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Development and optimization of ciprofloxacin-loaded gelatin microparticles by single-step spray-drying technique

Dina M. Morais da Silva
University of Wollongong

Heema Vyas
University of Wollongong, hv997@uowmail.edu.au

Martina L. Sanderson-Smith
University of Wollongong, martina@uow.edu.au

Vitor Sencadas
University of Wollongong, victors@uow.edu.au

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Abstract

Polymeric particles are a versatile class of local or systemic drug delivery systems, used to improve drugs pharmacokinetics and pharmacodynamics along with patient compliance. Herein, we report a rapid, scalable, and optimized method to encapsulate ciprofloxacin (CP_x), a poor water soluble antimicrobial agent, in gelatin microparticles by single step processing via spray-drying of an aqueous solution. The developed particles show mainly a wrinkle morphology with a unimodal distribution, with mean diameters ranging between 2 and 4 μm, depending on the processing conditions. The encapsulation of 1, 2 and 5 wt% CPx narrows the size distribution (1-3 μm). *In vitro* release experiments showed that up to 80% of encapsulated drug is released during the first 6 h, and the release kinetics was best fitted with the Korsmeyer-Peppas model, explained by the superimposition of drug diffusion and polymer chain relaxation. The minimal inhibitory concentrations against *S. aureus* and *E. coli*, obtained from pure and encapsulated ciprofloxacin, demonstrated that the spray-drying process does not inhibit the drug's bioactivity or the process feasibility. Thus, spray-drying of protein-drug particle systems is an advantageous method to produce microparticles with potential to lung delivery systems.

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Development and Optimization of Ciprofloxacin-Loaded Gelatin Microparticles by Single-Step Spray-drying Technique

Dina M. Silva[a*], Heema Vyas[b], Martina L. Sanderson-Smith[b], Vitor Sencadas[a,c*]

[a]School of Mechanical, Materials, Mechatronics and Biomedical Engineering, University of Wollongong, Wollongong, NSW 2522, Australia

[b]Illawarra Health and Medical Research Institute, School of Biological Sciences, University of Wollongong, Wollongong, Australia

[c]ARC Center of Excellence for Electromaterials Science, University of Wollongong, 2522 NSW, Australia

[*]Corresponding authors:

Vitor Sencadas: victors@uow.edu.au; vsencadas@gmail.com

Dina M. Silva: dsilva@uow.edu.au; dina.morais.silva@gmail.com

School of Mechanical, Materials, Mechatronics and Biomedical Engineering, University of Wollongong, Northfields Ave, Wollongong, NSW 2522, Australia

Ph: +61 242214614

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Abstract:

Polymeric particles are a versatile class of local or systemic drug delivery systems, used to improve drugs pharmacokinetics and pharmacodynamics along with patient compliance. Herein, we report a rapid, scalable, and optimized method to encapsulate ciprofloxacin (CPx), a poor water soluble antimicrobial agent, in gelatin microparticles by single step processing via spray-drying of an aqueous solution. The developed particles show mainly a wrinkle morphology with a unimodal distribution, with mean diameters ranging between 2 and 4 μm, depending on the processing conditions. The encapsulation of 1, 2 and 5 wt% CPx narrows the size distribution (1-3 μm). *In vitro* release experiments showed that up to 80% of encapsulated drug is released during the first 6 hours, and the release kinetics was best fitted with the Korsmeyer-Peppas model, explained by the superimposition of drug diffusion and polymer chain relaxation. The minimal inhibitory concentrations against *S. aureus* and *E. coli,* obtained from pure and encapsulated ciprofloxacin, demonstrated that the spray-drying process does not inhibit the drug`s bioactivity or the process feasibility. Thus, spray-drying of protein-drug particle systems is an advantageous method to produce microparticles with potential to lung delivery systems.

1. Introduction

Particulate systems are a class of drug delivery systems of great clinical and research interest for both local and systemic delivery, which are designed to improve drug bioavailability, stabilization, pharmacokinetics and selectivity, while avoiding toxicity issues raised by added excipients, continuous administration or tissue accumulation [1, 2]. Those systems are particularly appealing for pharmaceutical industry due to their versatility, enabling topical, pulmonary, nasal or parenteral administration [2, 3] in creams [4], hydrogels [5], dry powders and aerosols [6, 7] or injectable formulations [8].

Drug loaded polymeric particles fabrication often rely on drying of a droplet containing the formulated mixtures from dissolved, dispersed or emulsified substances [9, 10]. Most relevant techniques include spray-drying [7, 11], spray freeze drying [12], and electrospray [13], among others. Spray-drying is a well-established technology in the pharmaceutical and food industry, for microencapsulation [14, 15]. This a continuous, single step, cost-effective and scalable technique that allows for the tuning of the final particles properties [9, 16] and for the processing of heat sensitive molecules (proteins, peptides or drugs) which, in turn, enhance the bioactivity of the final formulations [17, 18]. This is due to the fast solvent evaporation of droplets with high surface area-to-volume ratio [18]. Furthermore, the encapsulation of hydrophobic molecules is also possible by the atomization of emulsified feed solution, introducing then an extra processing step [19, 20].

Natural polymers are often a smart choice when developing medical devices mainly due to their biocompatibility and biodegradability. Gelatin is a natural biopolymer, with a wide set of applications in the food, pharmaceutical and cosmetic industries,

since it is considered as a GRAS (generally recognized as safe) material by the FDA [21]. Despite its animal source, toxicity and antigenicity issues are minimal due to acid/alkali hydrolysis of collagen during processing [21, 22]. Other attractive features of gelatin for drug delivery systems include the available functional groups for the coupling of ligands, targeting or the stabilization by crosslinking, increasing circulation times [21]. In this work, an alternative source of gelatin – fish gelatin – was chosen, which, considering the targeted application, would reduce the concerns about prion transmission related with animal sources of gelatin [23].

Ciprofloxacin (CPx) is a broad-spectrum fluoroquinolone antibacterial agent indicated for the treatment of acute cystitis, chronic prostatitis, urinary and lower respiratory tract infections, acute sinusitis, skin infections, bone and joint infections, among others [24]. It is also administered to treat opportunistic *Pseudomonas aeruginosa* in cystic fibrosis patients [24]. CPx acts by inhibition of bacterial DNA gyrase and topoisomerase IV that disrupts bacterial DNA replication [25]. Side effects associated with CPx use, including gastrointestinal, cardiovascular and allergic reactions and nervous system complaints [26], along with its poor water solubility and bioavailability [24, 27] motivates the development of effective encapsulations systems.

This work reports the development and optimization of gelatin microparticles for CPx encapsulation using an orthogonal design by a single-step procedure using spray-drying. The developed microparticles were characterized by SEM, FTIR and thermal analysis and the *in vitro* release profile was fitted to the mathematical release models. The crosslinking effect (glutaraldehyde) on the microparticle diameter was also evaluated. CPx bioactivity was assessed by determining the minimal inhibitory and

bactericidal concentrations against susceptible strains of *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) comparing the CPx-loaded microparticles with the free drug.

2. Material and methods

2.1. Materials

Gelatin (gel) from cold water fish skin, glutaraldehyde solution (GA; 50 wt.%) and ciprofloxacin (CPx; $\geq 98.0\%$) were supplied by Sigma-Aldrich. All reagents and chemicals used were of analytical grade.

2.2. Polymer particle processing

Gel solutions were prepared in double distilled water (ddH_2O) at different concentrations (0.5, 1 and 2 % w/v) under magnetic stirring at 50 ºC and cooled to room temperature (25ºC) before use.

For gel-CPx formulations, a stock solution of CPx in 0.1N HCl was prepared separately and added to a gel solution (0.5 % w/v) for a final CPx concentration of 1, 2 and 5 wt%.

Spray-drying was performed using a home-made system (figure 1). The experimental parameters range for gelatin concentration, feed rate and inlet temperature are detailed in Table 1.

2.3. Experimental design

The influence of the processing parameters on gel microparticles produced by spray-drying was evaluated by an orthogonal design table L_9, using Orthogonal Design Assistant II software (Sharetop Software Studio). Each orthogonal table has its own mark denoted as (1):

$$L_n(t)^c \quad (1)$$

where L represents the orthogonal table of n experiments, t is the number of levels of each factor and c is the maximum number of factors. To produce gel microparticles the

defined factors of polymer concentration (A), feed rate (B) and inlet temperature (C), their levels and the experimental set up for the nine experimental conditions (A-I) are describe in Table 1.

The selection of optimal conditions was based on the orthogonal parameters *K* and *R*, which were calculated from the particle diameters as:

$$K_l^F = \frac{\sum evaluation\ indexes\ at\ same\ level\ of\ each\ factor}{3} \quad (2)$$

$$R^F = max\{K_l^F\} - min\{K_l^F\} \quad (3)$$

where K and R represents the mean and range values, respectively, for a factor F at the level l [28, 29]. The impact of the three factors (gel concentration, feed rate and inlet temperature) on the average particle diameter was evaluated by investigating the influence of different levels of each factor [28]. The determination of the factors order was achieved by calculating the range (R) for each factor upon evaluation indexes (K_i).

2.4. Rheology

The rheological behavior of gel solutions with different protein concentrations (0.5, 1 and 2 % w/v) were evaluated using a parallel plate ($\emptyset = 20\ mm$) geometry rheometer (MCR 301, Anton Paar) with a 0.3 mm gap to ensure adequate filling of the samples over the testing disk. Viscosity was measured as a function of shear rate within the range of 1-1000 s^{-1}, at 25 ºC.

2.5. Polymer particles crosslinking

Dry particles were crosslinked using 20 mL of a glutaraldehyde solution (50 % in H_2O, Sigma-Aldrich) in the vapor phase, in a vacuum chamber (VACUO-TEMP, JP Selecta, Spain), at room temperature, and under low pressure for 72 h.

2.6. Particles characterization

Particles were coated with a thin gold layer using a sputter coater (Smart Coater, JEOL) and their morphology was analyzed using a scanning electron microscope (JCM-6000PLUS Neoscope, JEOL), with an accelerating voltage of 10 kV. Particles average diameter and its distribution was calculated over 300 particles in SEM images (1,000 x magnification) using the Image J software [30]. Particles dispersity was quantitatively evaluated by the coefficient of variation (C_V), defined as:

$$C_V = \frac{\sigma}{\mu} \tag{4}$$

where σ represents the standard deviation of the mean particle size (μ) [31].

Infrared (FTIR) measurements were performed in a Shimadzu IRAffinity-1S apparatus in ATR mode from 4000 to 600 cm^{-1}, at room temperature. FTIR spectra were collected after 32 scans with a resolution of 2 cm^{-1}. Differential scanning calorimetry (DSC) measurements were performed with a TA Q100 apparatus (TAinstruments), between 30 and 200 ºC at a heating rate of 10 ºC.min^{-1}, under a nitrogen purge. Thermogravimetrical analysis (TGA) was conducted in a Q500 apparatus (TAinstruments) using a heating rate scan of 20 ºC min^{-1}, under a nitrogen atmosphere.

2.7. Determination of CPx loading

The CPx loading was determined by dissolving the dry gel-CPx microparticles in 0.1 M HCl, in ultrasonic bath for 6 h followed by mechanical stirring at 37 ºC. After 72 h, the suspensions were centrifuged (10,000 rpm, 10 min) to remove polymeric debris and the CPx was measured in the supernatants using a spectrophotometer (Shimadzu UV1800), using crosslinked drug-free gel particles as blank controls. The drug loading was calculated per equation 5, using a calibration curve of CPx in 0.1 M HCl (equation 6) at $OD = 277\,nm$, where x is the drug concentration ($\mu g/mL$) and R^2 is the coefficient of determination.

$$Drug\ loading\ (\%) = \frac{amount\ of\ CPx\ in\ particles}{amount\ of\ particles} \times 100 \quad (5)$$

$$OD_{277nm} = 0.1245x + 0.004768\ (R^2 = 0.999) \quad (6)$$

2.8. In vitro CPx release

Gel-CPx suspensions (10 mg mL^{-1}; 5 mL) were transferred to a tubular dialysis membrane with a MWCO of 1 kDa (Cellu.Sep H1, Fisher Biotec). The membranes were then immersed in 200 mL of PBS (pH 7.4), under smooth magnetic stirrer (100 rpm) at 37 ºC. At predetermined time intervals, 2 mL of the release medium was withdrawn and replaced with fresh PBS. The release medium was measured at $OD = 270\,nm$ using a UV-VIS spectrophotometer (Shimadzu UV1800) and the CPx amount was calculated using a calibration curve in PBS (7), where x is the drug concentration

($\mu g/mL$) and R^2 is the coefficient of determination. Results were expressed as % cumulative release relative to the initial weight of CPx loaded in the particles. Analysis was performed in triplicates and results were averaged.

$$OD_{270nm} = 0.09847x + 0.001677\ (R^2 = 0.998) \quad (7)$$

The experimental results were fitted to the first order (8), Higuchi (9) and Korsmeyer-Peppas (10) models to further evaluate the release kinetics.

$$\log C = logC_0 - Kt/2.303 \quad (8)$$

$$\frac{M_t}{M_\infty} = K\sqrt{t} \quad (9)$$

$$\frac{M_t}{M_\infty} = Kt^n \quad (10)$$

where C and C_0 are, respectively, the cumulative and initial amounts of drug, M_t/M_∞ is a fraction of drug released at time t, K is the rate constant, and n is the exponent that characterizes the release mechanism [32, 33].

2.9. Determination of the antibacterial activity of Gel-CPx particles

The susceptibility of *Staphylococcus aureus* (ATCC29213) and *Escherichia coli* (NCTC8196) to the gel-CPx (5 wt%) microparticles was evaluated by broth microdilution assay as previously described, with slight modifications [34]. Briefly, bacteria were streaked from glycerol-frozen stocks onto Luria-Bertani Agar (LB;

tryptone 10 gL^{-1}, yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}, agar 15 g L^{-1}, pH 7.4) plates and incubated overnight at 37 ºC. Overnight cultures of *S. aureus* and *E. coli* were prepared from single colonies in Brain-heart infusion (BHI; 37 g L^{-1}, pH 7.4) or LB broths, respectively, at 37 ºC in an orbital shaker (160 rpm).

Dry formulations of gel (uncross- and crosslinked) and gel-CPx (5 wt%) microparticles (crosslinked) were sterilized prior to incubation by UV exposure for 25 min. To ensure sterility of UV-irradiated samples, 100 µL of stock solutions was plated in LB agar and blood-agar media and monitored for bacterial growth. CPx concentration in formulations was calculated per the percentage of drug entrapment. Controls of pure CPx were prepared from a stock solution (30 µg mL^{-1}) in PBS (pH 7.4) and sterilized by filtration. All experimental formulations were then 2-fold serially diluted in 100 μl of culture medium to reach the tested concentrations. Each well was then inoculated with 100 μL of bacterial suspension containing $1x10^6\ CFU\ (colony\ forming\ units)\ mL^{-1}$.

Bacterial cultures were then incubated for 18-20 h at 37 ºC and the minimum inhibitory concentration (MIC) was determined as the lowest concentration with clear wells, using a microplate reader (SpectraMax Plus 384, Molecular devices, USA) at OD_{600} nm. Minimum bactericidal concentration (MBC) was determined by plating 10 µL of each well with growth inhibition (clear wells) on LB agar medium. Following incubation for 24 h at 37 ºC, MBC was determined as the lowest concentration where bacterial growth was absent.

2.10. Data analysis

Quantitative data are presented as mean ± standard deviation (SD). Significant differences were determined by one-way ANOVA analysis with Tukey`s HSD post hoc test using GraphPad Prism Software (v 6.01). Statistical differences were considered when $p < 0.05$.

3. Results and discussion

3.1. Optimization of gelatin microparticles

The average particle diameter was the measured response of the orthogonal design to produce gel microparticles by spray-drying technique, in a single step, avoiding intermediate steps, such as emulsification. Results of average particle diameter, coefficient of variation and orthogonal indexes are presented in table 2, for the experimental runs from A to I (table 1). The larger the R parameter, the more influence of the factor on the process, reflecting either valuable or detrimental effects [28, 29, 35, 36]. For the processing of gel microparticles by spray-drying, gelatin concentration is the most important factor followed by the feed rate and the inlet temperature ($A > B > C$). Figure 2 depicts the influence of tested factors and levels on the average particle diameter and coefficient of variance. Despite the narrow diameter range of the experimental runs (between 2.62 ± 0.78 and 3.65 ± 1.17 μm), it can be pointed out a concentration-dependent increase in particle diameter (figure 2a) that can be mainly addressed to the increased viscosity of the feed solution (figure 2b). Differences in the rheological behavior of gelatin solutions are obvious between the

lower (0.5 and 1 % (w/v)) and the highest concentration (2 % (w/v)); the first ones showed a typical Newtonian behavior, whereas the 2 % (w/v) solution behave as a shear thinning fluid. For instance, for lower shear rates the difference in the viscosity is up to 7 times for the 2 % (w/v) solutions (figure 2b – inset). The relationship between feed solution concentration and particle size has been reported by Elversson et al. for lactose powders, where the increase in concentration lead to larger particles, although the effect was not linear [37].

The influence of the tested factors and levels on the particles dispersity, quantitatively evaluated by the C_V, were almost negligible, and similar values were found for all experimental runs, and all below 0.5 (table 2). The water content in dry formulations is of outmost importance for the formulation preservation and for the improvement of shelf-life [38]. The overall variation in the moisture content for the orthogonal experiments (figure S1) was less than 2 % and, therefore, it is not influenced by the studied levels.

The selection of the optimal combination is determined by the smallest index and, for this work consisted of a feed concentration of 0.5% (w/v), using a feed rate of 308 ml h^{-1} at 115ºC. However, the optimal feed rate had to be adjusted for the subsequent experiments to the following level (177 ml h^{-1}) due to the moisture introduced in the sample collector, that dissolved the formed particles and leading to a non-continuous processing.

The samples collected after spray-drying presented a mixture of wrinkled (mostly) and a few smooth spherical particles, as depicted in figure 3, following a unimodal distribution (figure 4), in accordance with the lower C_V values (table 2). Irregular particle morphology was already reported for gelatin microspheres [19], and is usually

associated with high Peclet numbers, which relates convection and diffusion phenomena and is influenced by the solvent evaporation rate [16]. Nevertheless, wrinkle-shaped albumin particles showed better performance in aerosolization studies due to its lower contact area that decreases the powder cohesiveness [39].

3.2. *Characterization of Gel-CPx particles*

Ciprofloxacin (CPx)-loaded gelatin microparticles were produced by single-step spray-drying following the best experimental conditions determined in section 3.1. The gel crosslinking was performed in the vapor phase to improve particle stability, while diminishing possible cytotoxic effects associated with the crosslinking agent (GA) [19, 40]. Both uncross and crosslinked particles obtained by spray-drying presented a wrinkled surface morphology (figure 5 a – d) like the ones observed in the orthogonal experiments (figure 3). Overall, the presence of CPx induced a narrower particle size distribution (figure 5 e - f), presenting a higher impact on the particle size rather than the crosslinking itself. Statistical differences were found between uncross and crosslinked samples only for 0 and 1 % wt CPx. It was reported that the use of GA as a crosslinking agent for gelatin, did not changed significantly the sample fiber diameter [41, 42]. Furthermore, the moisture content for the CPx-loaded formulations (figure S2) was comparable to the drug free ones.

Average diameters for CPx-loaded particles ranged from $2.73 \pm 0.84\,\mu m$ to $2.93 \pm 0.86\,\mu m$ for the uncrosslinked samples, and from $2.61 \pm 0.70\,\mu m$ to $2.79 \pm 0.80\,\mu m$ for the crosslinked ones. The crosslinking also lowered coefficient of variation, except for the highest CPx concentration.

Considering the optimal particle size for the lung administration of small molecules, the obtained particle size shows potential to target the alveolar region. It was reported that particles in the narrow window of 1 – 5 μm, behave more like gas molecules and follow airflow all the way to the alveoli by a number of mechanisms, including diffusion, sedimentation, and electrostatic effects [43]. Particles in the range size of 1 – 3 μm are in peak of alveolar deposition [6, 44-46].

CPx loading was carried out according to the procedure detailed in the experimental section, and the efficiency achieved was above 94 % (Table 3). Spray-drying of an aqueous solution of gelatin proved to be suitable to incorporate CPx, and thus, avoiding the incorporation of any other organic solvents in the mixture, being also environmentally friendly.

The infrared spectrum of pure CPx, gel and gel-CPx (5 %wt.) particles is depicted in figure 6. The protein characteristic absorption bands were observed both in gel and gel-CPx particles at approximately 1650 cm^{-1} (amide I), 1540 cm^{-1} (amide II) and 1240 cm^{-1} (amide III), corresponding to the stretching vibrations of C=O bond, to the coupling of bending of N-H bond and stretching of C-N bonds, and to the stretching of C-N bonds plus the in-phase bending of N-H bonds, respectively [47, 48]. It was possible to observe the CPx absorption band at 1284 cm^{-1}, assigned to the C-N stretching [49], in the microparticles FTIR spectra, confirming its presence in the drug-loaded particles. Moreover, it can be observed that the particle processing does not suppress totally any absorption modes nor new vibrational modes seem to appear suggesting the absence of covalent bonds between gelatin and the drug.

3.3. Thermal analysis

DSC curves of microparticles and free CPx are reported in figure 7a. CPx showed to be stable within the range of the processing temperatures. DSC data recorded for the pristine gelatin and gelatin loaded with CPx, showed similar trends, suggesting that the spray dry process does not influence the polymer thermal properties. A broad endothermic peak at lower temperatures, between 30 – 120 ºC was attribute to the volatilization of absorbed and unbonded water present in the gelatin particles (figure 7a). The presence of water molecules in the particles is probably due to the storage and handling of the sample, at room conditions. Gelatin is a hygroscopic protein, and can trap up to 12 wt% of water from moisture [50, 51]. The characteristic helix-coil transformation of gelatin was detected at ~190 ºC [52]. Moreover, the enthalpy of the helix -coil decrease from 15.9 J g^{-1} for the pristine gelatin samples, down to 1.9 J g^{-1} for the sample loaded with 5 wt% of drug. These results suggest that the presence of CPx alters the formation of the characteristic helical structure, probably disrupting the hydrogen bonds between the polymer chains, by creating new hydrogen interactions between hydroxyl groups and amides present in both, drug and in the polymer chains [53]. Peña et al. reported that the incorporation of tannin in gelatin films, led to a decrease of the enthalpy of the helix-coil structure, from 15 J.g^{-1}, calculated for pristine gelatin films, down to 3 J g^{-1}, for a sample with 30 wt% tannin [53]. This behavior was attributed to the tannin ability to modify the hydrogen bonding between the protein chains, and creation of new hydrogen interactions between the tannin hydroxyl groups and gelatin polar groups.

Thermogravimetric analysis was performed to the prepared unloaded and loaded gelatin particles. Two different weight loss steps can be distinguished in figure 7b. The first one, between room temperature and 120 ºC, attributed to the loss of water

absorbed during sample storage and handling [50, 53]. The second degradation step, that occurred between 250 – 400 ºC, was attributed to the protein helix structure chain breakage, and peptide bonds rupture [53, 54]. The residual weight of the protein (≈35 wt%) agrees with the values reported in literature [54, 55].

3.4. In vitro release studies

The cumulative *in-vitro* release of CPx carried out at physiological pH is depicted on figure 8a. All tested formulations presented a similar release profile where the complete release was achieved within 24 hours. The release profiles of the gel-CPx microparticles with different amounts of drug showed a burst release within the first 6 hours, followed by a slower release rate (figure 8a).

Gelatin particles can be seen as a polymeric network swollen with solvent, where the water fills the space between the strands of the polymer chains [56, 57], and act as permeation channels. When the drug loaded particles are immersed in the release media, the polymer is capable to uptake the water based media, followed by relaxation of the macromolecular chains and volume expansion, and the CPx drug can diffuse to the external receptor medium from within the gelatin microparticle. The suggested mechanism for the CPx release is presented in figure 8b.

The obtained release profiles of the gelatin microparticles were in between of some gelatin systems reported in literature obtained via emulsion and desolvation techniques. The developed system had a more sustained release over time than a paclitaxel-loaded system with 90 % release in 2 h [58], but a faster release compared with methotrexate-gelatin nanoparticles with a release profile over 150 h, depending

on drug-gelatin ratio, and amount of poly(methyl methacrylate) present in the oil phase, during the preparation of the emulsion [56].

The release profile was further investigated, and the experimental data was modelled to the first order, Higuchi and Korsmeyer-Peppas (KP) models, and the kinetic parameters are presented in table 4. The best fittings were obtained for first-order and KP models with coefficients of determination higher than 0.98.

Despite having similar profiles, the calculated release rate tends to increase with the increasing of drug loading in the system, an effect already reported [56]. According to KP model, a statistically significant increase in release rate was obtained between 1 wt.% and 2 wt.% formulations, which could be due to the high distance that the drug must travel from the core of the polymeric particle to the release medium, and consequently decreasing the release rate. However, no significance was obtained between those formulations and the 5 wt.% ones, being more similar to the 2 wt.%, which indicates that for contents above 2 wt.% the release rate is not influenced by spatial effects.

Berkland et al. [59] reported the effect of particle size (10 to 100 μm) on drug release kinetics but such effect cannot be attributed to this gel-CPx system since the mean diameter differences are almost negligible (figure 5 - f). Thus, differences in k values can be mainly addressed to geometrical factors, since particles having smaller drug loadings provide a larger path to the drug molecules to reach the release media (figure 8b).

Drug release from a polymeric system can be due to relaxation of the polymer chains over time, resulting in additive movement out of the polymer matrix; due to diffusion, where the drug moves out of the polymer matrix due to a concentration gradient

within the polymer, or degradation controlled, where part of the polymer dissolves in the release medium which causes the release of the drug present in that part of the polymer network [60, 61]. For the spray dried gelatin microparticles, drug release due to erosion of the polymer matrix is unlikely, as crosslinked gelatin does not dissolve under existent experimental conditions and its biodegradation starts at a time longer than that taken into consideration [50]. Thus, the mechanism of CPx release could be either diffusion or relaxation controlled, and can be elucidated by the value of the diffusion exponent (n) predicted by the KP model (equation 10). The value of n, summarized in table 4, suggests that a n value between 0.69 up to 0.75, and therefore, the release of CPx may be considered as an anomalous transport and indicate a superimposition of the two mechanisms - drug diffusion and polymer chain relaxation ($0.43 < n < 0.85$, for spherical systems) [32].

3.5. MIC and MBC determination for CPx-loaded particles

The bioactivity of released ciprofloxacin was investigated by determining the minimal inhibitory and bactericidal concentrations against susceptible strains of *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). The dose-dependent curves of bacterial growth (figure 9) and MIC/MBC values (table 5) showed similar growth profiles for pure or encapsulated CPx for both bacteria. Unloaded gel particles (uncross- and crosslinked with GA) did not affect bacterial growth, mitigating any possible influence of GA in the MIC values. MIC and MBC values determined against *S. aureus* and *E. coli* were comparable for pure or encapsulated drug. Experimental MICs were consistent with those reported in literature for ciprofloxacin against non-resistant strains of *S. aureus* [7, 62-64] and *E. coli*. [62-65]. These results confirm the biological function of

ciprofloxacin after processing by spray-drying and suggest that the process does not induce any adverse effects on the structure of ciprofloxacin.

4. Conclusions

This work reports a facile and fast method of producing gelatin microparticles loaded with ciprofloxacin, a poorly water soluble antibacterial agent, with great potential for *scale-up*. Gelatin-ciprofloxacin particles with mean diameter between 2 and 3 μm were developed by single step procedure, with loading efficiencies above 94% and an *in vitro* release profile over the first 6 hours. Loaded ciprofloxacin activity was demonstrated against *S. aureus* and *E. coli*, showing MIC and MBC values comparable to the pure CPx.

Spray-drying of protein-drug particle systems could be an advantageous alternative to emulsions, providing higher production rates, and given their features might have potential to lung administration of drugs.

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Figure Captions:

Figure 1 – Spray dry apparatus used in the production of the gelatin microparticles.

Figure 2 – a) Relationship between the average particle diameter (black) and coefficient of variation (red) and the factors and levels of the orthogonal experimental design, and b) Viscosity of gelatin solutions (0.5, 1 and 2 % w/v) as a function of shear rate at 25ºC; inset: viscosity dependence on gelatin concentration at a shear rate of 10 s^{-1}.

Figure 3 – Morphology of gelatin particles obtained at experimental processing conditions from A to I analyzed by SEM.

Figure 4 – Particle (conditions A to I) diameter distribution obtained from, at least, 300 particles/sample using ImageJ software.

Figure 5 – Morphology of gelatin microparticles obtained by SEM analysis: (a) un-crosslinked gel-CPx 0 % wt, (b) crosslinked gel-CPx 0 % wt., (c) un-crosslinked gel-CPx 5 % wt., (d) crosslinked gel-CPx 5 % wt. Size distributions (e) and average diameter (f) for un-crosslinked (dash) and crosslinked (line) gel-CPx formulations (0, 1, 2 and 5 %wt). Inset: coefficient of variation (C_V) for the formulations. * represents differences by comparison with each control (un-crosslinked) and § represents comparisons between formulations ($^{*}p< 0.05$; $^{****}p<0.0001$; $^{§§§§}p <0.0001$; ns: non-significant).

Figure 6 – Fourier transformed infrared spectra of pure ciprofloxacin (CPx) and gelatin particles with 0 and 5 wt% of CPx.

Figure 7 – Thermal analysis of pure CPx and gel-CPx particles: (a) differential scanning calorimetry and (b) thermal degradation profile of recorded at 20 °C.min^{-1}.

Figure 8 – a) *In vitro* release of ciprofloxacin (CPx) from gel-CPx formulations with different loadings. Experiments were performed in triplicates in PBS buffer (pH 7.4) at 37 $\pm$ 0.5 ℃; b) proposed release mechanism for CPx.

Figure 9 – MIC measurements for pure ciprofloxacin (CPx) and gel-CPx formulations against *S. aureus* and *E. coli*. Unloaded gelatin particles uncross (gel-uncross) and crosslinked with glutaraldehyde (gel-cross) were used as controls.

Table Captions:

Table 1 – Factors (A, B and C), levels (1, 2 and 3) and experimental conditions of Orthogonal experimental design $L_9(3)^4$ for producing gelatin microparticles by spray-drying.

Table 2 –Diameter and coefficient of variation (CV) of gelatin particles and orthogonal indexes calculated from particles diameter.

Table 3 – Ciprofloxacin loading (%) in gelatin particles calculated by UV-VIS analysis.

Table 4 – Kinetic parameters for first order, Higuchi and Korsmeyer-Peppas models determined from fitting of release experiments for the three formulations of gel-CPx particles.

Table 5 – Comparison of MIC and MBC values for pure ciprofloxacin and gel-CPx formulations against *S. aureus* and *E. coli* using broth microdilution test. Results are expressed as mean standard deviation of three independent experiments.

Table 1

	Factors		
Levels	**A (% w/v)**	**B (ml h^{-1})**	**C (ºC)**
1	0.5	90	115
2	1	177	130
3	2	308	140
Experiment		**Levels**	
A	1	1	1
B	1	2	2
C	1	3	3
D	2	2	1
E	2	1	3
F	2	3	2
G	3	1	2
H	3	2	3
I	3	3	1

Table 2

Experimental runs	**Diameter ± SD (µm)**	**C_v**
A	2.63 ± 0.71	0.27
B	2.76 ± 0.75	0.27
C	2.62 ± 0.78	0.30
D	2.80 ± 1.09	0.39
E	3.14 ± 1.02	0.32
F	2.83 ± 1.05	0.37
G	3.65 ± 1.17	0.32
H	3.57 ± 1.33	0.37
I	3.25 ± 1.32	0.40

Indexes / Factors	**A (Concentration)**	**B (Feed rate)**	**C (Inlet temperature)**
K_1	2.668	3.139	2.894
K_2	2.925	3.045	3.081
K_3	3.492	2.901	3.110
Range R	0.824	0.237	0.217
Order		$A > B > C$	
Optimal combination		$A_1B_3C_1$	

Table 3

Samples	**Drug loading (%)**	**Efficiency (%)**
Gel-CPx 1 wt.%	1.0±0.03	100
Gel-CPx 2 wt.%	1.95±0.37	97.5
Gel-CPx 5 wt.%	4.73±0.44	94.6

Table 4

Formulations	First Order		Higuchi		Korsmeyer-Peppas[a]		
	k	R^2	k_H	R^2	k	n	R^2
1%	0.25±0.07*	0.986	33.99±3.69ns	0.984	35.00±4.35*	0.69±0.04	0.999
2%	0.49±0.01ns	0.986	43.46±5.12ns	0.944	52.61±7.76*	0.75±0.06	0.998
5%	0.54±0.07*	0.998	40.14±0.62ns	0.963	50.16±3.12ns	0.70±0.01	0.993

[a] calculation of kinetic parameters considered values below 60% release, following model assumptions [32]

* denotes statistically differences between marked formulations ($p < 0.05$)

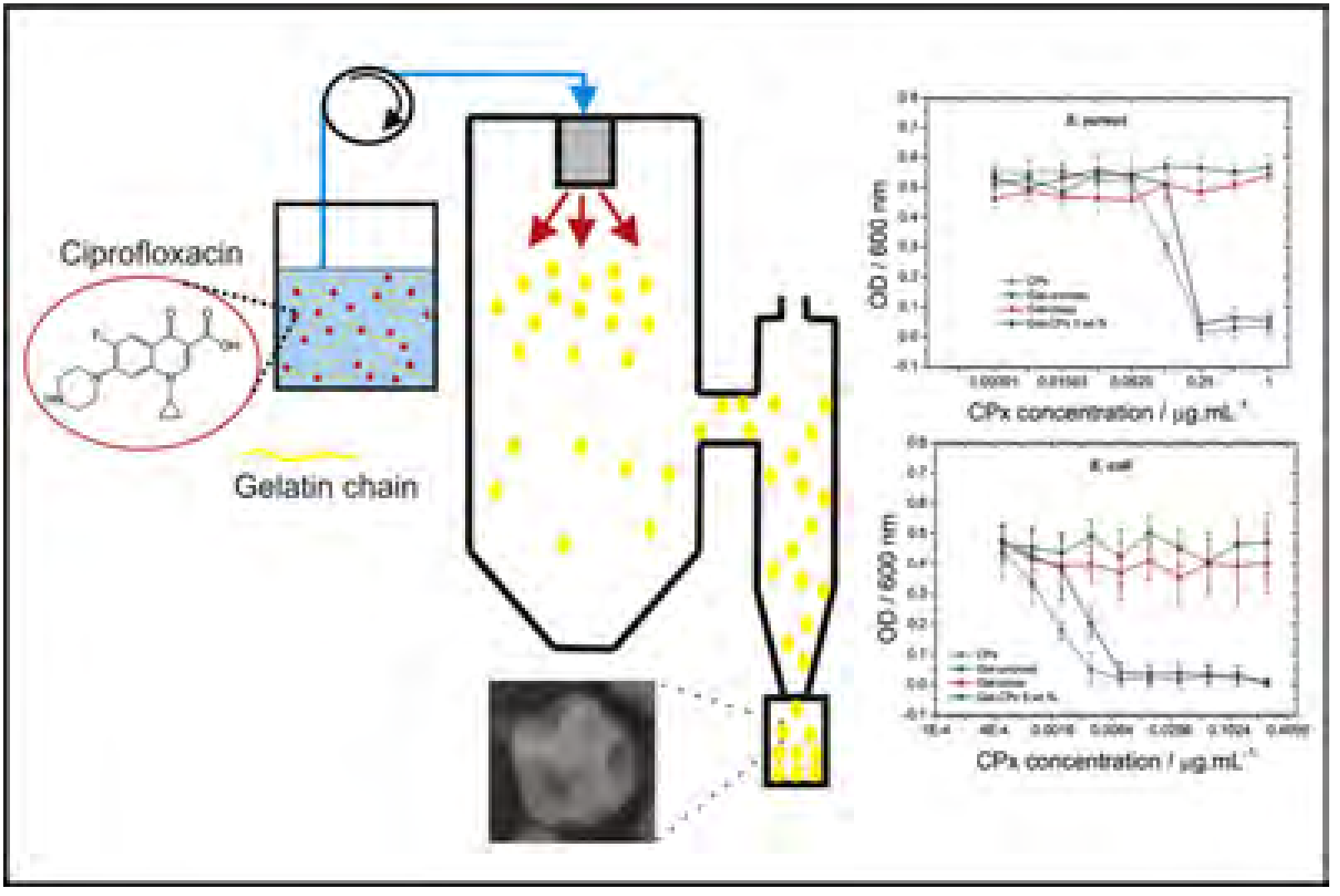
ns: non-significant

Table 5

	MIC ($\mu g/ml$)		MBC ($\mu g/ml$)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
Pure CPx	0.25	0.008	0.667$\pm$0.236	0.011$\pm$0.004
Gel-CPx	0.25	0.008	0.417$\pm$0.118	0.036$\pm$0.019

Highlights

- A single step method to produce gelatin-ciprofloxacin microparticles is presented.
- Spray-drying led to microparticles with suitable diameters for lung administration.
- 80 % of the encapsulated drug is released over 6 h, with MIC like the free drug.
- This scalable method shows potential for dry systems in pharmaceutical industry.



Graphics Abstract

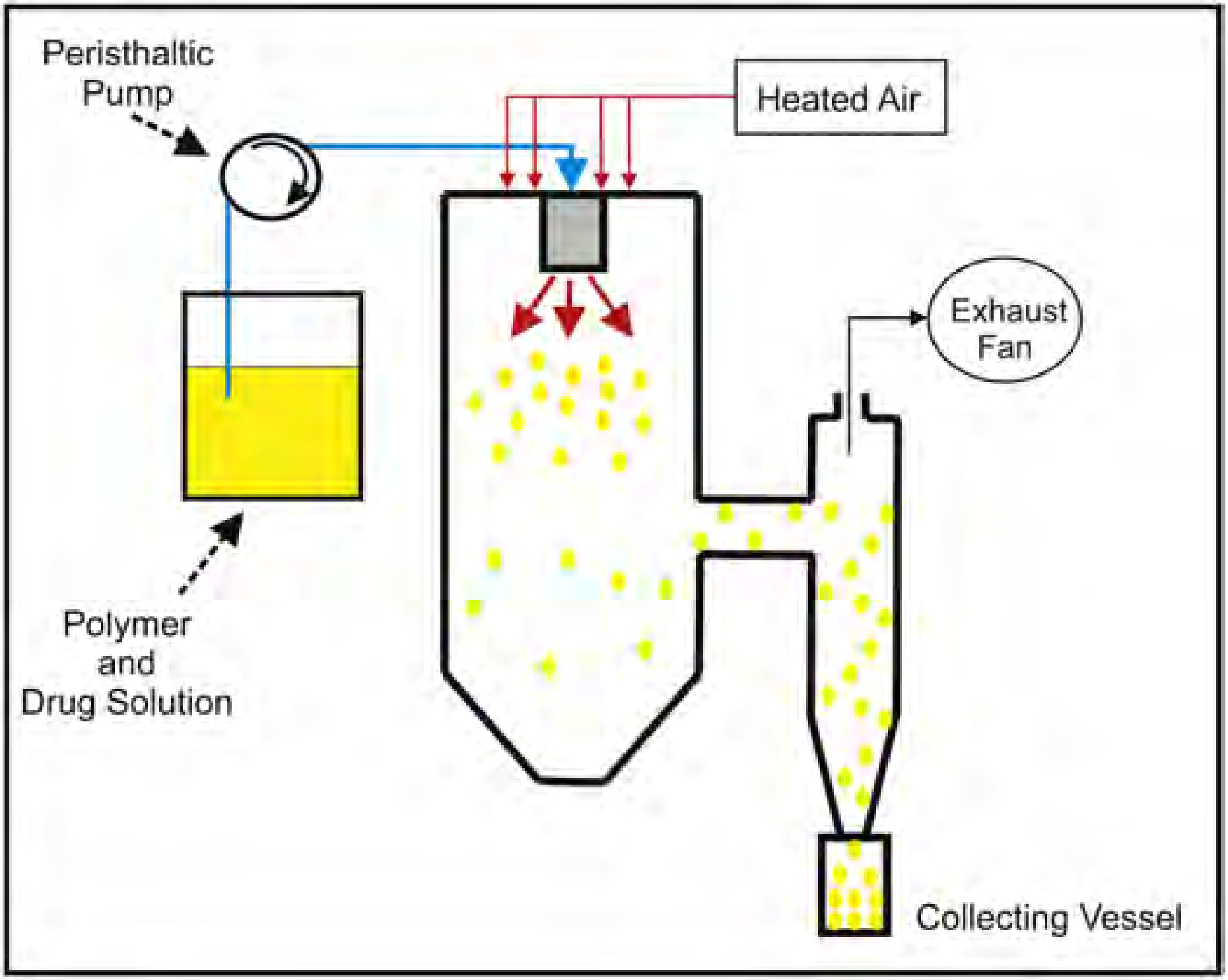

Peristhaltic
Pump
Heated Air
Exhaust
Fan
Polymer
and
Drug Solution
Collecting Vessel

Figure 1

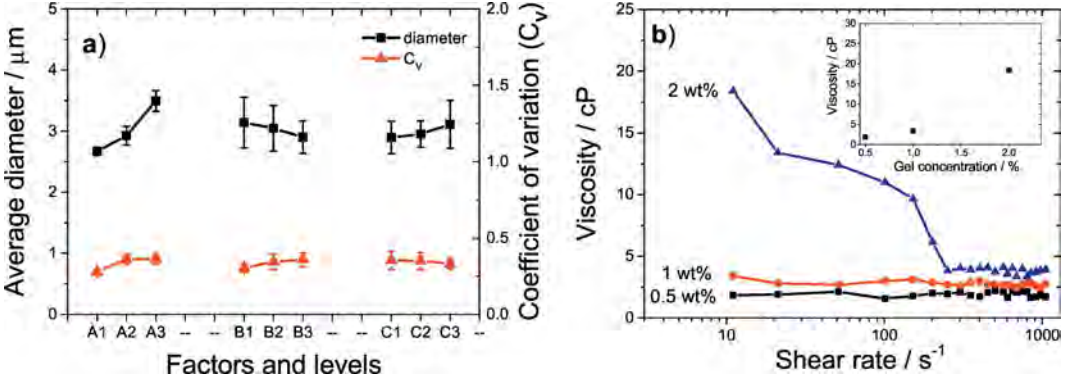

a)
diameter
C_v
Average diameter / μm
Coefficient of variation (C_v)
A1 A2 A3 -- -- B1 B2 B3 -- -- C1 C2 C3 --
Factors and levels
b)
2 wt%
1 wt%
0.5 wt%
Viscosity / cP
Shear rate / s^{-1}
Viscosity / cP
Gel concentration / %

Figure 2

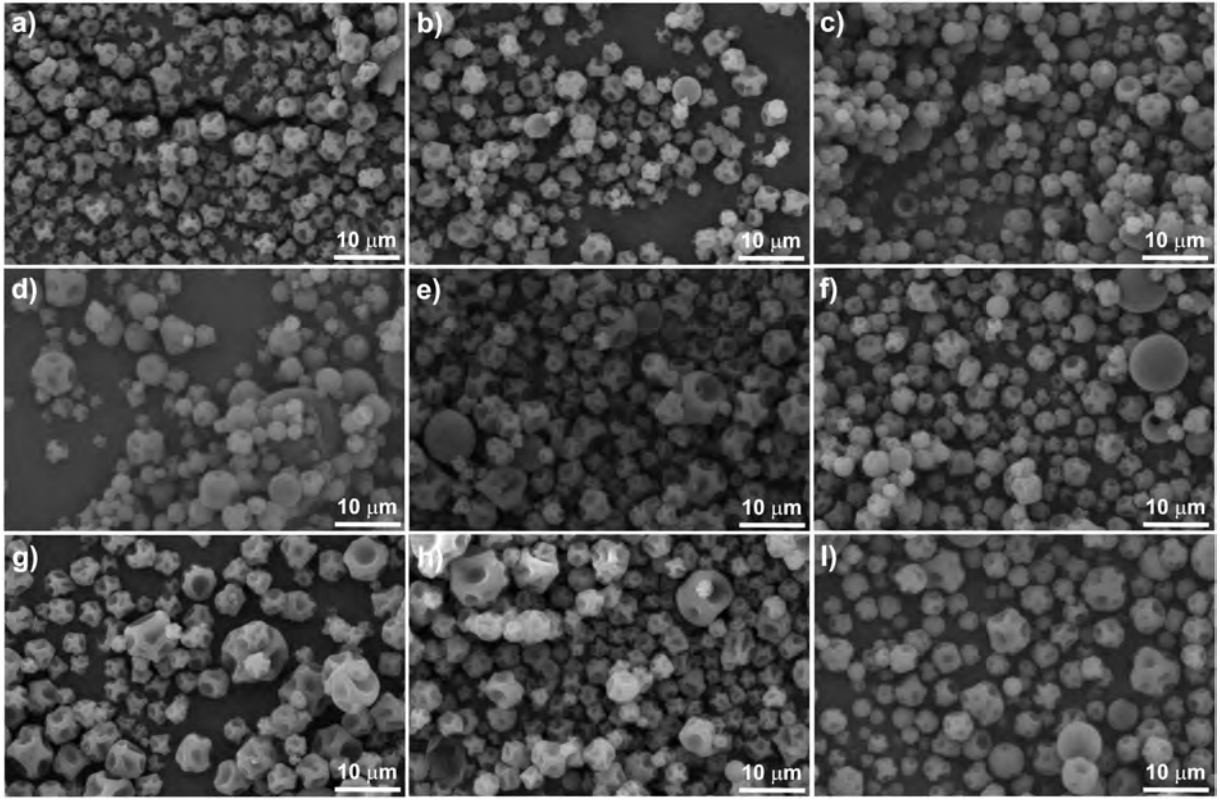


Figure 3

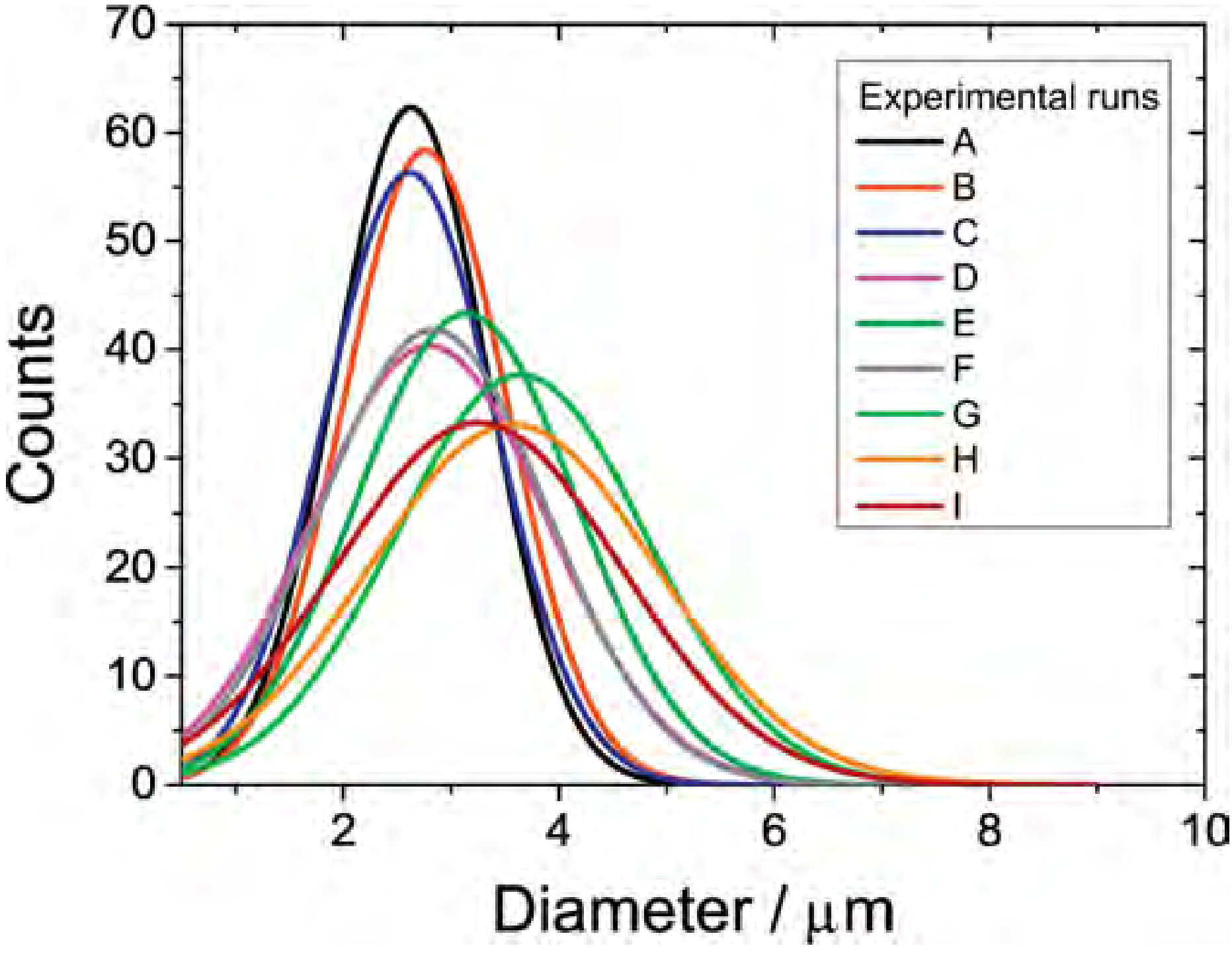

Counts
Diameter / μm
Experimental runs
A
B
C
D
E
F
G
H
I
0
10
20
30
40
50
60
70
2
4
6
8
10

Figure 4

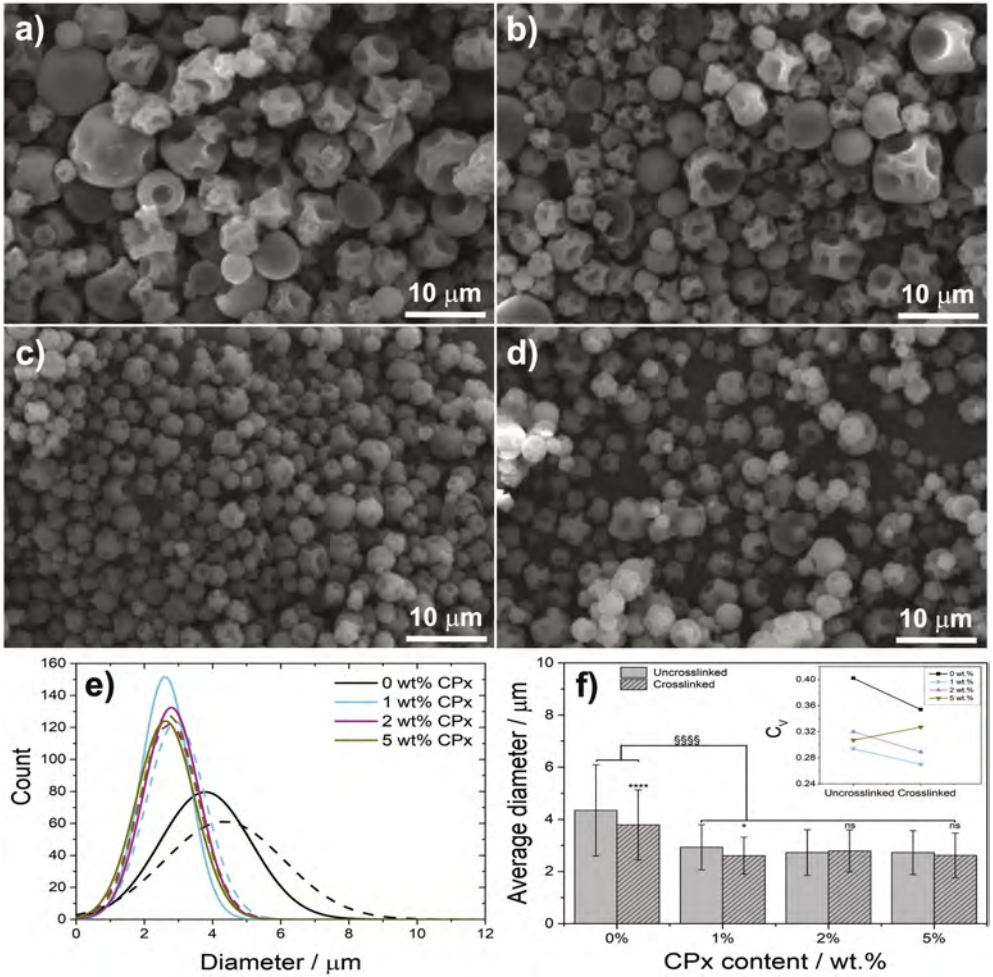

a)
10 μm
b)
10 μm
c)
10 μm
d)
10 μm
e)
0 wt% CPx
1 wt% CPx
2 wt% CPx
5 wt% CPx
Count
Diameter / μm
f)
Uncrosslinked
Crosslinked
§§§§
****
*
ns
ns
Average diameter / μm
CPx content / wt.%
0%
1%
2%
5%
C_V
Uncrosslinked Crosslinked

Figure 5

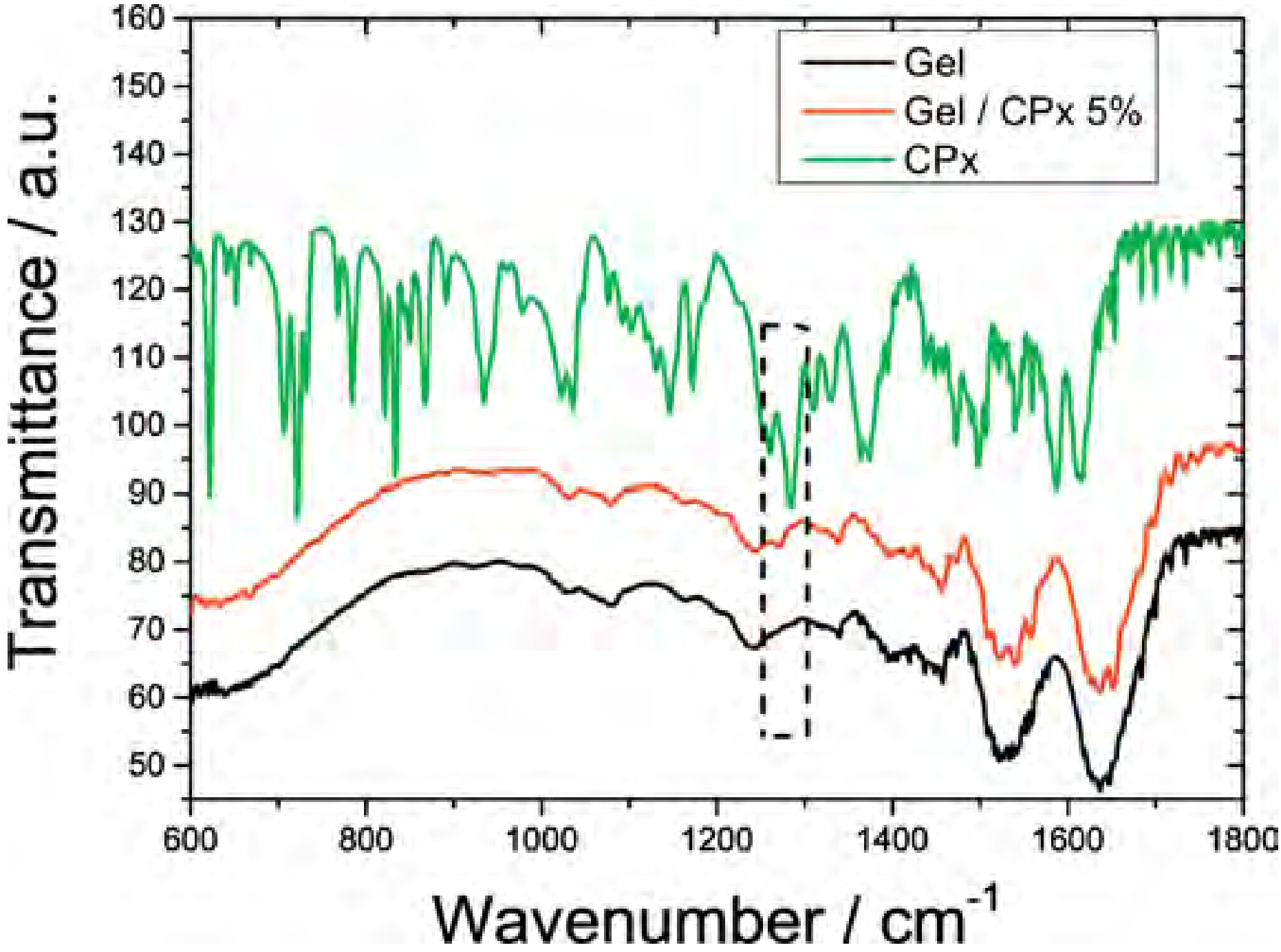

Gel
Gel / CPx 5%
CPx
Transmittance / a.u.
Wavenumber / cm-1
160
150
140
130
120
110
100
90
80
70
60
50
600
800
1000
1200
1400
1600
1800

Figure 6

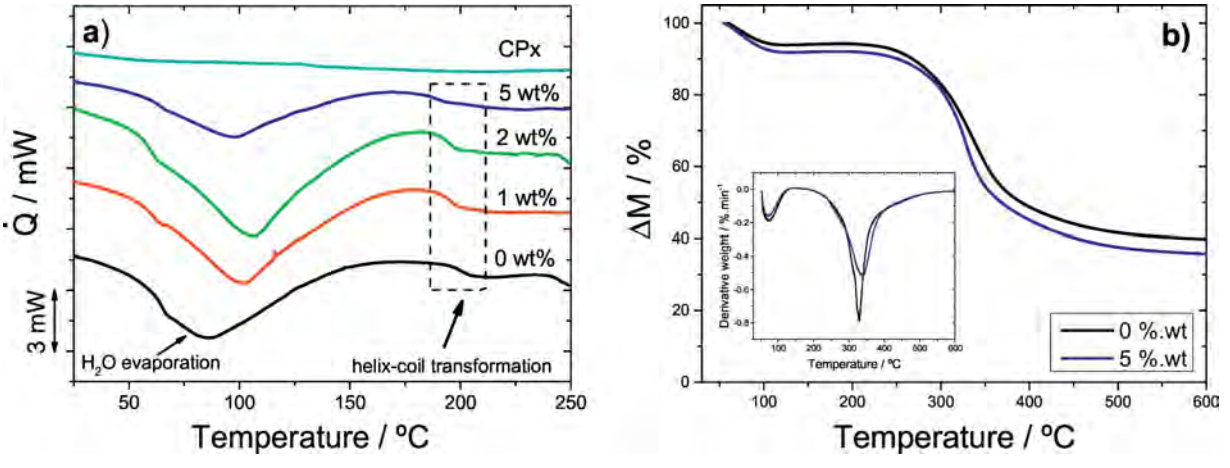

a)
CPx
5 wt%
2 wt%
1 wt%
0 wt%
Q̇ / mW
3 mW
H2O evaporation
helix-coil transformation
50
100
150
200
250
Temperature / ºC
b)
ΔM / %
100
80
60
40
20
0
Derivative weight / % min-1
0.0
-0.2
-0.4
-0.6
-0.8
100
200
300
400
500
600
Temperature / ºC
0 %.wt
5 %.wt
100
200
300
400
500
600
Temperature / ºC

Figure 7

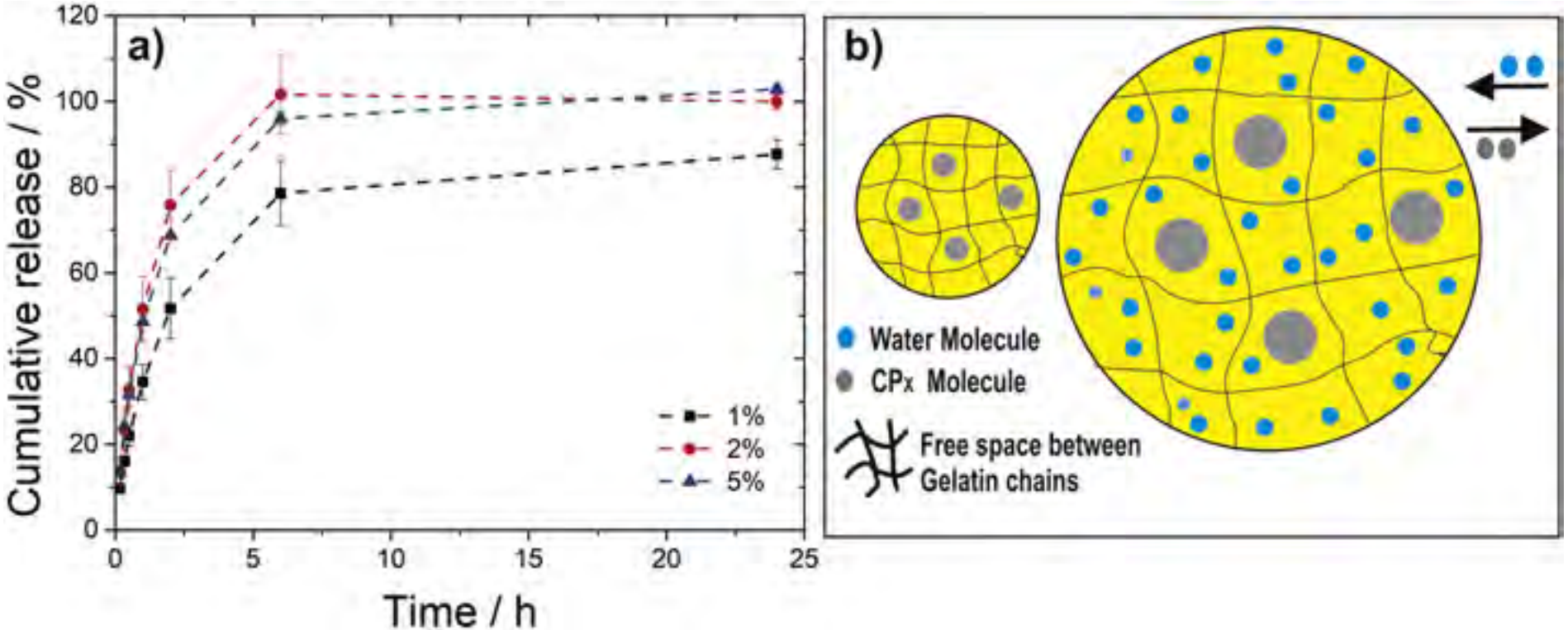
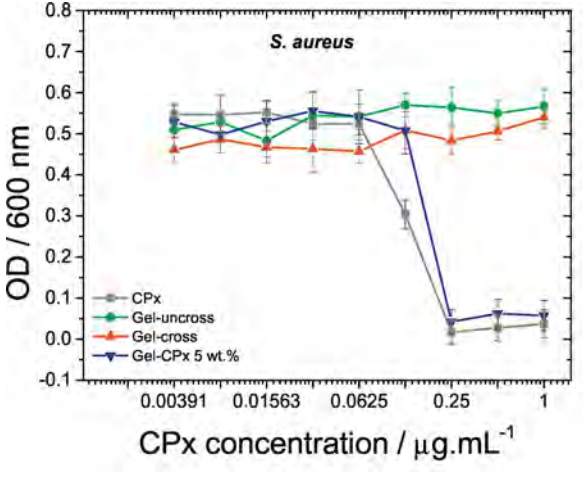

a)
Cumulative release / %
Time / h
0
20
40
60
80
100
120
0
5
10
15
20
25
1%
2%
5%
b)
Water Molecule
CPx Molecule
Free space between
Gelatin chains

Figure 8


S. aureus
OD / 600 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
CPx
Gel-uncross
Gel-cross
Gel-CPx 5 wt.%
0.00391
0.01563
0.0625
0.25
1
CPx concentration / µg.mL-1

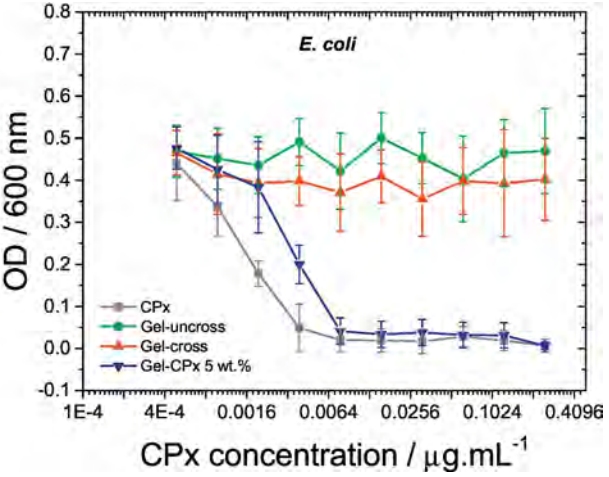

E. coli
OD / 600 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
CPx
Gel-uncross
Gel-cross
Gel-CPx 5 wt.%
1E-4
4E-4
0.0016
0.0064
0.0256
0.1024
0.4096
CPx concentration / µg.mL-1

Figure 9