methodology allows to develop broad range sizes (5-70 nm) [8]. Additionally, they can be functionalized with several materials such as polyethylene glycol (PEG) in order to make them more appropriate to be used biologically [9]. The FeNPs are suitable for biomedical applications [10] because they have unique properties, such as, superparamagnetic behaviour, external control, hypothermal behaviour when they are stimulated, and controllable parameters [11]. Researchers have attempted to use nanoparticles for tumour inhibition [12], and as antibacterial agents [13]. In this work, we hypothesized that the combination of erythromycin with FeNPs could improve the antibacterial effect (potency) and also to suggest that FeNPs as nanovehicles. In the future these findings help to propose new treatment schemes, which improve the quality of life of the patient.

Materials and Methods

Synthesis of FeNPs coating with PEG

This process needs 3 steps: synthesis of FeNPs coating with PEG, then coating erythromycin with the FeNP-PEG through alkoxide formation, the quantification of erythromycin. For the functionalization of the magnetite nanoparticles with the polymer polyethylene glycol (PEG); two solutions were necessary to prepare: the first solution 1.99 g of $FeCl_2.4H_2O$ and 3.24 g of $FeCl_3$ were dissolved in 50 ml of water and was added 25 ml of a solution containing 0.67 g of PEG. The second solution was prepared as follow: 30 ml of NH_4OH were spilled in 50 ml of water and mixed with 25 ml of a solution containing 0.67 g of PEG. Then, the second solution was added dropwise to first solution until the pH was around 9. The formation of the FeNPs was identified by the colour change. NH_4OH remanent was eliminated by several washes with water and the precipitate obtained was dried at room temperature for 24 h. Thereafter dried powder was dissolved in 50 ml of a solution containing 0.12 g of PEG. The final solution was sonicated and dried at 120°C for two h [8].

Coating FeNPs-PEG with eri by the alkoxide formation

A solution of eri was prepared dissolving 0.0075 g in water, it was deprotonated with NaOH [14,15]. After that, this solution was mixed with 0.0037 g of FeNPs-PEG sample. Then the mix was magnetically stirred at room temperature for 150 min to facilitate eri binding. The coating FeNPs-PEG with eri, were termed FeNPs-PEG-eri.

Quantification of eri in FeNPs-PEG-eri

The concentration of eri in the FeNPs-PEG-eri was determined by acid hydrolysis method, using a standard curve from a series of standard solutions ranging from 20 to 1280 µg of eri [16].

Bacterial growth conditions

S. pneumoniae of strain R6, TIGR4 and ATCC 49619 were stored at -70ºC in 10% glycerol and were routinely cultivated on Todd Hewitt agar supplemented with 5% sheep blood. The incubation was performed under microaerophilic growth conditions (5% CO_2) for 24 h at 37ºC [17].

Determination of the minimum inhibitory concentration

The MIC (minimum inhibitory concentration) values of eri and the FeNPs-PEG-eri against *S. pneumoniae* ATCC 49619, R6 and TIGR4 were determined using the broth microdilution method. The microbial suspensions were prepared as described CLSI (Clinical and Laboratory Standards Institute). The MIC was determined over a range from 128 to 0.015 µg/mL by the serial dilution method, as described CLSI [18].

The viability determination

In order to evaluate the effect of the FeNPs-PEG-eri over *S. pneumoniae* viability, the plate colony counting method was employed according to CLSI [18].

FeNPs-PEG are capable of support the cellular growth of *S. pneumoniae*

Cells of *S. pneumoniae* previously cultivated on Todd Hewitt agar were collected and inoculated in Todd Hewitt broth supplemented with 0.5% of yeast extract. This culture media was previously chelated with 700 µm 2,2-dipyridyl to starve the bacterium. Then, optical density was adjusted to 0.1 (600 nm) and the cellular growth was monitored each h. After 3 h under iron starvation the medium of culture was supplemented with $FeCl_3$ or FeNPs-PEG (tested as an alternative iron source). All growth cultures were performed under the follow conditions at 37°C with an atmosphere regulated of 5% CO_2.

Results

FeNPs-PEG were synthetized by a method based on iron salt coprecipitation

In order to investigate whether FeNPs can be utilized as nanovehicle of antibiotics, we synthetized FeNPs chemically by precipitation.

The FeNPs coated with PEG were characterized by Uv-Vis spectra and 3 bands were revealed, at 360 nm which corresponds to iron nanoparticles, the second at 240 nm that indicates the presence of PEG and the third at 260 nm that are FeNPs-PEG. As a control, the spectrum bands were tested individually FeNPs and PEG (Figure 1). This synthesis allowed us to obtain FeNPs functionalized with PEG (FeNPs-PEG) of 20 nm.

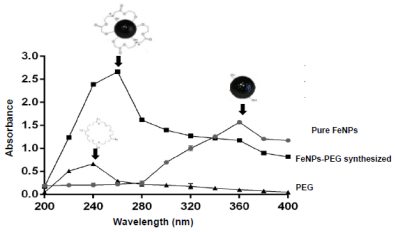
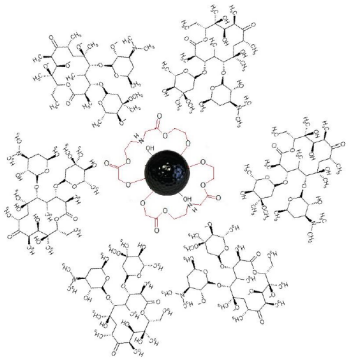


Figure 1: The absorption scanning of FeNPs-PEG, they were synthetized by iron salt coprecipitation method. Three absorption bands were detected by Uv-Vis at 240 nm which corresponds to PEG, the second at 360 nm that corresponds to FeNPs and the third at 260 nm that corresponds to FeNPs-PEG. The presence of both components was corroborated submitting PEG and FeNPs separately.

Coupling eri to FeNPs-PEG by alkoxide formation

In order to explore whether its antibacterial effect of eri can be improved by the usage of FeNPs-PEG, we decide to bind eri to FeNPs-PEG by alkoxide formation. eri was deprotoned with an alkaline solution in order to couple FeNPs-PEG, this solution had a pH 9 which allowed negative charge (O^-) to interact through hydrogen bonds with PEG (Scheme 1).



Scheme 1: Possible interaction between; erythromycin, polyethylene glycol and iron oxide nanoparticles. In the center the FeNPs are showed in black; PEG is indicated in red color and eri is showed in the periphery. The PEG and FeNPs interacted through a covalent bond while the eri and PEG interacted through a hydrogen bond.

Interestingly the interaction between PEG and FeNPs was through a covalent bond. Coupling eri was quantified by acid hydrolysis method. We got a yield around 86% at 150 min (Figure 2).

FeNPs optimize the antibacterial properties of eri

MIC was determined according to CLSI 2013 in order to demonstrate that FeNPs optimize the eri antibacterial effect. MIC for eri was 0.25 µg/mL however, when the eri was coupled (FeNPs-PEG-

eri) the potency diminished to 0.12 µg/mL. A control with FeNPs-PEG (without eri) did not show any effect. This result clearly showed that FeNPs-PEG optimize the antibacterial effect perhaps (Table 1) NPs permit eri to bind to the bacterium.

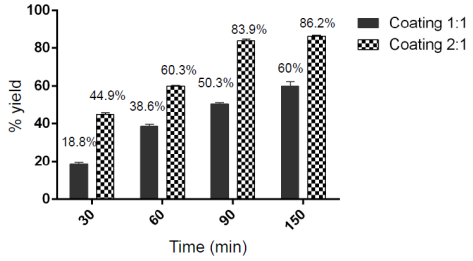


Figure 2: Percentage of eri coupled to FeNPs-PEG. (filled bars) Coating 1:1, (640 µg/mL of eri interacted with 640 µg/mL of FeNPs-PEG), (bars with squares: black and white) coating 2:1, (640 µg/mL of eri interacted with 320 µg/mL of FeNPs-PEG). The interaction was measured each 30 min during 150 min.

Treatment	*Streptococcus pneumoniae*		
	ATCC49619	TIGR4	R6
	MIC (µg/mL)	MIC (µg/mL)	MIC (µg/mL)
Erythromycin	0.25	0.25	0.12
FeNPs-PEG	No found	No found	No found
FeNPs-PEG-Eri	0.12	0.12	0.12

Table 1: Antimicrobial activity of eri, FeNPs-PEG and FeNPs-PEG-eri on three strains of *S. pneumoniae*.

The viability of *S. pneumoniae* is abated when eri is coupled to FeNPs

Since MIC, was lower when the eri was coupled to FeNPs, than itself, we decided to determine whether the viability also was diminished in presence of FeNPs-PEG-eri. Interestingly, when the viability was tested at 0.12 µg/mL no CFU (colony forming unit) was observed in strains analysed ATCC 49619, TIGR4 (both have capsule) or a mutant R6 (without capsule), however, when the eri was used by itself at the same potency (0.12 µg/mL), the viability was preserved because 5.3 X 10^{10} CFU for ATCC-49619 and 7.3 X 10^{10} CFU for TIGR4 strain were quantified. This result clearly showed that eri favoured its antibacterial effect when is coupled to FeNPs (Figure 3).

The capsule of *S. pneumoniae* diminishes the antibacterial effect of eri

Since *S. pneumoniae* is a bacterium that has capsule, we think that this structure could delay the enter of eri to the bacterium, to investigate this hypothesis the viability was analysed in a R6 strain (mutant without capsule) in this strain eri itself or coupled to FeNPs-PEG abated the viability at 0.12 µg/mL (Figure 3b). This result clearly showed that this bacterium can delay the entry of eri due to the presence of the capsule and in capsulated strain (ATCC 49619, TIGR4) the entry of eri is favoured by the presence of FeNPs.

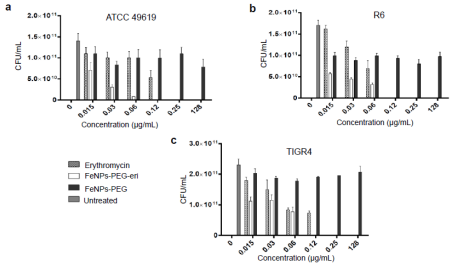


Figure 3: *Streptococcus pneumoniae* viability was abated by FeNPs-PEG-eri better than eri. The viability was determinated using concentrations according to CLSI 0.015, 0.03, 0.06, 0.12, 0.25 and 128 µg/mL. Bars with squares (black and white; erythromycin, empty bars; FeNPs-PEG-eri, filled bars; FeNPs-PEG and grey bars; untreated.

S. pneumoniae used FeNPs-PEG as iron source

Since FeNPs-PEG optimize the antibacterial effect, we think that the effect is favoured because the presence of Fe is attractive for *S. pneumoniae* and maybe this pathogen requires iron for covering its necessities and detects FeNPs-PEG as iron source. In order to investigate this hypothesis, we cultivate this pathogen under iron starvation and then broth media was supplemented with $FeCl_3$ or FeNPs-PEG as only iron source. The condition of iron starvation did not permit the bacterial growth, however when the media was supplemented with $FeCl_3$, the cellular growth was restored, Interestingly when the FeNPs-PEG were added as only iron source the cellular growth was supported (Figure 4).

Discussion

Antimicrobial resistance is a public health problem, because the resistance mechanisms developed by human pathogens are spread around the world [19]. Therefore, the discovery of new therapies is necessary and imperative [20]. In the present work, a strategy based in combining drug with iron oxide nanoparticles is suggested. Then, coupling of eri to FeNPs improved the antibacterial properties. The synthesis of FeNPs by a chemical methodology allowed us to obtain NPs coated with PEG [8], the protocol to couple eri to FeNPs allowed to have FeNPs-PEG coated with eri [14,15]. The production reached a yield of 86.2%, which was enough to test the antibacterial properties [21]. Antibacterial properties were improved when the eri was coupled to FeNPs [22], perhaps the iron present in the nanoparticles is attractive for the bacterium because when FeNPs were supplied as only iron source, *S. pneumoniae* supported its bacterial growth and therefore, the interaction between FeNPs-PEG-eri and bacteria was higher. This effect was demonstrated because only 0.12 µg/mL were necessary to inhibit the bacterial growth and viability, however, when the eri was tested itself, the potency must be increased up to 0.25 µg/mL [18], this result indicated that the presence of FeNPs improved the antibacterial effect. The capsule is a structure of *S. pneumoniae* that has an important role due to evade immune system [23] and is considered as a virulence factor, our results allowed to think that also

protects to the pathogen of the antibacterial effect because a mutant without capsule (R6) did not grow when the eri was tested itself at 0.12 µg/mL. Our overall results are the first efforts to propose that the antibacterial effect of eri could be improved using FeNPs. This knowledge can be used to the future in order to avoid the secondary effects in the patient caused by the drug, because the concentration of antibiotic could be less and additionally we could direct the antibiotic towards its specific target.

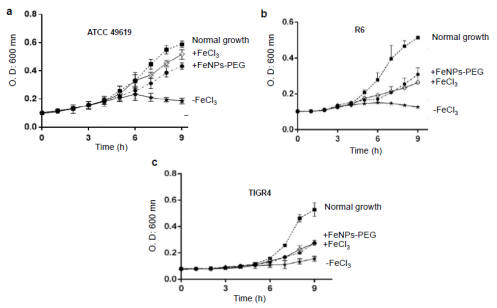


Figure 4: FeNPs-PEG support the cellular growth of *S. pneumoniae*. Bacteria were cultivated using different iron sources and the cellular growth was determined each h for 10 h by spectrophotometry at 600 nm. Cellular growth of ATCC 49619, R6 and TIGR4 with different sources of iron: squares; untreated, empty diamonds; +$FeCl_3$, filled circle; +FeNPs-PEG, filled stars; iron starvation.

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