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Nanoscale Kolliphor® HS 15 micelles to minimize rifampicin self-aggregation in aqueous media

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ABSTRACT

Tuberculosis is a highly-deadly disease that affects both children and adults. Rifampicin, one of the "first-line" anti tuberculosis drugs, self-aggregates in aqueous solutions where the critical aggregation concentration demonstrated a temperature-dependent behavior. Interestingly, drug self-aggregation could negatively affect the development of liquid aqueous rifampicin pediatric tuberculosis formulations. Therefore, our nanotechnological strategy to minimize this effect was the rifampicin encapsulation within polymeric micelles, employing the commercially available Kolliphor® HS 15, as the micelle-former biomaterial. The results show that Kolliphor® HS 15 is able to prevent rifampicin aqueous self-aggregation and precipitation, when used at certain concentrations. In this context, our work opens the possibility of developing aqueous liquid rifampicin dosage forms for pediatric patients to improve tuberculosis treatment.

1. Introduction

Tuberculosis (TB) is a highly-deadly disease, mainly caused by *Mycobacterium tuberculosis* that affects both adults and children. Indeed, it has been reported that 140,000 children died of this infectious pathology only in 2014 [1]. TB treatment involves the daily oral administration of a combination of "first line" anti-TB drugs usually commercialized as tablets or capsules. This represents a major drawback for pediatric adherence to TB treatment. Generally, children under 7 years old have difficulties at taking pills, which is why liquid formulations are required [2]. Rifampicin (RIF) (Scheme 1) represents one of the most effective "first line" anti-TB drugs employed along the short-term (6 months) standardized TB treatment [3]. It is a limit class II drug, according to the Biopharmaceutical Classification System (BCS) [4]. RIF exhibits amphiphilic properties due to its chemical structure (Scheme 1), with very low pH-dependent aqueous solubility, and poor stability in aqueous media [5,6]. Moreover, RIF is degraded in the stomach, due to the highly acidic environment [7]. Furthermore, RIF may undergo a significant self-aggregation in aqueous media over time, which hampers the development of stable RIF oral liquid formulations, especially for pediatric TB therapy. In this framework, the employment of polymeric micelles (PMs) could be an attractive nanotechnological strategy to overcome RIF instabilization/precipitation in aqueous media.

One commercially available amphiphilic copolymer employed as a parenteral excipient in aqueous formulations is known as Kolliphor® HS 15 (Kolliphor) [8,9]. It is an amphiphilic non-ionic emulsifying and solubilizer agent with low molecular weight (~963.25 g/mol) [10]. Kolliphor consists of polyglycol mono and di-esters of 12-hydroxystearic acid along with free poly(ethylene glycol) (PEG) (~30%) (Scheme 1) [11]. It has been approved by the United States Food and Drug Administration (U.S. FDA) and it has raised special interest as a micelle-former biomaterial in the last years. Interestingly, it is currently used for enhancing aqueous solubility of poorly aqueous soluble drugs [12]. Furthermore, nowadays Kolliphor is encoded in the United States, the European and the Germany pharmacopoeias [13].

In this context, one of the mains goals of the present

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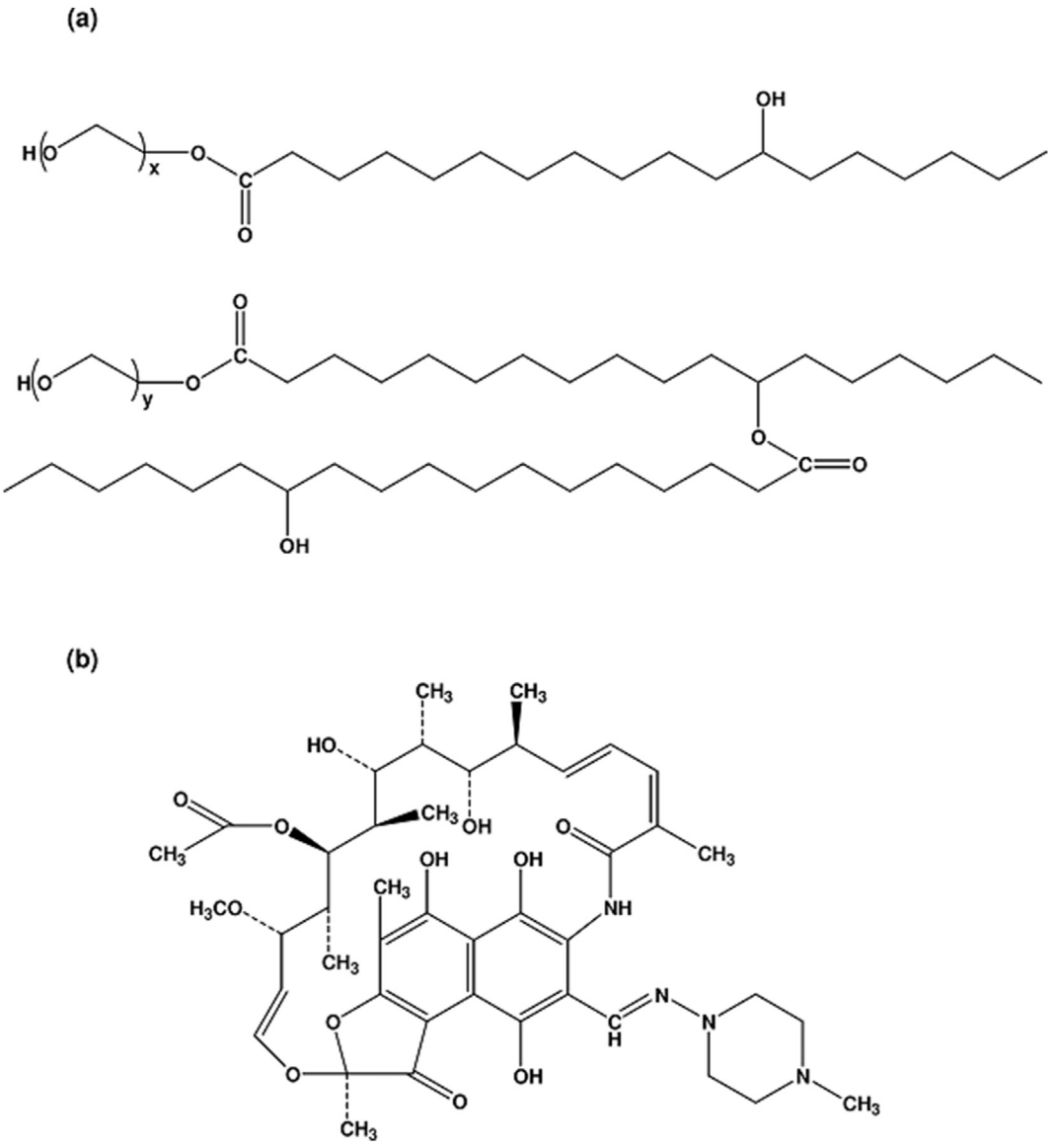


Scheme 1. Chemical structure of (a) Kolliphor and (b) RIF.

investigation was to improve RIF stability in aqueous formulations, minimizing its self-aggregation, by using PMs based on Kolliphor, for the potential development of novel pediatric liquid formulations for TB therapy.

2. Materials and methods

2.1. Materials

The materials employed consisted in polyoxyl 15 hydroxystearate (Kolliphor® HS 15, molar mass ~ 963.25 g/mol) kindly supplied by BASF (Argentina). RIF was purchased from Parafarm® (Argentina). Solvents, as acetone and *N-N*-dimethylformamide (DMF), were of analytical grade, and were used as indicated by manufacturers.

2.2. Preparation of RIF-loaded polymeric micelles

RIF-loaded PMs were prepared by a solvent-diffusion technique, employing acetone as organic solvent. Briefly, an organic solution of RIF (20–100 mg) was obtained in acetone (20 mL), with sonication to enhance its solubilization (Digital Ultrasonic Cleaner, PS-10A 50/60 Hz, China, 15 min, 25 °C). Subsequently, this solution was added dropwise to an aqueous Kolliphor dispersion (1–10% w/v) under magnetic stirring, using a programmable syringe infusion pump (1 mL/min, PC11UB, APEMA, Argentina) at room temperature. Magnetic stirring was maintained overnight to ensure acetone evaporation. Then, the volume of the resulting aqueous micellar dispersion was adjusted to 10 mL with distilled water in a volumetric flask. Immediately, samples were filtered by a clarifying filter (0.45 μm, cellulose nitrate, Microclar, Argentina) and stored in amber glass vials. Copolymer-free RIF (2 mg/mL) solutions and drug-free Kolliphor dispersions were used as controls.

On the other hand, RIF-loaded PMs were also prepared with different RIF concentrations (2.5–10.0 mg/mL), maintaining Kolliphor concentration (5% w/v), employing the same technique described above.

The concentration of RIF in every system assayed was determined by UV/Vis spectrophotometry (482 nm, 25 °C, UV-260, UV–Visible Recorder Spectrophotometer, Shimadzu, Japan)

employing a calibration curve of RIF solutions in DMF (3.125–50 μg/mL, R^2: 0.9998–0.9999). Aliquots (100–200 μL) of micellar dispersions and RIF solutions (without copolymer) were diluted with DMF (10 mL), and measured by UV–Vis. A drug-free copolymer dispersion in DMF was used as blank.

2.3. Dynamic light scattering

The average hydrodynamic diameter (D_h), size distribution (polydispersity index, PDI) and zeta potential of drug-free and drug-loaded PMs were determined by dynamic light scattering (DLS, scattering angle of $\theta = 173°$ to the incident beam, Zetasizer Nano-ZSP, Malvern Instruments, United Kingdom) at 25 °C. Samples were prepared and filtered by clarifying filters (0.45 μm, cellulose nitrate, Microclar, Argentina). Then, aliquots (1 mL) were equilibrated for three minutes at 25 °C, prior to the measurement. Formerly, the instrument was calibrated with standard latex nanoparticles, provided by Malvern Instruments, United Kingdom. The average of at least three measurements was used to express the D_h and the PDI values ± standard deviation (S.D.). In order to evaluate the physical stability of the RIF (2 mg/mL) control solutions and the RIF-loaded (2 mg/mL) systems (copolymer concentration 1–10% w/v) in aqueous media, samples (5 mL) were placed into sealed vials (10 mL) and stored at 25 °C for 14 days [14]. At different time points (0, 1, 2, 7, 9 and 14 days), the D_h and PDI values were evaluated by DLS as previously described. Further, the RIF concentration was monitored by UV–Vis spectrophotometry.

Additionally, DLS was used to establish the critical aggregation concentration (CAC) of RIF at different temperatures (20, 25 and 37 °C). Eleven RIF aqueous solutions, covering from 0.8×10^{-7} to 0.8 mg/mL, were developed and studied. Derivate counts rate were used to calculate CAC. Specifically, the CAC value corresponded to the concentration of the system at which a sharp increase in scattering intensity occurred. A similar method, at the same three temperatures, was used to determine the CMC of Kolliphor. Ten copolymer dispersions, covering a concentration range between 1×10^{-7} and 1% w/v, were prepared and analyzed. Every sample was equilibrated for five minutes before each measurement.

2.4. Determination of RIF melting point

Finally, RIF melting point was determined by means of a Thermo Scientific Fisher-Johns Melting Point Apparatus (220 V. USA). A sample (5 mg) of RIF precipitate isolated from stability assays was heated from room temperature (25 °C) to 200 °C employing a heating rate of 5 °C/min.

3. Results

Firstly, the RIF and Kolliphor aggregation behaviors in water were investigated at different temperatures by means of DLS. In this case, the drug CAC values observed were: 0.1540, 0.0140 and 0.0072% w/v at 20, 25 and 37 °C, respectively. Further the Kolliphor CMC values were 0.088, 0.022 and 0.012% w/v at 20, 25 and 37 °C, respectively.

In a second step, we developed RIF-loaded PMs to overcome the drug precipitation (self-aggregation). First we determined the micellar size, size distribution and zeta potential of blank Kolliphor micelles (1–10% w/v), where results demonstrated that there was a unimodal size distribution (regardless the copolymer concentration) with a peak range between 12.6 and 13.4 nm (PDI: 0.028–0.075) and neutral zeta potential values (−0.36 ± 0.04 mV).

On the other hand, for RIF (2 mg/mL) blank solutions (without Kolliphor presence) it was observed the presence of only one size population peak (232.6 ± 7.0 nm) with a size distribution of 0.589 ± 0.056 (Table 1) and zeta potential values of −19.80 ± 1.89 mV at 25 °C. Besides, similar Dh results were observed after the dilution (1/2) of the drug control solutions with distilled water (208.7 ± 4.0 nm; PDI: 0.423 ± 0.049).

Furthermore, RIF blank solutions (2 mg/mL) demonstrated a poor physical stability in aqueous media since it was observed an increment of the drug aggregate size from 232.6 nm (day 0) to 557.0 nm (day 14) at 25 °C as it is shown in Table 1. Moreover, RIF concentration decreased at 72% of the initial drug concentration at day 14, where a red precipitate was observed. Further, the melting

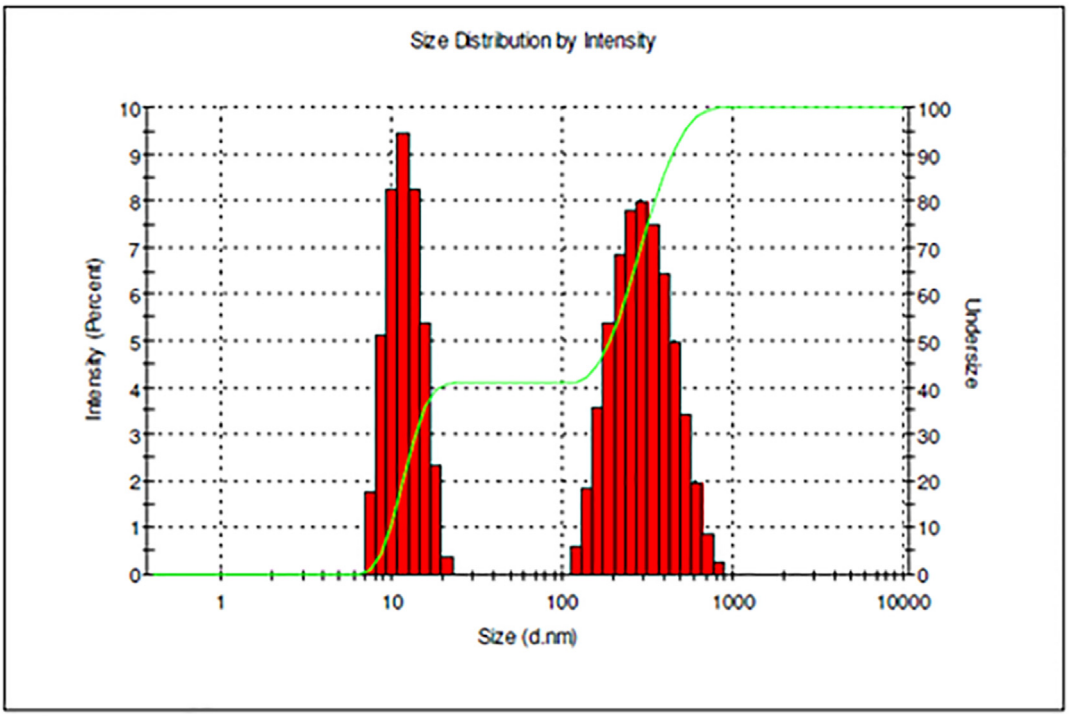
Table 1
Micellar size and size distribution of RIF-loaded (2 mg/mL) Kolliphor (1–10% w/v) PMs over 14 days at 25 °C.

Time (d)	Kolliphor (% w/v)	D_h (nm) (±S.D.)				
		Peak 1	%	Peak 2	%	PDI (±S.D.)
0	–	232.6 (7.0)	100.0	–	–	0.589 (0.056)
	1	11.0 (0.1)	39.0	234.5 (13.2)	61.0	0.949 (0.009)
	3	13.1 (0.1)	100.0	–	–	0.203 (0.001)
	5	12.8 (0.1)	100.0	–	–	0.085 (0.006)
	7	13.7 (0.1)	100.0	–	–	0.119 (0.011)
	10	14.2 (0.1)	100.0	–	–	0.205 (0.002)
1	–	216.5 (4.4)	100.0	–	–	0.635 (0.065)
	1	10.4 (0.1)	28.0	348.5 (17.4)	72.0	0.538 (0.022)
	3	12.4 (0.3)	100.0	–	–	0.225 (0.007)
	5	12.5 (0.1)	100.0	–	–	0.087 (0.001)
	7	12.9 (0.1)	100.0	–	–	0.090 (0.002)
	10	14.4 (0.2)	100.0	–	–	0.208 (0.005)
2	–	230.9 (46.9)	100.0	–	–	0.726 (0.093)
	1	10.1 (0.2)	29.0	393.3 (20.4)	71.0	0.601 (0.051)
	3	11.7 (0.2)	100.0	–	–	0.234 (0.004)
	5	11.9 (0.1)	100.0	–	–	0.080 (0.007)
	7	12.9 (0.1)	100.0	–	–	0.098 (0.004)
	10	14.0 (0.2)	100.0	–	–	0.206 (0.006)
7	–	534.8 (56.5)	100.0	–	–	0.271 (0.040)
	1	11.1 (0.2)	65.0	344.3 (73.7)	35.0	0.510 (0.127)
	3	11.4 (0.2)	100.0	–	–	0.252 (0.005)
	5	12.5 (0.2)	100.0	–	–	0.135 (0.015)
	7	13.0 (0.1)	100.0	–	–	0.128 (0.009)
	10	13.1 (0.2)	100.0	–	–	0.228 (0.004)
9	–	570.8 (78.7)	100.0	–	–	0.283 (0.034)
	1	11.5 (0.1)	68.0	370.2 (59.8)	32.0	0.427 (0.100)
	3	11.9 (0.1)	100.0	–	–	0.238 (0.002)
	5	12.5 (0.1)	100.0	–	–	0.144 (0.005)
	7	12.8 (0.1)	100.0	–	–	0.140 (0.009)
	10	12.5 (0.2)	100.0	–	–	0.238 (0.003)
14	–	557.0 (41.0)	100.0	–	–	0.319 (0.020)
	1	11.4 (0.2)	77.0	338.6 (36.1)	23.0	0.408 (0.122)
	3	12.4 (0.2)	100.0	–	–	0.224 (0.005)
	5	12.1 (0.2)	100.0	–	–	0.133 (0.008)
	7	12.4 (0.1)	100.0	–	–	0.142 (0.003)
	10	11.5 (0.1)	100.0	–	–	0.267 (0.100)

Table 2
Micellar size and size distribution of RIF-loaded (10 mg/mL) Kolliphor PMs (1–10% w/v) at 25 °C.

Kolliphor (% w/v)	D_h (nm) (±S.D.)				
	Peak 1	%	Peak 2	%	PDI (±S.D.)
–	232.6 (7.0)	100.0	–	–	0.589 (0.056)
1	11.1 (0.3)	6.1	262.7 (8.3)	93.9	0.484 (0.007)
3	11.8 (0.1)	40.4	210.1 (5.4)	59.6	0.859 (0.011)
5	12.2 (0.2)	40.9	320.0 (13.8)	59.1	0.945 (0.003)
7	13.0 (0.2)	95.7	3993.0 (64.1)	4.3	0.206 (0.003)
10	12.4 (0.1)	100.0	–	–	0.082 (0.004)

(a)



(b)

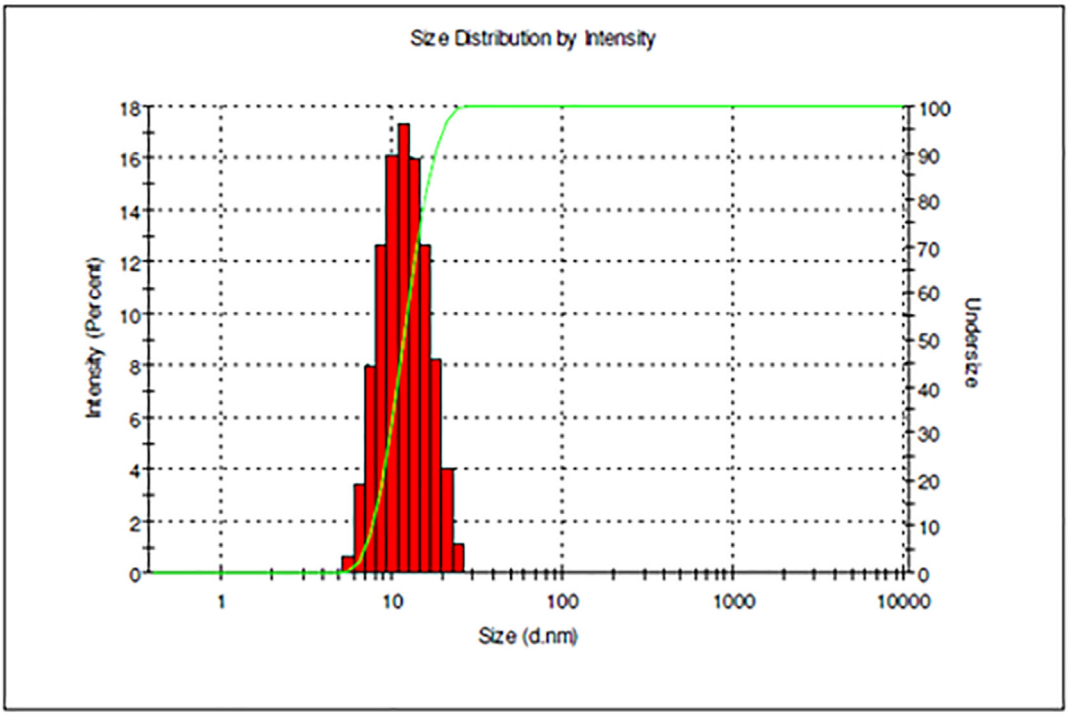


Fig. 1. Size and size distribution of RIF-loaded (10 mg/mL) − Kolliphor polymeric micelles, at 25 °C. Bimodal size distribution in 5% w/v Kolliphor micelles (a) and unimodal size distribution in 10% w/v Kolliphor micelles (b).

point of the precipitate was found to be 184 °C (followed by decomposition).

Interestingly, after the incorporation of growing Kolliphor concentrations (1–10% w/v), in RIF-loaded (2 mg/mL) PMs, a bimodal size distribution was observed only for 1% w/v. In this case there was one size population of 11.0 ± 0.1 nm (39% intensity) and a

second size population of 234.5 ± 13.2 nm (61% intensity) (Table 1). Furthermore, the bimodal size distribution was also observed over 14 days in aqueous media, as it is shown in Table 1. Conversely, as the copolymer concentration was increased from 1 to 10% w/v, a unimodal size distribution was observed (Table 1). Indeed, a monomodal size distribution was also observed over 14 days at 25 °C for those micellar dispersions with Kolliphor concentration >1% w/v.

Besides, the zeta potential values observed for those RIF-loaded Kolliphor dispersions which demonstrated an unimodal (3–10% w/v of copolymer) and a bimodal (1% w/v of copolymer) size distribution were found to be −1.80 ± 0.16 mV and −7.84 ± 1.57 mV, respectively.

For RIF-loaded (10 mg/mL) PMs (1–10 %w/v), two size populations were observed for copolymer concentrations between 1% and 5% w/v (Table 2 and Fig. 1). However, the intensity of the size peaks gradually decreased as the copolymer concentration was increased up to 5% w/v (Table 2). Moreover, only one size population (12.4 ± 0.1 nm) with a narrow size distribution (PDI: 0.082 ± 0.004) was observed for Kolliphor 10% w/v dispersions, corresponding to the RIF-loaded PMs (Table 2 and Fig. 1).

Finally, an additional assay was performed employing different RIF concentrations (2.5–10 mg/mL) for Kolliphor dispersions at 5% w/v. Results demonstrated the presence of two size populations for every RIF concentration assayed. We observed that the RIF aggregate would increase in its intensity (%), together with greater size and PDI values as the drug concentration was increased (Table 3). For instance, the aggregate size increased from 152.7 nm to 320.0 nm for RIF concentrations of 2.5 mg/mL to 10 mg/mL, respectively (Table 3).

4. Discussion

In recent years, the development of liquid pediatric dosage forms has become a pharmaceutical challenge since many poorly water-soluble/unstable drugs can not be formulated as liquid formulations (e.g. solutions and syrups).

Interestingly, several hydrophobic organic compounds exhibit amphiphilic properties, being the main reason why they can undergo self-agreggation in aqueous solutions at certain concentration, in a similar surfactant-manner [15]. Self-aggregation of these compounds possibly intends to lower to the minimum the direct contact between the aqueous medium and the hydrophobic residues of the molecule [16–18]. Particularly, RIF is a bulk molecule, with high molecular weight (822.95 g/mol) and amphiphilic properties (Scheme 1) [5,6]. Hence, RIF could also undergo self-aggregation in aqueous solutions where this behavior could negatively affect the development of aqueous RIF solutions, especially for pediatric use for a middle/long term TB treatment. Furthermore, elderly patients may also benefit from oral liquid dosage forms for anti-TB therapy.

In order to investigate this latter fact, we studied the RIF behavior in distilled water at different temperatures. It is noteworthy that light scattering is one of the best methods to study the CAC, since a sharp increment in the intensity of the scattered light occurs after the formation of the aggregate [19]. The CAC values observed for RIF are in good accordance with previous investigations using conductivity [20]. These results demonstrate that RIF can self-aggregate in aqueous solution with a temperature-dependent behavior, as it has been previously reported for amitriptyline hydrochloride [21]. Indeed, the aggregation tendency of RIF is greater at higher temperatures (lower CAC values). A similar behavior is observed for non-ionic monomers of amphiphiles, where their aggregation is related to the hydration of both the hydrophilic and the hydrophobic residues. At higher temperatures, aggregates formation could be enhanced due to the reduction of hydrogen bonds (hydrophilic portions) and the presence of solvation layers around the hydrophobic residues of the amphiphlic molecule [21].

With the aim to minimize RIF self-aggregation in water, one of the main objectives of our investigation was the drug encapsulation within PMs, employing Kolliphor as a micelle-former biomaterial. It is well known that the ability of PMs to encapsulate hydrophobic drugs is remarkably enhanced upon the CMC of the amphiphilic micelle-former biomaterial [22]. Remarkably, Kolliphor is able to easily self-assembly in an aqueous media. According to the results, there was a decrement in the CMC value as the temperature was increased. Similar results were obtained for other amphiphilic poly (ethylene oxide) and poly (propylene oxide) block copolymers [23]. Particularly, for non-ionic surfactants, CMC tends to decrease with the enhancement of temperature, as the hydrophilicity of the molecules diminishes [24].

Then, two different assays were performed for the RIF-loaded micellar dispersions. In the first place, micellar size and size distribution were studied at 25 °C for every micellar system (1–10% w/v) maintaining constant the RIF concentration (2 and 10 mg/mL) (Tables 1 and 2). Secondly, we studied different RIF concentrations (2.5–10 mg/mL) at a fixed Kolliphor concentration (5% w/v) (Table 3).

In this case, RIF blank solutions were investigated by DLS as an attempt to gain further insight on the drug self-aggregation. The size population observed (~232 nm) corresponds to RIF self-aggregate in the aqueous solution, where there is only a slight size difference (before and after dilution with distilled water) since RIF concentration is over the CAC (0.014 %w/v) in both cases at 25 °C. Furthermore, RIF aqueous blank solutions demonstrated negative zeta potential values.

These results suggest that RIF aqueous solutions are not formed only by individual drug molecules but also by drug aggregates since RIF is an amphiphilic drug [16]. Moreover, RIF blank solutions demonstrated a poor physical stability in water over time since there was an increment of the D_h value for the RIF aggregates. Indeed, the precipitate melting point was in good concordance with the RIF melting point [6]. These studies clearly show that RIF aggregate enlarges in aqueous media and precipitates over time, with a potential negative impact in the physical stability of RIF aqueous solutions for clinical use.

After the copolymer incorporation a bimodal size distribution (~11 nm and 235 nm) was observed for those micellar systems with Kolliphor 1% w/v. Probably, the smallest size population corresponds to the drug-loaded Kolliphor micelles since blank Kolliphor PMs (1% w/v) demonstrated a unimodal size distribution of 13.3 nm. On the other hand, the second size peak population probably corresponds to the drug aggregate, according with the D_h values observed for the RIF control solutions. These bimodal size distribution results demonstrated good correlation with the zeta potential values obtained since there was a decrement from −19.80 to −7.84 mV suggesting the presence of both, RIF-loaded polymeric micelles and drug aggregates. An increment of the copolymer concentration from 1 to 10% w/v led to the disappearance of the size population related to the RIF aggregate. This could possibly be associated with the drug encapsulation within the PMs, being this behavior also observed over 14 days (Kolliphor > 1% w/v). In this case, the reduction of the zeta potential values was more pronounced than for their counterparts with 1% w/v of copolymer, suggesting the incorporation of the hydrophobic drug inside the micellar core without the presence of drug aggregates (unimodal size distribution). Further, the polymeric micelles corona is

Table 3
Micellar size and size distribution of RIF-loaded Kolliphor PMs (5% w/v) at 25 °C.

RIF (mg/mL)	D_h (nm) (±S.D.)				
	Peak 1	%	Peak 2	%	PDI (±S.D.)
–	12.6 (0.1)	100.0	–	–	0.031 (0.011)
2.5	9.6 (0.1)	52.0	152.7 (2.7)	48.0	0.599 (0.004)
5.0	11.8 (0.1)	57.7	198.7 (1.6)	42.3	0.568 (0.001)
7.0	13.9 (0.1)	40.6	229.2 (4.5)	59.4	0.863 (0.003)
10.0	12.2 (0.2)	40.9	320.0 (13.8)	59.1	0.945 (0.003)

compounded by PEG (a non-ionic hydrophilic polymer), then neutral potential values were expected as it was observed for blank polymeric micelles (without RIF) (−0.36 ± 0.04 mV).

Therefore, the encapsulation of RIF within Kolliphor PMs could efficiently avoid drug self-aggregation and precipitation over time in aqueous media. These are promising results for the potential development of novel RIF pediatric liquid dosage form with an enhanced physical stability.

To gain further insight on the effect of PMs to avoid RIF self-aggregation, a higher RIF concentration (10 mg/mL) was assessed for Kolliphor PMs (1–10% w/v). In this case, the presence of the RIF aggregates was observed for those Kolliphor dispersions between 1 and 5% w/v with a bimodal size distribution. These results could be related with the increment of the RIF cargo. However, as the copolymer concentration was increased up to 10% w/v, the reduction of the size peak intensity of the drug aggregates (until its disappearance) suggests that higher Kolliphor concentrations were capable of preventing the aqueous self-aggregation of RIF.

Finally, an additional assay was performed in order to confirm that the encapsulation of RIF within PMs is indeed capable of minimizing its precipitation in water. For this purpose, we investigated Kolliphor dispersions (5% w/v) with growing RIF concentrations (2.5–10 mg/mL). It is important to remark that the PDI is the parameter of the broadness of the particle size distribution and it is related to the physical stability of the colloidal systems. We hereby chose the smallest concentration of Kolliphor (5% w/v) that presented the lowest PDI value (0.085 ± 0.006) (Table 1). In this case it was clearly demonstrated (according with the bimodal size distribution and the increment of the RIF aggregate size observed) that an increase in the RIF cargo led to RIF self-aggregation in a greater extend, since the copolymer concentration was maintained at a fixed concentration (Table 3).

Overall, we successfully avoided RIF self-aggregation in aqueous media by the drug encapsulation within PMs, employing Kolliphor as the micelle-former biomaterial. We obtained RIF-loaded (2 mg/mL) Kolliphor (5% w/v) micelles with an enhanced *in vitro* physical stability, capable of protecting RIF in the aqueous media and preventing its precipitation within time. Results suggest that Kolliphor micelles at certain concentrations are able to improve RIF aqueous stability. However, if there is an increase in RIF concentration, higher copolymer concentrations (10% w/v) will be required to improve drug physical stability (RIF concentration 10 mg/mL) in aqueous media. This novel RIF-loaded micellar system represents an attractive nanotechnological platform to improve the development of RIF liquid pediatric formulations for TB therapy with an enhanced physical stability. Furthermore, the possibility to employ a commercially available micelle-former biomaterial remains of clinical relevance.

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