Towards a better understanding of solid dispersions in aqueous environment by a fluorescence quenching approach

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Abstract

Solid dispersions (SDs) represent an important formulation technique to achieve supersaturation in gastro-intestinal fluids and to enhance absorption of poorly water-soluble drugs. Extensive research was leading to a rather good understanding of SDs in the dry state, whereas the complex interactions in aqueous medium are still challenging to analyze. This paper introduces a fluorescence quenching approach together with size-exclusion chromatography to study drug and polymer interactions that emerge from SDs release testing in aqueous colloidal phase. Celecoxib was used as a model drug as it is poorly water-soluble and also exhibits native fluorescence so that quenching experiments were enabled. Different pharmaceutical polymers were evaluated by the (modified) Stern-Volmer model, which was complemented by further bulk analytics. Drug accessibility by the quencher and its affinity to celecoxib were studied in physical mixtures as well as with in SDs. The obtained differences enabled important molecular insights into the different formulations. Knowledge of relevant drug-polymer interactions and the amount of drug embedded into polymer aggregates in the aqueous phase is of high relevance for understanding of SD performance. The novel fluorescence quenching approach is highly promising for future research and it can provide guidance in early formulation development.

1. Introduction

Solid dispersion (SD) is a widely employed approach to orally deliver poorly water- soluble drugs. The compound is mostly formulated in an amorphous high-energy state, which should be kinetically stabilized throughout the targeted shelf-life of the product. Especially critical for poorly soluble compounds is dispersion in aqueous medium, which comes naturally with the oral route of administration and bears a risk of drug crystallization from the amorphous state (Newmann, 2015). To fully benefit from SD formulations, physical instability must be therefore hindered, for example, by using polymers (Baghel et al., 2016; Chiou and Riegelman, 1971a, 1971b; Leuner and Dressman, 2000; Serajuddin, 1999; Serajuddln, 1999). The results of most studies indicate that polymers decrease the crystallization tendency of an amorphous drug due to a reduction of molecular mobility (Taylor and Zografi, 1997), as well as by breaking of the interconnections between drug molecules and the formation of specific drug-polymer interaction (Khougaz and Clas, 2000; Tantishaiyakul et al., 1996). These molecular interactions and their biopharmaceutical consequences are of major interest within the field of SDs. A majority of research focuses on drug-polymer interactions in the dry bulk state employing Fourier transform infrared (FT-IR), Raman spectroscopy, differential scanning calorimetry (DSC), X-ray powder diffraction (XRPD) and solid state NMR spectroscopy (Masuda et al., 2012; Matsumoto and Zografi, 1999; Newmann, 2015). Among the different characterization techniques to study the dry state of SDs, transmission electron microscopy was also deemed as highly relevant (Marsac et al., 2010),(Ricarte et al., 2015),(Ricarte et al., 2016),(Deng et al., 2008).

It seems more complex to understand and study drug excipient interactions upon aqueous dispersion because there is often a complex phase separation involved. Indeed, previous studies reported that in contact with an aqueous solution simulating the gastro-intestinal media, SDs

rapidly disperse and thereby provide a broad range of drug and excipient assemblies (Frank et al., 2014, 2012b; Friesen et al., 2008; Harmon et al., 2016; Taylor and Zhang, 2016). Release from these particles and colloids provide the free drug concentration that is the true supersaturation driving absorption (Friesen et al., 2008) (Frank et al., 2012a). The above investigations were conducted not only in simulated intestinal medium but also in mere buffer systems because simulated intestinal media make the interpretation more difficult due to the various colloidal states present even without dispersing ASDs (Tho et al., 2010). Already aqueous solution of lipophilic drug alone can exhibit complex behavior where a critical transition leads to a drug-rich and water-rich phase, which is known as a liquid-liquid phase separation (LLPS) (Ilevbare and Taylor, 2013; Sun et al., 2016; Taylor and Zhang, 2016). In this context, different authors (Mosquera-Giraldo and Taylor, 2015; Raina et al., 2014; Taylor and Zhang, 2016; Trasi and Taylor, 2015), have employed fluorescence probes as marker for the polarity of the molecular environment. This study is one of few reports on fluorescence as a tool in SD analysis. Solid drug particles were differentiated from a liquid and drug rich phase. As shown by Tho *et al*., nano- and micro-sized solid particles were formed (isolated and analyzed by X-Ray) on dispersion of SDs in buffer media (Tho et al., 2010). Moreover, Frank *et al*. reported a phase separation phenomenon during the dissolution of a commercial SD, including the formation of solid amorphous particles, which were isolated, dried, and analysed by XRPD.

Given the wide range of established applications of fluorescence in the life sciences, it is rather surprising that fluorescence methods have not been more harnessed in pharmaceutical analysis of SDs. A notable exception is the very recent work on fluorescence lifetime and steady-state fluorescence spectra measurements, which were successfully employed to differentiate and characterize phase transformations in supersaturated aqueous solutions of poorly water-soluble

drug (Tres et al., 2017). Interesting is in the context of fluorescence analysis of poorly soluble compounds also another work that employed pyrene to elucidate a drug dissolution enhancement effect of stevia-G (Uchiyama et al., 2011). Moreover, a work on fluorescence resonance energy transfer (FRET) is noteworthy, which aimed at differentiation of compound distribution in SD as either in the form of molecular dispersion or as larger amorphous clusters (Van Drooge et al., 2006). Fluorescence analysis is highly sensitive and can provide valuable information of a probe molecule regarding its immediate environment (*i.e.* polar molecules in polar solvents), rotational diffusion, distances between the sites on biomolecules, conformational changes, and binding interactions. It seems that fluorescence analysis could be further exploited in the field of solid dispersions and it may particularly help with the scientific challenges of analyzing the formulations on release in aqueous media.

In aqueous dispersion, the evolving complex multiphase systems of SDs are inherently difficult to study. There are different approaches reported in the literature to study release from SDs (Meng et al., 2015), but no single technique alone appears to be sufficient to characterize both the solid particles as well as the aqueous colloidal phase that is formed during release. The evolving phases from SDs could therefore be analyzed separately using complementary analytical approaches. This work reports on a fluorescence analysis to assess the drug-polymer interactions in the aqueous colloidal phase on drug release. In particular, we introduce a method based on fluorescence quenching and size-exclusion chromatography to investigate such systems. Celecoxib (CX), a native-fluorescent poorly soluble compound was studied in physical mixtures with various polymers (at different concentrations) as well as with SDs where the 1:1 CX: polymer ratio was selected in order to have a high drug loading. The combined analysis of the (modified) Stern-Volmer plots and size-exclusion chromatography enabled unique insight

into how the selection of polymer affected the accessibility of drug by the quencher as well collisional affinity in the aqueous colloidal phase. Such information is highly attractive to learn about the molecular interactions of drug with formulation components that take place during the dissolution in the aqueous colloidal phase.

2. Materials and methods

2.1. Materials

Celecoxib (CX) was purchased from AK scientific, Inc. (USA), hydroxypropyl methyl cellulose acetate succinate, L grade (HPMCAS-LG) was obtained from Shin-Etsu AQUOT, polyvinylpyrrolidone vinyl acetate (PVP VA64) and Soluplus® were purchased from BASF, Poloxamer 188 and potassium iodide (KI) were purchased from Sigma Aldrich. PD MidiTrap G-25 M was purchased from GE healthcare life science. All solutions were prepared using Mill-Q water (18.2 MΩ cm^{-1}).

2.2. Methods

2.2.1. Preparation of solid dispersions and physical mixtures

SDs were prepared by using a solvent evaporation method as described in literature (Chiou and Riegelman, 1969). Briefly, CX and polymer were taken in ratio of 50:50 w/w and dissolved in an adequate amount of methanol. The solvent was then rapidly evaporated under reduced pressure using a mild heating bath (up to about 50 °C) to form a uniform solid mass. The co-precipitate was crushed and desiccated under vacuum for 24 h, then pulverized and vacuum desiccated again for a day. In case of the physical mixtures, CX and the different polymers were mixed in a

ratio of 90:10, 80:20, 70:30, 50:50 and 30:70 (w/w) by trituration with a pestle-mortar, and were then stored in a desiccated environment. CX based SDs and physical mixtures were prepared using HPMCAS-LG, PVP VA64, Poloxamer 188 and Soluplus.

2.2.2. Powder x-ray diffraction (XRPD)

Powder X-ray diffraction was used to characterize the solid form of the physical mixtures and of SDs at ambient temperature using a Bruker D2 PHASER (Bruker AXS GmbH, Germany) with a PSD-50 M detector and EVA application software version 6. Samples were prepared by spreading powder samples on PMMA specimen holder rings from Bruker. Measurements were performed with a Co Kα radiation source at 30 kV voltage, 10 mA current and were scanned from 10–35 2θ, with 2θ being the scattering angle at a scanning speed of 2θ/min.

2.2.3. Differential scanning calorimetry (DSC)

A DSC 4000 System, from PerkinElmer (Baesweiler, Germany) was calibrated for temperature and enthalpy using indium. Nitrogen was used as the protective gas (20 mL/min). Samples (approximately 5 mg) were placed in 40 µL aluminium pans with pierced aluminium lids. The midpoint glass transition temperatures (T_g), was determinate by a single-segment heating ramp of 5 °C/min from 25 °C to a maximum temperature of 200 °C. All DSC measurements were carried out in triplicate.

2.2.4. Dynamic light scattering (DLS) for particle sizing

The size of the obtained aggregates was measured with NanoLab 3D (LS instruments, Freiburg, Switzerland) equipped with a 45 mW at 685 nm, vertically polarized laser, having the detector at 180° with respect to the incident beam at 37 ± 0.1 °C. Disposable polystyrene cuvettes of 1 cm optical path length were rinsed several times (at least five) with the solutions to be analyzed and finally filled with the same solution under laminar flow hood to avoid dust contamination. At least three independent samples were taken, each of which was measured 10 times. Measurements were done in auto correlation mode and the obtained values are reported as an average ± standard deviation (STDV). Each measurement had a duration of 30 seconds with the laser intensity set on 100%. For the fitting of the correlation function, third order cumulant fits were performed with the first channel index and the decay factor being 15 and 0.7 and analyzed according to the cumulant method (Frisken, 2001).

2.2.5. Diffusing wave spectroscopy (DWS)

DWS RheoLab (LS Instruments AG, Fribourg, Switzerland) was used as optical technique for microrheological measurements as reported previously (Reufer et al., 2014). The theory of DWS-based microrheology was already explained in detail in our previous work (Niederquell et al., 2012). The DWS was calibrated prior to each measurement with a suspension of polystyrene particles, PS, (Magsphere Inc., U.S.A) in purified water (10 wt. %). The PS particles have a mean size of 250± 25 nm with a solid content of 0.5 wt. % in dispersion. This suspension was filled in cuvettes with a thickness L of 5 mm prior to measuring for 60 s at 25 ˚C. The value of the transport mean free path, l^* (microns) was determined experimentally as reported previously (Negrini et al., 2017). The transmission count rate was measured several times until a constant value was reached and the cuvette length, L, was considerably larger than the obtained values for

l^* ($L \gg l$) ensuring diffusive transport of light. The transport mean free path of the sample l^* is needed for the determination of the correlation intensity function and thus for the microrheological characterization. Viscosity measurements were performed on 0.5 mg/mL HPMCAS-LG, PVP VA64, Soluplus and Poloxamer 188 solutions in PBS at pH 6.5. Thus, 0.5 wt. % polystyrene (PS) nanoparticles were added to the clear samples to ensure the correct regime (guarantee a L/l^* ratio larger than 7) (Reufer et al., 2014). 5 mm quartz cuvettes were employed and data acquired for 60 s and each sample was measured 5 times. The viscosity measurement of polymer solutions was determined in a broad frequency range by DWS, where an average reference viscosity (expressed as G''/frequency) at high frequencies (from 100000 to 150000 rad/s) is reported in table S1.

2.2.6. Preparation of aqueous colloidal phase

10 mL of PBS at pH 6.5 were added to 10 mg of freshly prepared SD or physical mixture of CX and different amounts of polymers. The obtained mixtures were kept under stirring (400 rpm) at 37 °C for 4 hours in the dark. The time period was arbitrarily selected to represent a pseudo-equilibrium that is of physiological relevance for the absorption process. The aqueous phase of the dispersed samples containing the solubilized CX and polymer was then collected as supernatant (called here aqueous colloidal phase, ACP) and separated from the above mentioned mixtures. Subsequently, an aliquot from the ACP was taken out and used for further experiments. The amount of solubilized drug and its concentration in the ACP (CX concentration in aqueous phase) was based on high- performance liquid chromatography (HPLC) and calculated by using a calibration curve (both HPLC method and calibration curve are shown in SI). Measurements were carried out in triplicate and the results are shown (Table 1 to 5) as mean

± standard deviation (STDV). It has to be noted that such percentage values refer to the solubilized amount of drug in the aqueous colloidal phase (in the pseudo-equilibrium after 4h), while the residual part of the total drug amount was unreleased in a solid phase.

2.2.7. Fluorescence quenching experiments

Fluorescence quenching experiments on the above mentioned ACP were performed using iodide (I^-) as collisional quencher. All fluorescence experiments were carried out at 25°C on solutions with optical densities smaller than 0.05 to minimize inner filter effects. Fluorescence quenching experiments were performed by adding small aliquots of 1 M KI (containing small amount of $Na_2S_2O_4$ to avoid the oxidation of the quencher) solution to the samples. Decrease of the CX fluorescence intensity was monitored at 380 nm by exciting at 250 nm using Greiner® UV-transparent microplates and a SpectraMax® M2 plate reader (Molecular devices, San Jose, CA, USA). Quenching of fluorescence is described by the Stern-Volmer equation and quenching data were presented as plots of F_0/F versus quencher concentration [KI], were F_0 and F are the fluorescence intensity in absence or in presence of the quencher, respectively (Lakowicz, 2006). The plot of F_0/F versus [KI] is expected to be linearly dependent upon the concentration of quencher and it yields an intercept of one on the y-axis and a slope equal to the Stern-Volmer quenching constant K_D (1/M × s) when the quenching process is dynamic. The K_D is given by $kq \times \tau_0$ where kq is the bimolecular quenching constant and τ_0 is the lifetime of the fluorophore in the absence of quencher. When the Stern-Volmer plots deviate from linearity toward the x-axis (i.e. downward curvature) a modified Stern-Volmer equation (Equation 1) was used to calculate the amount of accessible fraction (fa) and its affinity to the quencher (Ka, 1/M) (Lakowicz, 2006). A plot of F_0/F_0-F versus 1/ [KI] yields fa^{-1} as the intercept on the y-axis and $(fa \times \mathrm{Ka})^{-1}$ as

214 the slope. The K_D, *Ka* and *fa* values are the coefficient of the curves obtained from six point's
215 linear regression fitting and the coefficient of determinations, *i.e*. R-squared (R^2) of the fitting is
216 reported in Tables; the errors for each coefficient shown in tables represent their standard error
217 (SE) as obtained from the fits determined using Sigma Plot (Systat Software, Inc. San Jose, CA,
218 USA).
219
220 $$F_0/\Delta F = 1/(f_a \times K_a \times [\mathrm{KI}]) + 1/f_a \qquad \text{EQ.1}$$
221
222 ***2.2.8. Size exclusion chromatography***
223 0.5 mL of the ACP containing only the solubilized CX and polymer was filtered at 25°C through
224 a PD MidiTrap G-25 M, a Sephadex G-25 packed column. According to size exclusion
225 chromatography (SEC), small molecules (such as the free CX) that are able to enter into the resin
226 pores are retained longer in the column, while large molecules (such as aggregates) which are
227 bigger than the pore size are eluted firstly. Therefore, this technique enables to discriminate
228 between free dug and the dug embedded in to aggregates. The elution profile was retrieved
229 plotting either the value of the mean count rate (Kcps), obtained by DLS measurements or the
230 percentage of CX present in the fractions eluted from the column *vs* the elution volumes (mL).
231 The percentage of CX in the fractions (% CX) was evaluated according with equation 2.

232 $$\%\ \mathrm{CX} = (F_{fr}/F_{nf}) \times 100 \qquad \text{EQ. 2}$$

233 $$\%\ \mathrm{CX\ free} = 100 - \%\ \mathrm{CX\text{-}Polymer} \qquad \text{EQ. 3}$$

234 Where F_{fr} is the fluorescence intensity value of the fractions eluted from Sephadex filtration and
235 F_{nf} is the fluorescence intensity value before Sephadex filtration. The total percentage of CX

236 embedded in polymer aggregates (% CX-Polymer) is given by the sum of the percentage of CX
237 (% CX) present in the fractions where DLS shown presence of aggregates. On the other hand, the
238 percentage of free CX (% CX free) was calculated according to the equation 3. All experiments
239 were carried out consecutively (n = 3) at 25 °C, the % CX free is reported as mean ± standard
240 deviation.

241

242 ## 3. Results

243 ### *3.1. Bulk characterization of physical mixtures and solid dispersions*

244 Prepared SDs were analyzed by powder X-ray diffraction (PXRD) at 25°C to verify the
245 amorphous nature of the dispersions and the results were compared with those of the
246 corresponding physical mixtures. As shown in Figure 1A, CX based SDs manufactured with
247 HPMCAS-LG, PVP VA64, and Soluplus (at 50% (w/w) drug loading) were X-ray diffraction
248 amorphous. However, the SD prepared with Poloxamer 188 showed diffraction peaks and a
249 substantial crystallinity was verified in the physical mixtures as well as with pure drug. The SDs
250 were further characterized by DSC to confirm the physical state of drug in the matrix. As shown
251 in Figure 1B, the SD with HPMCAS-LG, PVP VA 64 and Soluplus display a single glass
252 transition temperature (T_g) and the absence of a drug melting temperature (T_m). On the other
253 hand, the SD based on Poloxamer 188 shows two different thermal events, one corresponding to
254 melting of the eutectic mixture and the second to the T_m values indicate suspended CX present in
255 the eutectic melt. As very different types of polymers were selected deliberately, it was expected
256 that not all SDs of CX would result in an entirely amorphous system.

257

258 ### *3.2. Characterization of drug-polymer interactions*

Fluorescence quenching experiments were employed to gain information about the molecular environment of the model drug CX in the aqueous colloidal phase. The study of accessibility of drug to the quencher was therefore of interest in the physical drug-polymer mixtures as well as in SD formulations.

The freshly prepared SDs or physical mixtures of CX with different amounts of polymers were added to PBS at pH 6.5 and kept under stirring at 37 °C for 4 hours in the dark. The duration was selected as physiologically-relevant time scale which typically allows a SD to reach a pseudo equilibrium. The obtained aqueous colloidal phase, containing the solubilized CX (CX concentration in aqueous phase) and polymers were used to study drug-polymer interactions that take place in the aqueous solution. Even though the study focused on this aqueous phase, one should keep in mind that this phase contained only a part of the dose since 4 h release (i.e. pseudo- equilibration) resulted in multiphase system in which some drug was either not released or it precipitated from supersaturation. Therefore, any given percentages in the fluorescence experiments are understood as relative to the drug amount solubilized in the aqueous phase. However, the exact amount of drug (CX concentration in aqueous phase) present in the aqueous colloidal phase (and the percentage of dose) used for quenching experiments were evaluated by HPLC and the results are shown in tables from 1 to 5.

Moreover, CX quenching is established to be dynamic and the fluorescence of the drug decreased linearly with the concentration of KI that is a commonly used collisional quencher (see SI, Figure S2).

As shown in Figure 2A and summarized in Table 1, the quenching of fluorescence is described by the Stern-Volmer equation in the cases of CX alone (black circles) and the physical mixtures with 10 (black upper triangles) and 20 (white diamonds) %, w/w of HPMCAS-LG. Thus,

fluorescence quenching data, presented as plots of F_0/F versus [KI], show a linear behavior. Already the presence of 10 or 20 w/w % polymer led to a decrease CX quenching as seen from decreased values of the quenching constant (K_D). Interestingly, addition of 30 w/w % polymer or more (see Figure 2B) resulted in Stern-Volmer plots that clearly deviated from linearity. Indeed, CX quenching decreases by an increasing amount of HPMCAS-LG from 30 (white upper triangles) to 70 (black down triangles) w/w %. As shown in Figure 2C and summarized in Table 1, a modified Stern-Volmer equation was used to calculate the amount of accessible fraction (*fa*) and its affinity to the quencher (*Ka*).

On the other hand, when in the physical mixture, HPMCAS-LG is replaced with PVP VA64, Soluplus, or, Poloxamer 188, the Stern-Volmer quenching plot did not deviate from linearity by a clear downward curvature (see SI, Figure S3) even not at highest polymer concentration (*i.e.* 70 w/w %). Similar as for HPMCAS-LG, was for PVP VA64 (Table 2) or Soluplus (Table 3) that a decrease of the quenching constant (K_D) was noted with added CX in physical mixtures. By contrast, Poloxamer 188 did not exhibit any changes in the quenching of CX and the obtained K_D values for different drug-polymer mixture ratios are comparable with the one for CX alone (Table 3).

Moving from the physical mixtures to SDs of drug and polymer revealed that except for the Poloxamer 188 based SD, the Stern-Volmer plots deviated from linearity (downward curvature) for all the other formulations (see SI, Figure S4). As summarized in Table 5, the HPMCAS-LG based SD shows the lowest value of *Ka* and *fa,* while using Soluplus in SD, the accessible fraction rises up close to 0.9. As already mentioned in the case of Poloxamer 188, the obtained K_D value (Table 5) was comparable with those obtained in the physical mixture and in the case of CX alone. This was different in the case of HPMCAS-LG (Figure 3) because the quenching

constant in the physical mixtures was higher than in the SD while *fa* was about the same. Differences between SD and physical mixture were found also in the case of PVP VA64. As shown in Figure 4, in the case of the physical mixture, the quenching of fluorescence is described by the Stern-Volmer equation. This plot in case of SD deviated from linearity and the modified Stern-Volmer equation (inset Figure 4) was used to calculate the amount of accessible fraction (*fa*) and its affinity to the quencher (*Ka*). The same behavior was also observed in case of Soluplus (see SI, Figure S5).

After the fluorescence quenching experiments, the ACP was filtered at 25°C through a Sephadex G-25 packed column (Figure 5) to quantify the amount of free dug (% CX free) as well as the drug embedded in polymer aggregates (% CX-Polymer). Drug percentages obtained in the size exclusion chromatography experiments are again understood as relative to solubilized compound in ACP, which holds only for a part of the dose.

As summarized in Table 6, the HPMCAS-LG based SD shows the lowest value of free drug, indicating that most of the drug is embedded in polymer aggregates. Moreover, the HPMCAS-LG aggregates, analyzed by DLS, were the biggest with respect to the SD prepared with the other polymers. By contrast, in the case of SD of Poloxamer 188, the aggregates were about three times smaller than in the SD using HPMCAS-LG and almost the entire compound was in the free drug fraction. It has to be noted that the values of accessible fraction (*fa* in Table 5) and the values of percentage of free CX (% CX free in Table 6) were comparable for all the investigated SDs.

4. Discussion

328 Formulations based on SD technology generally target enhanced dissolution and sustained
329 supersaturation of drug for optimal performance following oral administration (Baghel et al.,
330 2016). However, the aqueous formulation dispersion leads to phase changes and emergence of
331 different particle species from which drug release takes place. The mechanisms of how polymers
332 affect such drug release from SDs are still not thoroughly understood. Much current research is
333 directly toward individual mechanistic aspects, for example how polymers can sustain drug
334 supersaturation (Chauhan et al., 2013), (Usui et al., 1997), (Raghavan et al., 2001). Interesting is
335 further the mechanism that an enhanced dissolution rate was found to be partly due to the
336 stabilization of drug in nanosized particles formed by precipitation (Kanaujia et al.,
337 2011),(Alonzo et al., 2011). These different mechanisms of drug release provide a better
338 understanding of drug-polymer interactions in aqueous environment. To gain such insights into
339 the aqueous phase of SDs in a pseudo-equilibrium at a physiologically relevant time scale (4h)
340 was the primary objective of the present work.

341 Celecoxib (CX) a Biopharmaceutics Classification System (BCS) class II drug was selected as
342 model because it exhibits fluorescence. We introduced quenching analysis as a tool to explore
343 drug-polymer interactions in SDs that take place in the aqueous colloidal phase during release,
344 which was meant to complement existing analytics for this type of drug delivery systems. (Guo
345 et al., 2013) First, we analyzed different SDs by means of XRPD and DSC to determine their
346 amorphous nature.
347 Within this work, the ratio between CX and polymer (50:50 w/w %) was selected arbitrarily to
348 reflect a rather high loading. In the case of SDs prepared with HPMCAS-LG, PVP VA64, and
349 Soluplus, no distinct peaks were observed in the diffraction patterns. The case of SD prepared
350 with Poloxamer 188 was different (but in agreement with previous results in the literature

(Homayouni et al., 2014)), because peak positions similar to CX were evidenced, indicating that notable amounts of drug were crystalline. These results were confirmed by DSC studies. As shown in Figure 1B in the SD formulated with HPMCAS-LG, PVP VA64, and Soluplus, the absence of melting point (T_m) of CX and the presence of single peak of glass transition temperature (T_g) indicate the conversion of drug to an amorphous state and its miscibility with the polymer. A broad peak in the case of PVP VA64 SD is likely due to strong interaction between the carrier matrix and CX. On the other hand, as already reported in literature, (Serajuddin, 1999) Poloxamer 188 and CX form an eutectic, which exhibits a T_m at 40 °C and the second broad peak at 88 °C was attributed to the excess amount of the suspended CX present in the molten eutectic.

It has to be noted that the four polymers have been selected to cover a broad variety of excipients: from the most hydrophobic and negatively charged at pH 6.5 HPMCAS-LG, to the nonionic triblock copolymers Poloxamer 188 that shows a rather high water solubility (>100 g/l) (Bodratti and Alexandridis, 2018). Therefore it was already expected that not all of them would result in completely amorphous dispersions of CX.

Fluorescence quenching was then used to obtain information about the environment that surrounds the model drug in the aqueous colloidal phase (ACP). Quenching of fluorescence is presented as a Stern-Volmer plot where the ratio F_0/F is plotted versus the quencher concentration [KI] (Lakowicz, 2006). The extrapolated quenching values, such as K_D or fa, are independent from the absolute values of F and F_0 and therefore also from the concentration of CX in ACP. However, the exact CX concentration in the ACP used for the quenching experiment was evaluated by HPLC. As already mentioned, it only contained a part of the dose because any pseudo-equilibrium of drug release from solid dispersion typically results in either some

unreleased or precipitated drug in the course of supersaturation (Huang and Dai, 2014). Any given percentages in the fluorescence experiments are understood as relative to the drug amount solubilized in the ACP. The reference value of crystalline CX (4 h pseudo-equilibrium) was in line with literature (Gupta et al., 2004). The physical mixtures showed drug concentrations in ACP that were higher than solubility of pure CX, which was attributed to excipient solubilization effects (Tables 1 to 4). This effect was particularly notable for Poloxamer 188 (Table 4). As for SD formulations Table 5 indicates elevated concentrations of CX with exception of Soluplus. Perhaps the Soluplus (at least at the CX/polymer ratio used here) resulted in extensive drug precipitation after the equilibration time in accord with literature (Tsinman et al., 2015). An increase in Soluplus/ CX ratio could have decreased drug precipitation (Shamma and Basha, 2013).

In the case of CX alone, the solubilized drug is totally accessible to the quencher and its fluorescence intensity decreased by increasing [*KI*]. However, when a polymer is added, different scenarios are observed and quenching measurements reveal important information about the polymer spatial arrangement around the drug.

As already known from literature (Negrini et al., 2017) and as experimentally evaluated herein, the presence of polymers at the same concentration used within this work (see SI, Figure S6) increases the viscosity of the system. The quenching, a diffusion-limited process, is inversely proportional to the viscosity of the solution (Alberty and Hammes, 1958), since an increase of viscosity decreases the mobility of the quencher and therefore the number of collisions with the drug (Eftink and Ghiron, 1987).

In the case of physical mixtures of CX with HPMCAS-LG, PVP VA64, and Soluplus either the drug-polymer interactions or the increase of viscosity could lead to a decrease in quenching

efficiency. However, it has to be noted that even though Poloxamer 188, PVP VA64 and Soluplus solutions exhibit comparable viscosity values (0.83, 0.84 and 1.1 mPa s respectively), the extent of quenching did not decrease by using Poloxamer 188. Furthermore, the most viscous HPMCAS-LG (2.12 mPas s) displays a comparable decrease of quenching with the less viscous PVP VA64 (0.84 mPas s). This suggests that drug-polymer interactions predominantly contributed to the fluorescence quenching decrease, whereas viscosity was a factor of lesser importance.

Given that a polymer can form aggregates able to surround the drug, the latter would be totally protected from the quencher and hence quenching cannot occur. Additionally, two populations of drug in the aqueous phase can be present simultaneously: one which is accessible to quencher (*fa*) while the other one is inaccessible or buried in polymer aggregates. In this scenario, *fa* is the drug fraction that is not sequestered by the polymeric network. As a consequence, the more the polymer is able to bury the drug by forming aggregates surrounding it, the more the *fa* decreases. Interestingly, increasing the HPMCAS-LG concentration up to 30 %w/w in the physical mixture, the excipient was able to surround a fraction of CX. The drug interacting with polymer could have either become buried due to conformational change of the macromolecule or because of polymer aggregation. By contrast, the other polymers were not able, at least as physical mixtures, to protect CX from the quencher either by conformational change or by forming aggregates even not at a higher amount (70 % w/w). CX was likely to interact with either hydrophobic side chains as well as via polar interactions, or hydrogen bonding with HPMCAS-LG (Baghel et al., 2016). Especially the comparatively lipophilicity of polymer led in combination with the lipophilic model drug was likely to result in pronounced drug embedding (Ueda et al., 2014).

As known from the literature, electric charge either on the quenchers or on the polymers' surface can have a dramatic effect on the extent of quenching (Zinger and Geacintoov, 1988). In general, charge effects might be present with charged polymers such as HPMCAS-LG, and might be absent for neutral like PVP VA64 (Ando and Asai, 1980). For instance, a negative charge on HPMCAS-LG could prevent a negatively charged quencher from coming in contact with the drug. However, it is clear from our results (see Table 1 to 4) that the decrease of quenching was not mainly due to the electrostatic repulsion, because the neutral PVP VA64 showed almost the same extent of quenching as the negatively charged HPMCAS-LG.

Interestingly, except for Poloxamer 188, SDs in aqueous environment displayed at least two drug populations: one which is accessible to the quencher and the second that was inaccessible as it was buried in a polymeric conformation or in aggregated macromolecules. In the case of HPMCAS-LG (see Figure 3) the physical mixture showed a higher *Ka* as compared to SD. The quenching constant measures the stability of the quencher-fluorophore complex, and it is related to the accessibility of the fluorophore to the quencher, in particular to the separation distance within the excited-state complex, affected by diffusion and steric shielding of the fluorophore (Bombelli et al., 2010). Therefore, despite of the same values for *fa* (0.3 for both SD and physical mixture), in the case of SD, the drug was bound to a microenvironment less suitable for the interaction with the quencher compared to the physical mixture. This was obviously the results of different spatial arrangement of drug in polymer matrix as the SD was prepared by a solvent-evaporation method. This preparation must have facilitated a higher extent of polar interactions and hydrogen bonding of drug-HPMCAS-LG compared to physical mixture (Gupta et al., 2005). However, also more frequent hydrophobic interactions (due to succinoyl substituent) could have occurred (Ueda et al., 2014). In the case of PVP VA64 (Figure 4) and

443 Soluplus (SI, Figure S5), the polymer was able to embed the drug only when it was formulated
444 as SD. Even in this case, a possible explanation can be the capability of the polymer to strongly
445 interact with the drug trough H-bonds between amide protons of CX and carbonyl C=O of
446 polymers only in an amorphous state, as reported in literature (Lee et al., 2013), (Obaidat et al.,
447 2017).
448
449 A problem of classical drug release studies from SDs is that drug free in aqueous solution or
450 interacting colloids in different forms is typically not differentiated at all. Few research articles
451 emphasized the different drug forms emerging from SDs in aqueous environment (Frank et al.,
452 2012b),(Friesen et al., 2008),(Frank et al., 2014). Especially interesting is the percentage of drug
453 that is embedded into drug nanoparticles.
454 By using SEC method, it was possible to discriminate between the percentage of drug embedded
455 in polymer aggregates (% CX-polymer) and the percentage of free drug (% CX free) present in
456 the aqueous phase (i.e. ACP). As for the quenching experiments, it has to be kept in mind that a
457 part of the initial drug was not in the colloidal aqueous phase and hence, the term free drug refers
458 to the amount of solubilized drug in ACP, which was not buried or embedded in polymer
459 aggregates. This should not be confused with the total amount of free CX relative to an initially
460 administered dose.
461 As shown in Table 6, the HPMCAS-LG is able to entrap around 76 % of the drug (% CX-
462 polymer) and only 24% of CX is free (% CX free). It has to be noted that the values of accessible
463 fraction (*fa* in Table 5) and the values of percentage of free CX (Table 6) were comparable for all
464 the investigated SDs. In the case of SD, the polymer aggregate protected the drug and therefore
465 only the free fraction was reachable by the quencher.

However, drug release is a dynamic process and different populations of drug can coexist. The amount of free CX present in the ACP, will change over time, since a percentage of it can be either released or sequestered by the polymer. We considered a rather long but reasonable equilibration time for oral drug absorption so that the percentage of free CX would be either at or comparatively close to a pseudo- equilibration in the case of SDs. Studying the accessibility of the drug to a fluorescence quencher is a powerful and new method to investigate and elucidate the drug-polymer interactions upon drug release from SDs. In one formulated solubilization mechanism, the drug particles dissolve rapidly generating a highly supersaturated solution followed by the formation of drug nanoclusters within the polymer matrix (Kanaujia et al., 2011), (Marasini et al., 2013). It has been emphasized, for example by Ricarte et al. (Ricarte et al., 2017) (who studied SDs of HPMCAS) that emergence of nanostructures from polymeric SDs can determine the kinetics of drug supersaturation. Accordingly, the present study suggests the presence of the polymer aggregates in the aqueous colloidal phase, which is able to interact and embed a solubilized drug fraction. We know that absorption is driven by free drug but it is unclear if buried drug in polymer from the aqueous solution phase is lost for absorption or if it merely acts as a reservoir of drug in the sink of absorption. It will be a matter of individual colloidal partitioning kinetics regarding how much of the drug in the solubilized form is finally available for intestinal permeation.

5. Conclusions

The molecular and supramolecular interactions of drug and excipients are of critical relevance for the performance of oral solid drug dispersions. Traditional release testing offers only limited

characterization and more recent approaches attempted to better understand particles and colloids formed in aqueous environment. Various physical methods can be used to either study the solid phase that is typically formed on release from SDs or an aqueous colloidal phase is studied following a physiologically-relevant equilibration time. The current work introduced a fluorescence quenching method to study drug-polymer interactions in such an aqueous phase. Information was obtained regarding the accessible fraction of drug by the quencher and about the affinity to the quencher, which offered insights into molecular interactions with the polymer. An improved understanding of solubilization behavior was achieved by a comparison with results from size exclusion chromatography and dynamic light scattering. Depending on the polymer, a fraction of drug can obviously be buried in the macromolecule. This reduces free drug in solution, which leads to lower absorptive flux but also reduces the risk of undesired drug precipitation. Thus, it will depend on the partitioning kinetics of a given system between buried and free drug if such embedded drug can act as a favorable reservoir of drug absorption or if it adds to the dose fraction that is lost for absorption. There is certainly more research needed but it seems that fluorescence quenching analysis can greatly contribute to a better understanding of drug -polymer interactions *in vitro*, which ultimately can guide development of oral solid dispersions.

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Figure captions

Fig. 1. Powder X-ray diffraction (XRPD) plots of CX alone (a), physical mixtures with HPMCAS-LG (b), PVP VA 64 (c), Soluplus (d), and Poloxamer 188 (e). CX solid dispersions (SD) are shown with HPMCAS-LG (f), PVP VA 64 (g), Soluplus (h), and Poloxamer 188 (i) (A). DSC thermograms of CX alone (a), SDs with HPMCAS-LG (b), PVP VA 64 (c), Soluplus (d) and Poloxamer 188 (e).

Fig. 2. Physical mixtures: Stern–Volmer plots (A and B) and modified Stern–Volmer plots (C) for fluorescence quenching of CX in the presence of 0 (black circles), 10 (black upper triangles), 20 (white diamonds), 30 (white upper triangles), 50 (white circles) and 70 (black down triangles) w/w % of HPMCAS-LG.

Fig. 3. Modified Stern–Volmer plots for fluorescence quenching of CX with HPMCAS-LG as either SD (black circles) or physical mixture (white circles).

Fig. 4. Stern–Volmer plots for fluorescence quenching of CX/ PVP VA 64 as either SD (black circles) or physical mixture (white circles). The inset in the figure shows the modified Stern–Volmer plots for a comparative fluorescence quenching of CX/ PVP VA 64 SD

Fig. 5. Elution profile obtained by SEC: Percentages of CX (A) and the mean count rate, expressed by Kcps (B) present in the eluted fractions were plotted *vs* the elution volumes. CX SD with HPMCAS-L (black circles), PVP VA 64 (white triangles), Soluplus (black squares), and Poloxamer 188 (white circles).