

## Chitosan–dextran sulphate nanocapsule drug delivery system as an effective therapeutic against intraphagosomal pathogen *Salmonella*

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**Objectives:** The ability to target conventional drugs efficiently inside cells to kill intraphagosomal bacteria has been a major hurdle in treatment of infective diseases. We aimed to develop an efficient drug delivery system for combating infection caused by *Salmonella*, a well-known intracellular and intraphagosomal pathogen. Chitosan–dextran sulphate (CD) nanocapsules were assessed for their efficiency in delivering drugs against *Salmonella*.

**Methods:** The CD nanocapsules were prepared using the layer-by-layer method and loaded with ciprofloxacin or ceftriaxone. Antibiotic-loaded nanocapsules were analysed *in vitro* for their ability to enter epithelial and macrophage cells to kill *Salmonella*. *In vivo* pharmacokinetics and organ distribution studies were performed to check the efficiency of the delivery system. The *in vivo* antibacterial activity of free antibiotic and antibiotic loaded into nanocapsules was tested in a murine salmonellosis model.

**Results:** *In vitro* and *in vivo* experiments showed that this delivery system can be used effectively to clear *Salmonella* infection. CD nanocapsules were successfully employed for efficient targeting and killing of the intracellular pathogen at a dosage significantly lower than that of the free antibiotic. The increased retention time of ciprofloxacin in the blood and organs when it was delivered by CD nanocapsules compared with the conventional routes of administration may be the reason underlying the requirement for a reduced dosage and frequency of antibiotic administration.

**Conclusions:** CD nanocapsules can be used as an efficient drug delivery system to treat intraphagosomal pathogens, especially *Salmonella* infection. This delivery system might be used effectively for other vacuolar pathogens including *Mycobacteria*, *Brucella* and *Legionella*.

**Keywords:** intravacuolar pathogens, typhoid, layer-by-layer (LbL), polyelectrolyte capsules, ciprofloxacin/ceftriaxone/gentamicin

### Introduction

Intracellular pathogens, ranging from bacteria to parasites,<sup>1–5</sup> are notorious as causative agents of a number of diseases worldwide, including tuberculosis, typhoid, hepatitis and AIDS. The intracellular nature of the pathogens provides them with effective protection against antibiotics present in the extracellular milieu, thereby creating hurdles in the treatment of these diseases. The failure of conventional antibiotic therapies may be due to a lack of efficiency of penetration of the antibiotics (aminoglycosides and  $\beta$ -lactams) or to the poor retention of antibiotics inside the cells (fluoroquinolones or macrolides).<sup>1</sup> With an increase being seen in multidrug resistance, the treatment of these diseases has become more

challenging.<sup>2–7</sup> This calls for the design of smart drug delivery systems to improve the targeting of antibiotics to the intracellular niche. Apart from being able to cross the cell membrane barrier, these systems should retain and release the drug intracellularly over specific durations at a therapeutic level.

*Salmonella enterica* is a Gram-negative intracellular pathogen that can infect a wide range of hosts including reptiles, birds and mammals. *Salmonella enterica* serovar Typhi (*Salmonella* Typhi) and Paratyphi (*Salmonella* Paratyphi) can cause typhoid and paratyphoid fever, respectively, in humans, with contaminated food and water being the major sources of infection. These bacteria establish an intracellular niche in macrophages after translocating across the epithelial barrier of the gut. In infected macrophages,

*Salmonella* survives within *Salmonella*-containing vacuoles and disseminates to different organs such as the liver and spleen.<sup>8</sup> *Salmonella* Typhi infection was initially treated with the antibiotic chloramphenicol and then, with the emergence of chloramphenicol-resistant bacteria, with fluoroquinolones.<sup>9</sup> Recently, several multidrug-resistant salmonellae have been reported worldwide.<sup>10,11</sup> In order to surmount these problems, we made use of a novel polyelectrolyte capsule-based drug delivery system to improve antibiotic efficacy in *Salmonella* infection.

Polyelectrolyte capsules have been used as a unique method of drug encapsulation<sup>12–14</sup> to ensure a sustained release of the drug coupled with increased efficiency and decreased side effects.<sup>15,16</sup> The capsules are fabricated by employing the layer-by-layer (LbL) adsorption of oppositely charged polyelectrolytes onto the surface of a template such as calcium carbonate,<sup>17</sup> silica<sup>18</sup> or melamine formaldehyde,<sup>19</sup> or another biological template,<sup>12</sup> followed by dissolution of the core.<sup>20</sup> Electrostatic interaction between the adjacent layers forms the driving force for LbL adsorption. Permeability of the capsules can be manipulated by factors such as pH, ionic strength, the concentration of the polymer solution and the number of layers.<sup>21,22</sup>

The present study focuses on nanocapsules based on chitosan (CH) and dextran sulphate (DS) as the oppositely charged polyelectrolytes, and biocompatible silica particles as the template. Chitosan [ $\beta$ -(1–4)-2-amino-2-deoxy-D-glucose] is a natural polycationic polysaccharide derived from the exoskeleton of insects, the shells of crustaceans and fungal cell walls. It is the deacetylated form of chitin and is made up of *N*-acetyl-D-glucosamine and D-glucosamine. CH is found to possess several properties such as antimicrobial activity, biodegradability and biocompatibility, making it suitable for use in biomedical and pharmaceutical formulations.<sup>23–25</sup> However, the applicability of CH is limited as it cannot function in an acidic pH.<sup>26</sup> In order to combat this issue, the LbL technique has been pursued by making use of a biocompatible polyanionic polymer, DS. This is a branched-chain polysaccharide with 1–6 and 1–4 glycosidic linkage. It has also been reported to play a significant role in enzyme inhibition and as a drug conjugate.<sup>27,28</sup>

The hollow capsules thus produced were loaded with ciprofloxacin and ceftriaxone, which is the current drug of choice for *Salmonella* infection.<sup>29</sup> Apart from the prolonged antibiotic treatment required for clearing *Salmonella* infection,<sup>30</sup> the emergence of ciprofloxacin-resistant *Salmonella* has become a major threat.<sup>31–33</sup> Different adverse effects of ciprofloxacin have also been observed because of the increased dosage needed.<sup>34,35</sup> A reduced dosage, without compromising efficacy, could have great potential in antibiotic therapy. Very few studies have shown the application of nanoparticle therapy against *Salmonella* infection. Gentamicin-loaded nanoparticles have demonstrated an increased uptake of these particles in macrophages.<sup>36</sup> In this study, we have shown the effective ciprofloxacin treatment of *Salmonella* infection using CH–DS nanocapsules. Using this delivery system, a reduced dosage and frequency of dosing were achieved. Therefore, the drug delivery system may be used to improve patient adherence and reduce the cost and duration of treatment, which are the key points to be addressed to prevent drug resistance.

## Materials and methods

CH ( $M_w=150$  kDa), DS ( $M_w=500$  kDa), ciprofloxacin ( $M_w=331$ ), gentamicin ( $M_w=694$ ), PBS and FITC were purchased from Sigma Aldrich.

Ceftriaxone sodium salt ( $M_w$  554.58) was purchased from SRL (Bangalore, India). Hydrofluoric acid (HF) was obtained from Thomas Baker Ltd (Bangalore, India). Acetic acid ( $\text{CH}_3\text{COOH}$ ), sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl) and citrate buffer were obtained from Rankem, RFLC Limited (Bangalore, India). Double-autoclaved Milli-Q water (Millipore, Billerica, MA, USA) was used for all the experiments.

## Bacterial strains

*Salmonella* Typhimurium 14028 and *Salmonella* Typhi Ty2 were used in this study.

## Preparation of hollow capsules

The capsules were prepared using the LbL technique. This involves the sequential adsorption of oppositely charged polyelectrolytes onto silica ( $\text{SiO}_2$ ) nanoparticles, which function as a sacrificial template. Negatively charged  $\text{SiO}_2$  was incubated in the positively charged CH solution (1 mg/mL in 1 M NaCl at pH 5.6) for about 30 min at room temperature. The sample was washed three times by centrifugation at 4000 rpm for 5 min (MIKRO 200R; Hettich Zentrifugen, Tuttlingen, Germany) to wash out the unadsorbed CH. Subsequently, a layer of DS (1 mg/mL in 1 M NaCl) was deposited under similar conditions. The process was continued until the desired number of layers had been laid down, which in this case was four bilayers of the polyelectrolytes [(CD)<sub>n</sub>] in this experiment. The coated particles were then treated with 1 M HF for 1.5 h to remove the silica core without affecting the polymeric layers, thus forming the hollow capsules. The samples were subsequently washed six times with water (pH 5.6) by centrifugation at 2000 rpm for 8 min to ensure removal of the HF.

## Drug-loading studies

The loading studies were carried out by incubating 200  $\mu\text{L}$  (2 mg/mL) of hollow capsules in 400  $\mu\text{L}$  (1 mg/mL) of ciprofloxacin or ceftriaxone sodium salt or gentamicin sulphate or FITC for 12 h by changing the pH of the sample to 8.0 at room temperature by the addition of 0.1 M NaOH. Having attained a loading saturation, effective locking of the capsule layers and retention of the ciprofloxacin or ceftriaxone or gentamicin or FITC was ensured by changing the pH of the sample to pH 4 by the addition of 0.1 M HCl. The amount of ciprofloxacin loaded was calculated as the difference between the initial drug concentration and the drug concentration in the supernatant after loading. Using a spectrophotometer (Nanodrop ND1000; Thermo Scientific, Wilmington, DE, USA), the standard concentration curve for ciprofloxacin was plotted by measuring the absorbance at 276 nm, from which the slope and intercept were obtained for the equation  $y = a + bx$ . The extinction coefficient was found to be  $1.25 \times 10^{-6} \text{ M}^{-1} \text{ cm}^{-1}$ . The amount of ciprofloxacin in the supernatant was subsequently calculated by substituting for the corresponding values in the equation after measuring the absorbance. Ceftriaxone-loaded capsules (CD-Cef) were concentrated by centrifuging to achieve a higher concentration.

## Drug-release studies

After the loading studies, the sample was washed twice by centrifugation at 2000 rpm for 5 min to ensure the removal of unloaded ciprofloxacin. Studies of the release of ciprofloxacin were carried out by incubating the loaded nanocapsules in citrate buffer (pH 4.8) and PBS (pH 7.4) at 37°C. The mixture was then placed on a shaker at 100 rpm and the supernatant was removed at stipulated time periods (0.5, 1, 2, 4, 8, 16, 24, 48 h). The amount of ciprofloxacin release was quantified by measuring the absorbance at 276 nm in the supernatant using a spectrophotometer. The supernatant that had been removed for analysis was replaced by buffer (pre-warmed to 37°C).

## Characterization of nanocapsules using scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Samples containing the capsules were dropped onto a clean silicon wafer and dried overnight. In order to ensure electrical conductivity, the samples were subjected to gold sputtering (JEOL JFC-1100E ion sputtering device; JEOL, Tokyo, Japan) and analysed by field emission-SEM (FEI Sirion, Eindhoven, The Netherlands). A similar experiment was carried out by placing the sample on a 300 mesh carbon-coated copper grid (Toshniwal Bros SR Pvt Ltd, Bangalore, India) for field emission-TEM (Tecnaï F30; FEI, Eindhoven, The Netherlands).

## Zeta potential and size distribution measurements

Particle size distribution and zeta potential were measured using a Zetasizer Nano ZS (Malvern, Southborough, MA). In order to ensure the alternate deposition of CH and DS, the zeta potential of the outer surface was measured after deposition of each layer. Each measurement was taken as the average of three separate readings.

## Confocal microscopy

Confocal images were taken using a Carl Zeiss LSM Confocal scanning system (Carl Zeiss, Jena, Germany) equipped with a 100× oil immersion objective with a numerical aperture of 1.4. The capsules were visualized by electrostatic adsorption of FITC, which gives an indication of the size and degree of filling. RAW 264.7 (a kind gift from Professor Anjali Karande, Department of Biochemistry, Indian Institute of Science, India) and Intestine 407 (obtained from the National Center for Cell Science, Pune, India) cell lines were treated with FITC-loaded CH-DS nanocapsules (CD-FITC) for 30 min, repeatedly washed, fixed with 4% paraformaldehyde and visualized under confocal microscopy.

## MTT assay

The *in vitro* cytotoxicity of bare and ciprofloxacin-loaded CD nanocapsules was assessed by MTT assay in RAW 264.7 (a mouse macrophage-like cell line) and Intestine 407 (a human epithelial cell line) cells. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Sigma) at 37°C and 5% CO<sub>2</sub>. Volumes of 5 × 10<sup>4</sup> cells were seeded in a 96-well plate and incubated for 14 h. The cells were incubated with various concentrations of empty nanocapsules, ciprofloxacin-loaded nanocapsules and ciprofloxacin for 48 h. A volume of 20 μL of MTT dye (5 mg/mL) was added to each well and left for 4 h at 37°C. Depending on the viability of the cells, MTT is reduced to insoluble formazan crystals, which are subsequently dissolved by DMSO into a purple-coloured solution. The percentage cell viability was determined by spectrophotometry at 570 nm relative to non-treated cells.<sup>37</sup>

## In vitro effect of ciprofloxacin-loaded nanocapsules

### Intracellular proliferation assay

A total of 1 × 10<sup>5</sup> RAW 264.7 or Intestine 407 cells were seeded in each well of a 24-well plate, 24 h prior to infection. *Salmonella* Typhimurium was grown to stationary phase in Luria Bertani broth (LB) to infect the RAW264.7 cells. Stationary-phase cultures (*Salmonella* Typhimurium or *Salmonella* Typhi) were diluted 1:33 in LB and grown for 3 h (late-exponential phase) for infection of the epithelial (Intestine 407) cells. This was done to induce the *Salmonella* pathogenicity island 1 (SPI-1)-encoded genes required for the invasion of non-phagocytic cells.<sup>8</sup> The gentamicin protection assay was carried out as previously described.<sup>38</sup> Briefly, RAW 264.7

and Intestine 407 cells were infected with *Salmonella* (moi of 10) and incubated for 30 min. After repeated washing with PBS, DMEM containing gentamicin (100 μg/mL) was added and the mixture was incubated for 1 h. Ciprofloxacin-loaded CD nanocapsules (CD-Cipro), empty nanocapsules (Empty CD) and ciprofloxacin were then added in DMEM containing gentamicin (25 μg/mL), and the cells were washed with PBS after 1 h of incubation. After washing, the cells were incubated in DMEM containing gentamicin (25 μg/mL) for the rest of the experiment to avoid any extracellular *Salmonella* growth. At different timepoints, cells were washed three times with PBS and lysed with 0.1% Triton-X 100 and plated onto LB agar for bacterial counting. The fold intracellular replication of *Salmonella* was calculated by dividing the intracellular bacterial load at 16 h by the bacterial load at 2 h.

## Ciprofloxacin and FITC release into the cells

RAW 264.7 and Intestine 407 cells were treated with ciprofloxacin, FITC, CD-Cipro or CD-FITC (50 μg/mL) for 1 h. After the incubation, the mixtures were repeatedly washed to remove the extracellular capsules, extracellular ciprofloxacin and FITC. The concentrations of released ciprofloxacin or FITC in the cells were determined at different timepoints (2, 4, 8 and 16 h). Capsules containing ciprofloxacin or FITC (unreleased capsules) were locked in an acidic pH (pH 4.0) and removed, along with cell debris, by centrifugation. The concentration of released ciprofloxacin or FITC in the supernatant was determined using a fluorimeter (TEKAN, Infinite M200 Pro, Switzerland) with excitation at 280 nm and emission at 450 nm for ciprofloxacin, and excitation at 490 nm and emission at 525 nm for FITC; this was compared with the results for PBS-treated cells (which were used as a blank).

## In vivo experiments

BALB/c mice were bred and housed at the Central Animal Facility at the Indian Institute of Science. The mice used for the experiments were 6–8 weeks old. All procedures with animals were carried out in accordance with the institutional rules for animal experimentation. All animal experiments were approved by the Institutional animal ethics committee and national animal care guidelines were followed strictly. Mice were infected with *Salmonella* Typhimurium orally (10<sup>7</sup> cfu/mouse, five mice in each group). Ciprofloxacin, CD-Cipro, Empty CD and PBS control were given intravenously at different concentrations and different intervals for 3 days. The mice were sacrificed after 4 days, and the liver, spleen and mesenteric lymph nodes (MLNs) were aseptically isolated, weighed and homogenized in sterile PBS. The homogenate was plated in serial dilutions on *Salmonella*-Shigella (SS) agar to determine the bacterial load in various organs. Ceftriaxone, CD-Cef and PBS control were given intravenously for 3 days, and the bacterial burdens in the organs were calculated as described. The samples were plated onto SS agar with ceftriaxone (2 mg/mL) to determine the presence of *Salmonella* with decreased susceptibility to the antibiotic. Different sets of mice (eight per group) were treated with ciprofloxacin (2.5 mg/kg) and ceftriaxone (25 mg/kg) intravenously at 24 h intervals for 4 days after *Salmonella* Typhimurium infection (10<sup>8</sup> cfu/mouse—orally) as described. The animals were checked for morbidity and mortality twice daily for 15 days.

## Distribution of nanocapsule-encapsulated ciprofloxacin in vivo

Mice were assigned to two groups ( $n = 18$ ) and injected with 10 mg/kg of ciprofloxacin or CD-Cipro (1.12 mg/mL) by intravenous injection into the tail vein. Blood was collected at different time intervals (1, 2, 4, 8, 12 and 24 h after the injection,  $n = 3$  at each timepoint), and serum was collected by centrifugation at 2500 rpm for 20 min before being frozen at -20°C until assay. MLNs, spleen and liver were isolated, weighed and homogenized. Serum and tissue homogenate were diluted with an equal volume of

acetonitrile—0.1 M sodium hydroxide (1:1, v/v) in the microseparation system equipped with a 3000 Da molecular mass cut-off filter. Samples were vortex-mixed and centrifuged for 30 min at 4000 g. Supernatant was collected, and the concentration of released ciprofloxacin was measured by fluorescence with excitation at 280 nm and emission at 450 nm. Ciprofloxacin concentration was calculated from a standard curve, plotted by adding a known quantity of ciprofloxacin to the serum and carrying out a similar extraction procedure.

### Pharmacokinetic and pharmacodynamic analysis

Bioavailability from 0 to 24 h was calculated from the area under the curve (AUC) of the blood or tissue concentration versus time curve ( $AUC_{0-24}$ ) using the linear trapezoidal rule in GraphPad Prism 5 software. The half-life was determined using non-linear regression analysis and subsequently used to estimate the goodness of fit for each parameter using GraphPad Prism 5 software. Clearance and volume of distribution ( $V_d$ ) were calculated as follows: Clearance = Dose/AUC;  $V_d = (\text{Clearance} \times \text{Half-life})/0.693$ .  $AUC/MIC$ ,  $C_{max}/MIC$ ,  $T_{max}$  and  $T_{>MIC}$  were calculated using GraphPad Prism 5 software.

### Statistical analysis

The data were subjected to statistical analysis by applying the Student's *t*-test and Mann–Whitney *U*-test using commercially available Graph Pad Prism 5 software. A *P* value of <0.05 was considered significant.

## Results

### SEM and TEM characterization

CD nanocapsules were produced by the LbL technique described by Decher *et al.*<sup>13,14</sup> The morphology and topography of the bare silica template, coated particles and hollow capsules are clearly seen in Figure S1(a–c, available as Supplementary data at JAC Online). TEM images of the capsules are shown in Figure S1(d), available as Supplementary data at JAC Online. It was observed that the size of the template was in the range  $220 \pm 30$  nm, while that of the hollow capsules was on average  $180 \pm 20$  nm, indicating shrinkage in the capsule layers after dissolution of the template (Figure S1e, available as Supplementary data at JAC Online).

### Energy dispersive spectroscopy (EDS) and zeta potential measurement

In order to prove the removal of the silica core from the hollow capsule, EDS was carried out. Initially, EDS was done for the coated particles, which clearly indicated the presence of the silica template, as shown in Figure S1(f) (available as Supplementary data at JAC Online). Similarly, EDS was also carried out for the hollow capsule, and absence of the silica peak was demonstrated, as shown in Figure S1(g) (available as Supplementary data at JAC Online). The stability of the nanocapsules was studied by zeta potential measurements and was found to be around  $-42$  mV, indicating good stability. Zeta potential measurements were carried out after the deposition of each layer in order to ensure that all the four bilayers had been deposited. The zig-zag pattern shown in supplementary Figure S2(a) (available as Supplementary data at JAC Online) clearly indicates the alteration in the zeta potential with the deposition of the oppositely charged polyelectrolytes CH and DS.

### UV spectroscopy-based loading and release studies

The influence of factors such as pH and salt concentration plays a significant role in altering the capsule properties to produce efficient ciprofloxacin-loaded nanocapsules.<sup>39</sup> Here, 1 M NaCl was used as it ensured screening of the charges without any thickening of the walls, whereas a pH of 5.6 was chosen taking into account the *pK*<sub>a</sub> of the polyelectrolytes. The initial concentration of the drug and nanocapsules was 1 mg/mL and 0.4 mg/mL, respectively. The loading was performed by incubating 400  $\mu$ L of drug with 200  $\mu$ L of hollow capsules. The efficiency of drug encapsulation was determined by measuring the absorbance of the supernatant at 276 nm, indicating that 78% of the drug had loaded (312  $\mu$ g out of 400  $\mu$ g) into the hollow nanocapsules (mass of hollow nanocapsules was 80  $\mu$ g).

Subsequently, release studies were carried out at pH 4.8 and pH 7.4 as indicated in Figure S2(b) (available as Supplementary data at JAC Online). It was observed that there was a burst release in the first 30 min followed by a sustained release of ciprofloxacin over a period of 48 h. At the end of the 48 h, it was observed that about 70% of the drug, i.e. 156.8  $\mu$ g, had been released at neutral pH. Similar studies carried out at pH 4.8 indicated a release of 51%, i.e. 114.24  $\mu$ g, of the drug. The final ciprofloxacin concentration after loading was 1.12 mg/mL, which was subsequently used for *in vitro* and *in vivo* studies.

### Cytotoxicity studies using MTT assay

The RAW 264.7 and Intestine 407 cell lines used for this study are the model system for *Salmonella* infection. The biocompatibility of the fabricated nanocapsules was ascertained by carrying out the MTT assay in the RAW 264.7 and Intestine 407 cell lines. In both the RAW 264.7 (Figure S2c, available as Supplementary data at JAC Online) and Intestine 407 (Figure S3a, available as Supplementary data at JAC Online) cells, empty capsules did not affect the viability of these cell lines up to a concentration of 40  $\mu$ g/mL, which corresponds to 100  $\mu$ g/mL of ciprofloxacin. Ciprofloxacin does not have any toxic effect up to a concentration of 100  $\mu$ g/mL.

### In vitro studies

Intracellular survival assay was carried out in the RAW 264.7 and Intestine 407 cells to check the efficiency of the system to deliver the antibiotic intracellularly. *Salmonella* Typhimurium and *Salmonella* Typhi were used to infect the cells. After infection, cells were treated with CD-Cipro, Empty CD and ciprofloxacin for 1 h, and the cells were washed and incubated in medium containing gentamicin (25  $\mu$ g/mL) for the rest of the experiment to avoid the growth of any extracellular *Salmonella*. At 2 h and 16 h, the cells were lysed and the intracellular *Salmonella* burden was estimated by plating. The Empty CD did not have any antibacterial effect, whereas the CD-Cipro was able to enter the cells and release ciprofloxacin, resulting in a reduction in bacterial replication (Figure 1a–c). A similar reduction was observed in both cell lines with *S. Typhimurium* and *S. Typhi* infection (Figure 1a–c). The aminoglycoside gentamicin was used to kill extracellular bacteria in these experiments, and the entry of gentamicin into the cells was minimal. Gentamicin was loaded into the CD nanocapsules and the killing of the bacteria inside the Intestine 407 cells was assessed. The results show that the encapsulated gentamicin could kill *Salmonella* inside the cells even at lower concentrations

(10 µg/mL), whereas the higher concentration of gentamicin that was present extracellularly (25 µg/mL) could not kill the *Salmonella* in the control (Figure 1d).

In order to evaluate the drug-release pattern, ciprofloxacin release *in vitro* was studied. The ciprofloxacin concentration reached a maximum at the initial timepoint for the ciprofloxacin-treated cells, whereas the maximum concentration was obtained at a later timepoint for the CD-Cipro-treated cells (Figure 1e and f). A similar phenomenon was observed for both the RAW 264.7 and Intestine 407 cell lines. Furthermore, there was a reduction in ciprofloxacin concentration at 16 h in the case of the ciprofloxacin-treated cells. This may be due to the clearance of ciprofloxacin at later timepoints. Similar results were obtained when FITC and CD-FITC were used to determine the intracellular concentration of fluorescence released from the nanocapsules (Figure S3b and S3c, available as Supplementary data at JAC Online).

The entry of nanocapsules was confirmed by confocal microscopy. CD-FITC nanocapsules are shown in Figure 2(a). The RAW 264.7 and Intestine 407 cell lines were incubated with CD-FITC for 30 min before being fixed and observed under confocal microscopy. The particles were seen to be present in the cells. Most of the particles seemed to be present in the cell membranes (Figure 2b and c). Interestingly, the CD-FITC remained surrounding the membrane and was never seen to traffic inside the cells. The nanocapsules remained intact for 30 min and hence much less fluorescence was observed in the cytoplasm as FITC was released slowly into the cytoplasm at later timepoints (Figure S3b and c).

### In vivo studies

To check the *in vivo* efficiency of the delivery system against *Salmonella* infection, a murine salmonellosis model was used. *Salmonella* Typhimurium, which can cause typhoid-like systemic infection in a murine model, was used to infect BALB/c mice. The recommended dosage of ciprofloxacin for *Salmonella* infection is 20 mg/kg for oral dosing and 10 mg/kg for intravenous administration to maintain a therapeutic concentration of ciprofloxacin.<sup>40</sup> Apart from the prescribed dose (10 mg/kg) for intravenous administration, a reduced dose (2.5 mg/kg) was given intravenously in the case of CD-Cipro. All the dosages were given for 3 days continuously at 12 h intervals. The *Salmonella* burden was calculated by plating the homogenized tissues. A similar antibacterial effect was observed with a 5-fold reduction in the dose of CD-Cipro when compared with the recommended dose (Figure 3a) in all the organs (MLNs, spleen and liver).

To determine the effect of dosage frequency of ciprofloxacin and CD-Cipro on *Salmonella* infection, each drug was administered intravenously at different time intervals (12 h and 24 h). A significant reduction in *Salmonella* burden was observed even though the frequency of administration was 24 h in the case of CD-Cipro instead of 12 h in the case of ciprofloxacin (Figure 3b). When ciprofloxacin was administered after a 24 h interval, the *Salmonella* burden increased compared with the recommended dosing interval (12 h).

Ceftriaxone, a third-generation cephalosporin used against typhoid, was also assessed for its efficacy when delivered as CD-Cef (Figure 4a and b). The burden of *Salmonella* was significantly reduced in all the organs studied when CD-Cef was administered at a reduced dosage (Figure 4a). We further looked for the presence of *Salmonella* with decreased susceptibility to the antibiotic, if any,

by plating the organ lysates onto SS agar medium containing ceftriaxone (2 mg/mL). Higher values for *Salmonella* cfu were obtained in antibiotic media from the organ lysates of ceftriaxone-treated than CD-Cef-treated mice (Figure 4c). Although the percentage of bacteria with resistance to ceftriaxone at 2 mg/mL was found to be similar (data not shown), the number of bacteria showing decreased susceptibility was high in ceftriaxone-treated mice (Figure 4c). These data clearly suggest that there is a risk of development of bacterial resistance when a reduced antibiotic dose is given. When the antibiotic is encapsulated in the delivery system, the therapeutic level is maintained, and hence the development of resistance is significantly reduced. All the mice treated with a reduced dosage and frequency of drug encapsulated in a nanocapsule survived after *Salmonella* Typhimurium infection, whereas the free drug (at a reduced dosage and frequency) failed to save the mice (Figure 4b and Figure 5a). These results confirm the efficiency of the delivery system for *Salmonella* infection.

For further confirmation of the observed effect, the concentration of ciprofloxacin in the serum and tissues was measured. In case of CD-Cipro, the therapeutic level of ciprofloxacin was maintained for a prolonged time compared with ciprofloxacin (Figure 5b and Figure 6a–c). The maximum ciprofloxacin concentration was observed at 1–2 h for both ciprofloxacin and CD-Cipro, but with CD-Cipro the ciprofloxacin concentration was maintained for up to 8 h, whereas the concentration of ciprofloxacin had fallen to < 1 µg/mL before 5 h with ciprofloxacin alone. From these results, it was confirmed that the CD nanocapsule system may be a suitable option for delivering antibiotics and other drugs for *Salmonella* infection, with more beneficial effects.

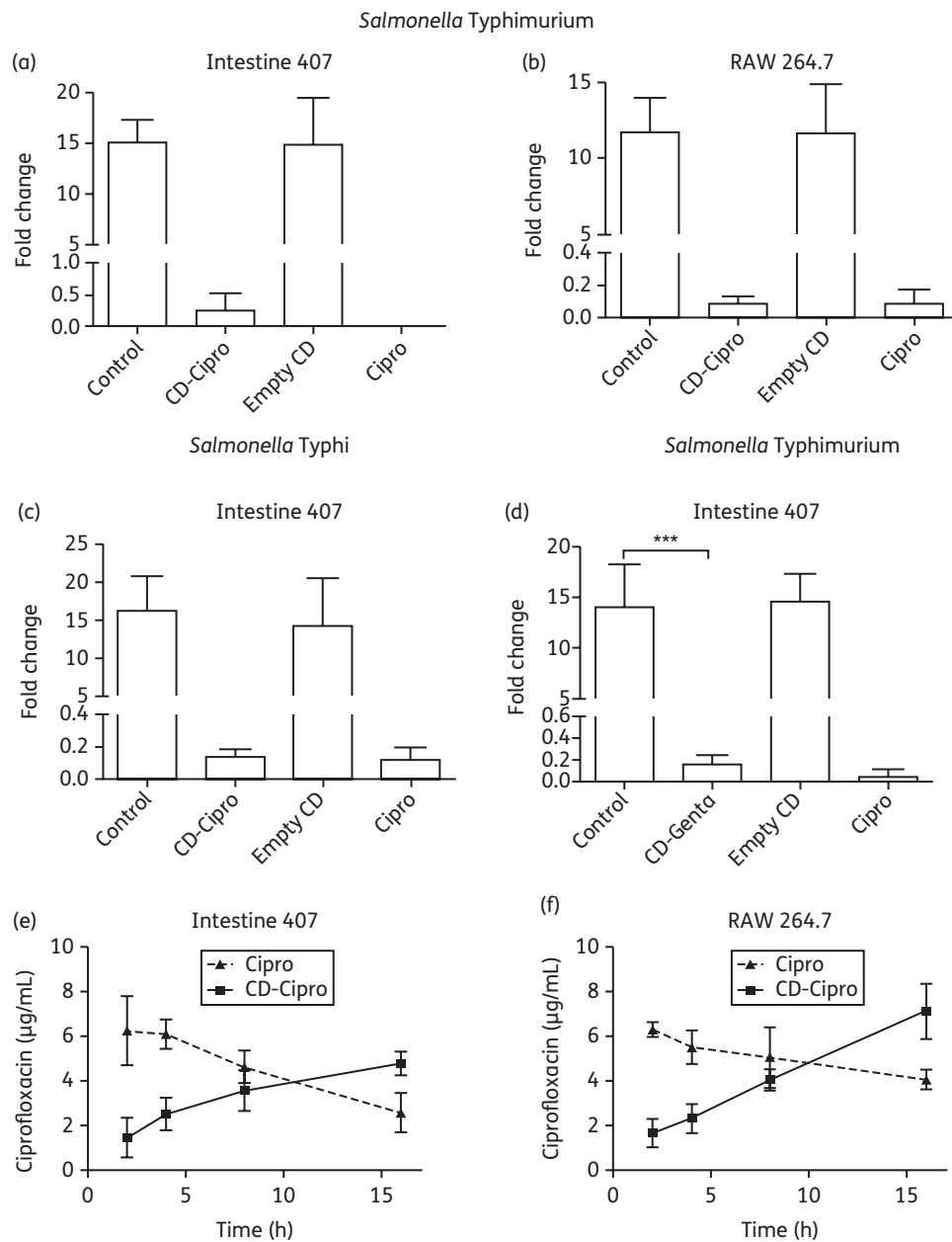
### Discussion

Conventional therapeutic regimens have been beset with problems as they have invariably been found to lead to toxicity and adverse effects whenever the dosage or frequency of dosing was increased. Here, we discuss the production of a capsule system composed of the bio-polymers CH and DS based on the principle of electrostatic interaction between the polyelectrolytes encapsulating ciprofloxacin, the drug of choice for *Salmonella* infection.

CD nanocapsules were formed by electrostatic interaction between the polyelectrolyte layers deposited onto a silica template. The key factor that plays a vital role in this interaction is the pKa of CH and DS, which is 6.5 and 2, respectively. Therefore, the reaction is carried out at a working pH of 5.6, ensuring that there is a high concentration of NH<sub>3</sub><sup>+</sup> and SO<sub>4</sub><sup>2-</sup> to produce a strong interaction. The size of the capsules formed in this way naturally depends on the silica template. After dissolution of the template, hollow capsules are formed that are found to attain a much smaller size owing to shrinkage of the capsule layers.

The hollow capsules formed after dissolution of the template were incubated in 1 mg/mL of ciprofloxacin solution. Entry of the drug molecules depends on the size of the pores in the capsules, which is in turn related to the pKa of the polyelectrolytes. Since the pKa of CH is 6.5, the loading was done at a pH of more than 6.5 as NH<sub>3</sub><sup>+</sup> are converted to NH<sub>2</sub>, which essentially decreases the interaction between the layers. This causes the pores to open up and allows the ciprofloxacin into the nanocapsules.

The same principle is exploited in order to ensure the release of the drug at various pH values. As the pH of the solution is altered,

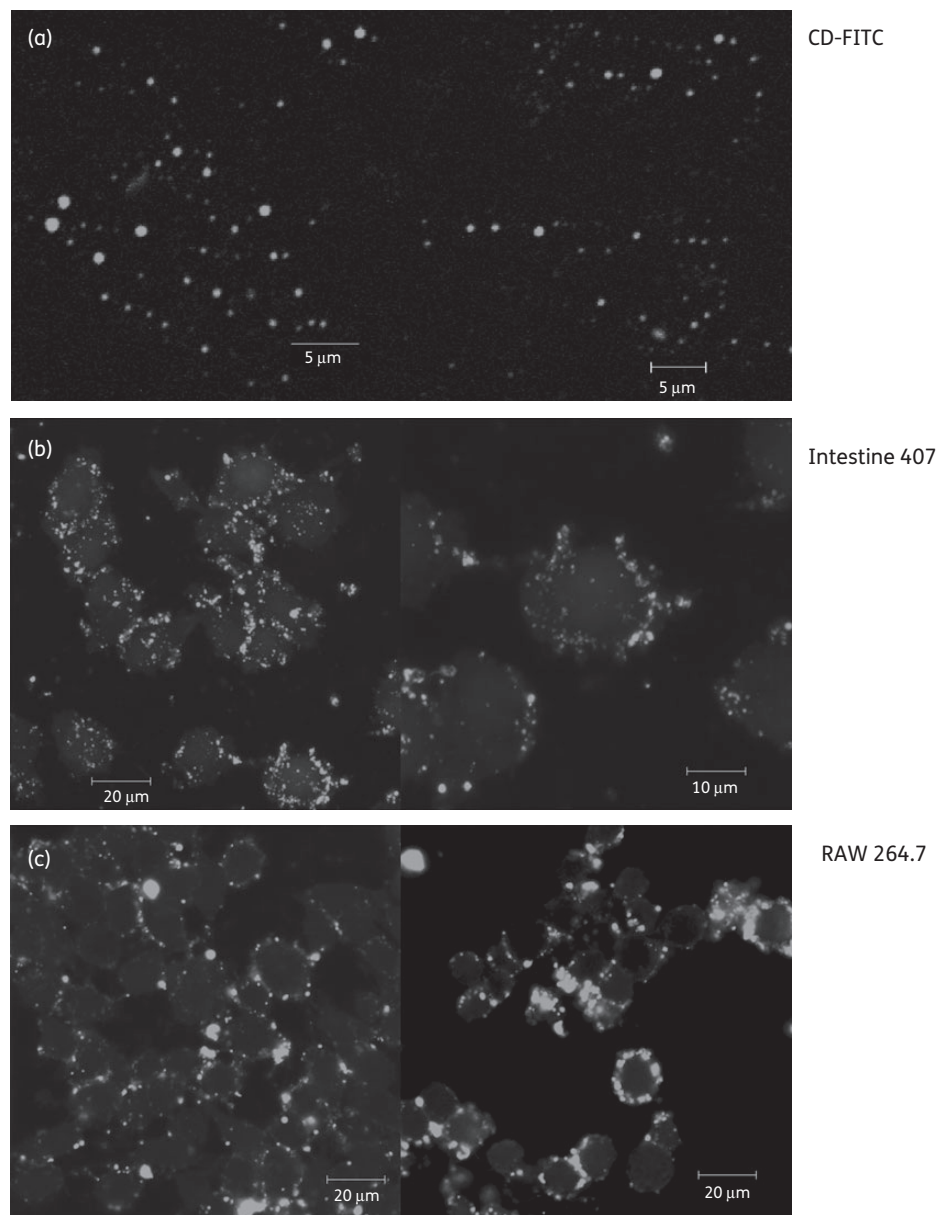


**Figure 1.** *In vitro* model system for CD-Cipro nanocapsules. Intracellular replication of *Salmonella Typhimurium* inside epithelial cells (Intestine 407) (a) and a mouse macrophage-like cell line (RAW 264.7) (b). Hollow nanocapsules (Empty CD), CD-Cipro and ciprofloxacin (Cipro; 50 µg/mL) were incubated with the cells for 1 h after infection, and the intracellular fold change in *Salmonella* burden was calculated by lysis and plating at 2 h and 16 h. (c) Intestine 407 cells infected with *Salmonella Typhi*. The fold change in *Salmonella* burden was determined by lysing the infected cells and plating at 2 h and 16 h post infection. (d) Gentamicin nanocapsules (CD-Genta; 10 µg/mL) and ciprofloxacin (50 µg/mL) were incubated with the cells for 1 h after infection, and the intracellular fold change in *Salmonella* burden was calculated by lysis and plating at 2 h and 16 h. CD-Cipro nanocapsules were incubated for 1 h at different timepoints, after locking the capsules in an acidic pH, and the concentration of ciprofloxacin was determined in Intestine 407 cells (e) and RAW264.7 cells (f) by measuring the fluorescence with excitation at 280 nm and emission at 450 nm. Graphs are representative of three independent experiments, each with samples in triplicate. Data shown as mean ± SD. Values that are statistically significantly different are indicated: \*\*\*,  $P < 0.0005$  by Student's *t*-test.

the drug that is adsorbed onto the surface and adjacent to the surface will be released quickly, providing a possible reason for the burst release observed. In comparison, it can be seen that the release was faster at neutral pH as opposed to an acidic pH. The faster drug elution might be due to the swelling of the

polymer matrix, which is brought about by deprotonation of amine group of CH.

From ciprofloxacin release studies *in vitro* (in RAW 264.7 and Intestine 407 cell lines), it was observed (Figure 1e and f) that entry or uptake was very rapid in case of ciprofloxacin, whereas the

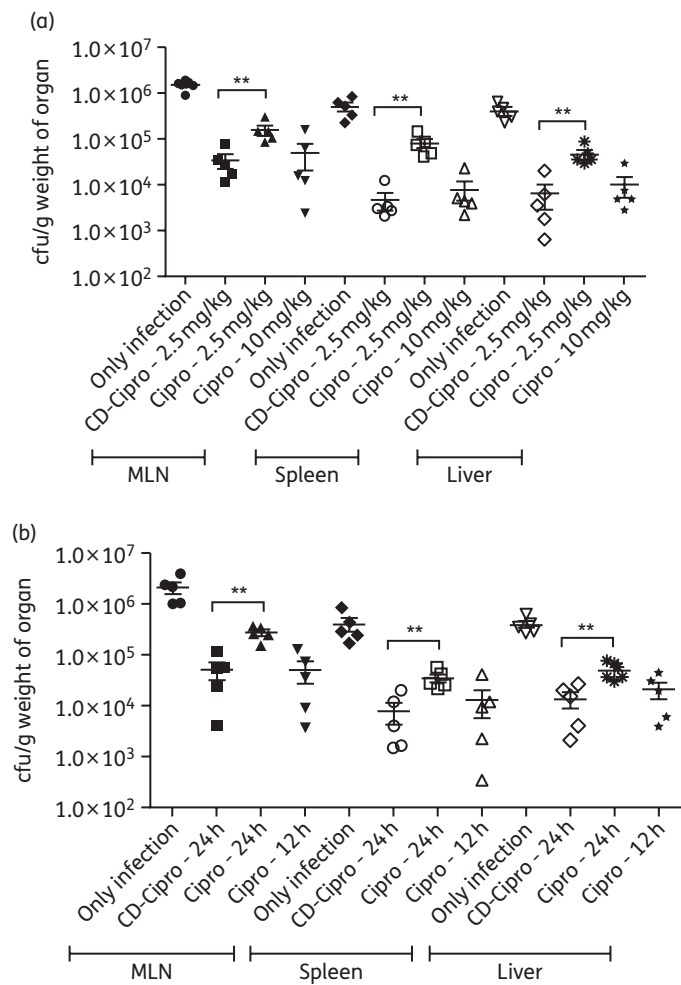


**Figure 2.** CD-FITC visualization. (a) The FITC-loaded capsules were visualized under confocal microscopy. (b) Intestine 407 and (c) RAW 264.7 cell lines were incubated with CD-FITC for 30 min and visualized under confocal microscopy.

CD-Cipro showed a slower release inside the cells. This result was not similar to the release observed in neutral pH where burst like release profile was observed (Figure S2b, available as Supplementary data at JAC Online). This may be due to the presence of nanocapsules mostly in the membranous part of the cells (Figure 2b and 2c). The slow release of ciprofloxacin into the cells may be achieved if the nanocapsules encounter the acidic compartment inside the cells. However, the reason for a slow release in the cells is not very clear. There was no difference in the killing potential of ciprofloxacin and CD-Cipro for *Salmonella*-infected RAW 264.7 and Intestine 407 cells (Figure 1a–c), although the release of ciprofloxacin from the nanocapsules was slower (Figure 1e and f). The concentration of released ciprofloxacin

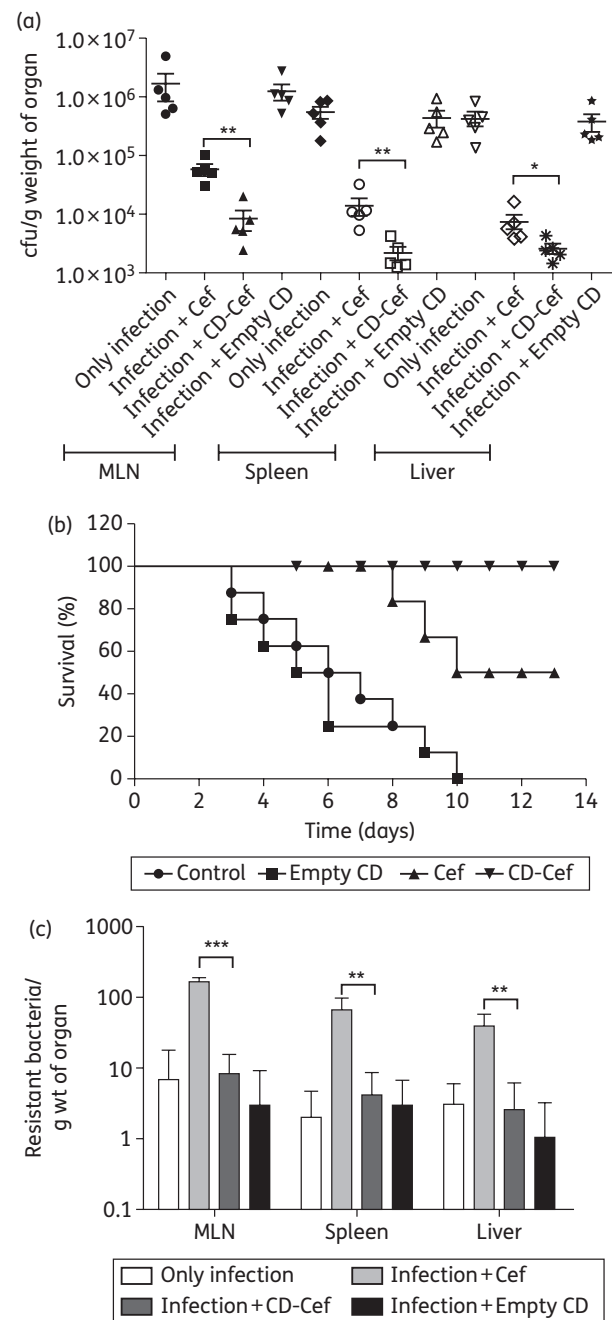
inside the cells was  $\sim 4 \mu\text{g/mL}$  at 10 h in both conditions (ciprofloxacin and CD-Cipro). Since replication of *Salmonella* in the cells was calculated from 2 h to 16 h, the ciprofloxacin concentration achieved inside the cells is sufficient to kill the intracellular *Salmonella*.

*Salmonella* Typhi causes typhoid in humans, whereas *Salmonella* Typhimurium can infect mice and cause typhoid-like symptoms.<sup>41</sup> This murine salmonellosis model is a well-established model of typhoid<sup>38</sup> and was used to determine the effect of CD-Cipro *in vivo*. From the results, it was observed that the reduced dosage of ciprofloxacin (2.5 mg/kg body weight) is sufficient to give a similar effect to the prescribed dosage (10 mg/kg body weight). When intravenously injected CD-Cipro reaches the



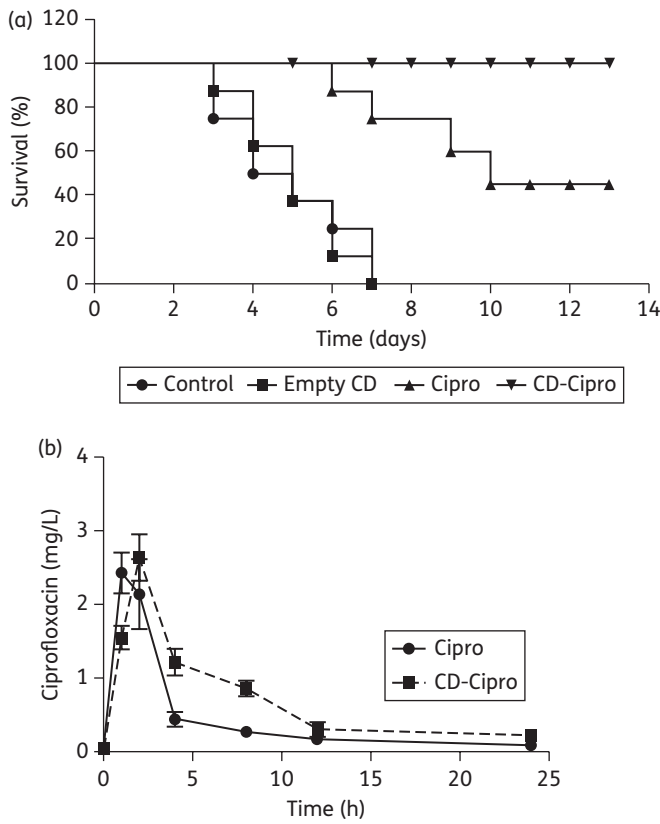
**Figure 3.** *In vivo* antibacterial effects of CD-Cipro and Empty CD in different mouse organs. (a and b) Mice were infected with *Salmonella* Typhimurium ( $1 \times 10^7$  cfu) and treated with CD-Cipro, Empty CD or ciprofloxacin (Cipro) intravenously from the second day onwards. After 3 days of treatment (the fifth day after infection), *Salmonella* burdens were calculated in different organs by homogenization and plating. (a) Different concentrations of ciprofloxacin were given for 3 days at a frequency of 12 h. (b) CD-Cipro (2.5 mg/kg) and ciprofloxacin (10 mg/kg) were given intravenously at different intervals (12 h and 24 h) for 3 days and the *Salmonella* burden was calculated. Values that are statistically significantly different are shown: \*\*,  $P < 0.005$  by the Mann–Whitney *U*-test.

organs, a slow release of ciprofloxacin takes place. The reduction in *Salmonella* burden was observed in all the secondary lymphoid organs studied. The ciprofloxacin levels in different organs were also higher when CD-Cipro was injected compared with ciprofloxacin (Figure 6a–c). The CD nanocapsule-mediated delivery system for ciprofloxacin needs only one quarter of the recommended dosage of ciprofloxacin compared with the conventional routes of administration. When CD-Cipro was given at 24 h time intervals, the burden of *Salmonella* was reduced as efficiently as with a frequency of 12 h (the recommended dosage frequency). Considering these observations (Figure 3a and b), one eighth of the recommended dosage is sufficient for a therapeutic effect of ciprofloxacin if ciprofloxacin is given as CD-Cipro.



**Figure 4.** *In vivo* antibacterial effects of CD-Cef and Empty CD. (a) Mice were infected with *Salmonella* Typhimurium ( $1 \times 10^7$  cfu) and treated with CD-Cef, Empty CD and ceftriaxone (Cef; 25 mg/kg and 24 h interval) intravenously from the second day onwards for 3 days. On the fifth day after infection, the *Salmonella* burden was calculated in different organs by homogenization and plating. Values that are statistically significantly different are shown: \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  by the Mann–Whitney *U* test. (b) For survival assay, mice were treated (25 mg/kg and 24 h interval) with ceftriaxone after *Salmonella* ( $1 \times 10^8$  cfu) infection and assessed for morbidity and mortality. (c) CD-Cef and ceftriaxone (25 mg/kg) were given intravenously for 3 days, and on the fifth day tissues were homogenized and samples were plated onto SS agar containing ceftriaxone (2 mg/mL) to determine the development of resistance in *Salmonella*. \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$  by Student's *t*-test.



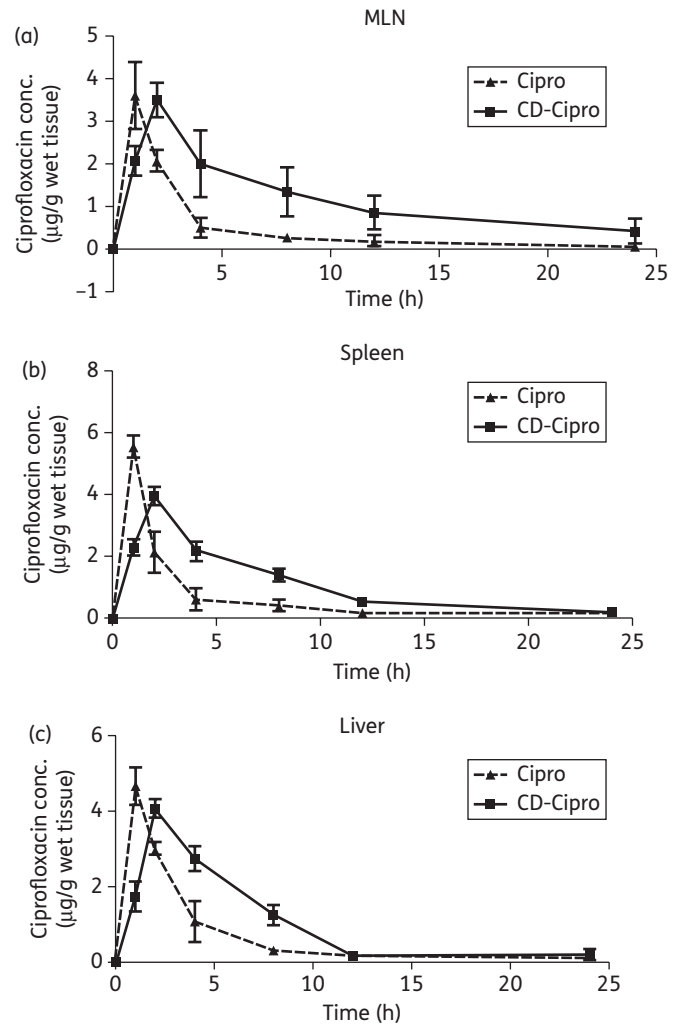


**Figure 5.** *In vivo* antibacterial effects of CD-Cipro and Empty CD. (a) Survival assay of mice treated with ciprofloxacin and CD-Cipro (2.5 mg/kg) along with Empty CD after *Salmonella* infection ( $1 \times 10^8$  cfu). (b) A single dose of 10 mg/kg ciprofloxacin was given intravenously and serum was collected at different timepoints. Ciprofloxacin was extracted from the serum, and ciprofloxacin concentration was measured using a fluorimeter.  $AUC_{0-24}$  was calculated using GraphPad Prism software.

To estimate its biological distribution, ciprofloxacin was extracted from serum at different timepoints after the intravenous administration of ciprofloxacin and CD-Cipro, and quantified. It was observed that the therapeutic concentration was maintained for a longer period of time for CD-Cipro. The  $AUC_{0-24}$  was calculated with a linear trapezoidal rule using GraphPad Prism software. The  $AUC_{0-24}$  for CD-Cipro was 16.26  $\mu\text{g}\cdot\text{h}/\text{mL}$ , which is >1.5 times (a 63.83% increase) higher than the  $AUC_{0-24}$  of ciprofloxacin, which was 9.93  $\mu\text{g}\cdot\text{h}/\text{mL}$  for serum. Similarly, there is also a significant increase in bioavailability in other tissues where *Salmonella* can be found (Table 1).

To support the suggestion CD-Cipro remains in the system for a longer period of time, the half-life for ciprofloxacin was found to be 0.62 h, as opposed to a half-life of 3.17 h for CD-Cipro (a 411.29% increase). Similarly, ciprofloxacin was found to have a clearance of 16.79 mL/min/kg, whereas CD-Cipro had a relatively lower clearance of 10.25 mL/min/kg (a 38.96% decrease). Ciprofloxacin was found to have a  $V_d$  of 0.9 L/kg, whereas that for CD-Cipro was 4.58 L/kg, clearly indicating that CD-Cipro is more effectively taken up by the tissues.

In the case of concentration-dependent killing, the higher the concentration, the higher the kill rate. However, in the case of ciprofloxacin and CD-Cipro, there was no significant difference in



**Figure 6.** Distribution of ciprofloxacin in the tissues. A single dose of 10 mg/kg of ciprofloxacin was given intravenously, and MLNs (a), spleen (b) and liver (c) tissue were collected at different timepoints and homogenized. Ciprofloxacin was extracted from the tissues, and its concentration was measured using a fluorimeter.  $AUC_{0-24}$  was calculated using GraphPad Prism5 software. Data are shown as mean  $\pm$  SD.

**Table 1.** Comparison of pharmacokinetic parameters of ciprofloxacin and CD-Cipro *in vivo*

Sample	$AUC_{0-24}$ ( $\mu\text{g}\cdot\text{h}/\text{mL}$ )		Increase in AUC (%)
	ciprofloxacin	CD-Cipro	
Serum	9.93	16.26	63.83
MLNs	11.22	27.97	149.29
Spleen	14.26	25.84	81.21
Liver	15.24	23.44	53.81

$C_{\text{max}}/\text{MIC}$ , with values of 2.7 and 3, respectively. The sustained release characteristic of CD-Cipro was demonstrated by a  $T_{\text{max}}$  of 2 h, whereas ciprofloxacin reached  $T_{\text{max}}$  in 1 h. It was also observed that CD-Cipro had a higher  $T_{>\text{MIC}}$  (25%) compared with

**Table 2.** Pharmacokinetic (PK) and pharmacodynamic (PD) parameters of ciprofloxacin and CD-Cipro in serum

PK and PD parameters	Ciprofloxacin	CD-Cipro	Increase (%)
Half-life (h)	0.62	3.17	411.29
Clearance (mL/min/kg)	16.79	10.25	38.96
V <sub>d</sub> (L/kg)	0.9	4.58	408.69
AUC/MIC	9.93	16.26	63.83
C <sub>max</sub> /MIC	2.7	3.0	11.11
T <sub>max</sub> (h)	1.0	2.0	100
T <sub>&gt;MIC</sub> (%)	12.125	25	106.19

ciprofloxacin (12.125%), showing a 106% increase that indicated time-dependent killing. The concentration of ciprofloxacin in the serum was maintained at >1 µg/mL for a longer time, which can indeed reduce the chance of developing antibiotic resistance.<sup>42</sup> AUC/MIC is a combination of T<sub>>MIC</sub> and C<sub>max</sub>/MIC in which the rate of bacterial killing is related to the time above the MIC and the total exposure of the organism to ciprofloxacin. In the case of ciprofloxacin, AUC/MIC was found to be 9.93, whereas CD-Cipro demonstrated a value of 16.26, thereby clearly indicating that the frequency of dosage of ciprofloxacin was reduced when it was encapsulated in CD (Table 2).

These properties of CD-Cipro make it an ideal treatment of choice since a prolonged treatment is required for *Salmonella* infection, with a relapse rate as high as 10%.<sup>30</sup> Hence, effective targeted delivery that will reach the organs and the vacuoles to deliver the drug is high on the agenda. In addition to *Salmonella*, many other intracellular pathogens can be controlled in an effective manner by using drug delivery systems. Phosphatidylserine-specific ligand-anchored nanocapsules have been developed to target specialized macrophages, which can be used as a drug delivery system in leishmaniasis.<sup>43</sup> Our in-depth experiments comprising both an *in vitro* and *in vivo* animal model suggests a novel drug delivery system that could achieve a better management of infectious diseases. This system could probably also be used for other intraphagosomal pathogens such as *Mycobacteria*, *Brucella* and *Legionella*.

## Conclusions

There are plethora of other important infectious diseases caused by intracellular pathogens such as *Mycobacterium tuberculosis*, hepatitis C virus and HIV. However, the treatment process is becoming difficult due to the emergence of antibiotic resistance. Apart from the emergence of multidrug resistance, targeting the drug within the cell is a major challenge in the treatment of intracellular pathogens. The drug delivery system could be used to deliver these drugs and hence the development of resistance could be delayed. In future, drug delivery systems targeted to different niches of intracellular pathogens could be developed for better efficiency. It may be possible to target the *Salmonella*-containing vacuoles to kill the pathogen so that complete cure can be achieved without any relapse.<sup>30</sup>

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## Transparency declarations

None to declare.

## Supplementary data

Figures S1–S3 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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