School of Pharmacy, Department of Pharmaceutics, University of Oslo, Norway

Influence of cosolvents, ionic strength and the method of sample preparation on the solubilization of curcumin by Pluronics and HP-γ-cyclodextrin

Studies of curcumin and curcuminoids, XLIV

R. Singh, S. Kristensen, H. H. Tønnesen

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Ravinder Singh, School of Pharmacy, Department of Pharmaceutics, University of Oslo, P.O. Box 1068, Blindern, 0316 Oslo, Norway
ravinder.singh@farmasi.uio.no

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Curcumin was solubilized by Pluronics and the concentration of dissolved curcumin seemed to be related to the number of propylene oxide units in the Pluronic polymer. All Pluronics showed a maximum solubilizing capacity at a certain curcumin: Pluronic molar ratio and exceeding this molar ratio resulted in precipitation of curcumin when following the samples for 356 hours. PEG 400 could to a certain extent stabilize the supersaturated samples, while ethanol physically destabilized the samples. Ionic strength did not influence the solubilization of curcumin by the Pluronics. Supersaturation and precipitation inhibition caused a higher concentration of curcumin in samples prepared by SEM compared to samples prepared by SFM (i.e. the thermodynamic solubility).

1. Introduction

Curcumin (Fig. 1) has a potential as photosensitizer for topical photodynamic therapy, used against neoplastic cells (PDT) and bacteria (aPDT). However, curcumin is practically insoluble in aqueous solutions (< 50 nM in phosphate buffer 0.05 M pH 5) (Tønnesen et al. 2002). The solubility can be improved by alkalization of the aqueous solution, but then the compound will be rapidly degraded (Tønnesen et al. 1985; Tønnesen et al. 2002). Curcumin is also photochemically unstable (Tønnesen et al. 1986; Tønnesen et al. 2002). To allow administration of the photosensitizer at a sufficient concentration to attain a proper effect, solubilization and stabilization of curcumin is decisive.
Various formulations of curcumin have previously been developed and evaluated to increase its aqueous solubility and stability (Singh et al. 2010; Tomren et al. 2007; Tønnesen 2002; Tønnesen et al. 2002; Tønnesen 2006). Formulations of curcumin with cyclodextrins have been most extensively studied. Inclusion into cyclodextrins increases water solubility and hydrolytic stability of curcumin. The solubility of curcumin in the presence of 10% (w/v) 2-hydroxypropyl-γ-cyclodextrin (CD) was determined to 5.35 mM, i.e. an increase in solubility by a factor of $> 10^5$ compared to phosphate buffer pH 5 (Singh et al. 2010; Tomren et al. 2007; Tønnesen et al. 2002). However, solubilization by CD increased the photodecomposition rate compared to curcumin dissolved in organic solvents (Tønnesen et al. 2002).

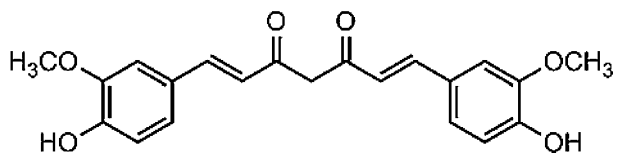
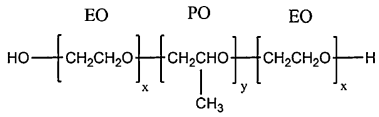


Fig. 1: Chemical structure of curcumin

Addition of the biopolymer alginate increased the solubility by a factor of $> 10^4$, but no thermal or photochemical stabilization was observed (Tønnesen 2006). Solubilization by a surfactant micellar systems resulted in an increase in water solubility by a factor of $> 10^5$. Hydrolytic stability was better than with CD, but photodecomposition was increased compared to curcumin in hydrogen bonding media (e.g. ethanol and methanol, and mixtures of ethanol and buffer) (Tønnesen 2002). Curcumin has also been solubilized by block copolymers such as β-lactam functionalized poly(isoprene-b-ethylene oxide) (Gardikis et al. 2010), poly (ethylene oxide)-block-poly (ε-caprolactone) (Ma et al. 2008) and by methoxy poly (ethylene oxide)-block-poly (caprolactone) (Letchford et al. 2008). Reported solubility of curcumin in 1% (w/v) (≈ 0.34 and 2 mM respectively) of the two latter block copolymers is approximately 2–4 mM, while solubility in 2% (w/v) (≈ 0.59 mM) of β-lactam functionalized poly(isoprene-b-ethylene oxide) is approximately 7 mM, increasing solubility by a factor of $> 10^5$. To our knowledge, this is the highest concentration of aqueously dissolved curcumin reported in the literature. However, none of these compounds are approved by the FDA as pharmaceutical excipients (FDA 2009).
Pluronic is a trademark for block copolymers which consist of hydrophilic ethylene oxide (EO) and hydrophobic propylene oxide (PO) blocks arranged in an A-B-A structure: EO_x – PO_y - EO_x forming amphiphilic molecules (Table 1). By varying the number of EO (x) and PO (y) one can attain different properties of the Pluronics, such as defoaming, detergency, foaming, emulsification, gel formation and wetting (BASF 2009).
Several Pluronics are approved by the FDA as pharmaceutical excipients for various administration routes (FDA 2009). The Pluronics are good solubilizers for aromatic compounds and show thermally reversible gel formation at high concentrations (Kabanov et al. 2002). The Pluronics are also reported to increase

Table 1: Chemical structures, molecular weight (Mw) and critical micelle concentration (CMC) of selected Pluronics



Pluronic	A-B-A structure	Mw	CMC	Method	Reference
P85	$EO_{26}-PO_{40}-EO_{26}$	4600	230 µM	Fluorescence probe method with pyrene at 25°C	(Sezgin, et al. 2006)
F127	$EO_{100}-PO_{65}-EO_{100}$	12600	69 µM	Fluorescence probe method with pyrene at 25°C	(Sezgin, et al. 2006)
P123	$EO_{20}-PO_{70}-EO_{20}$	5750	49 µM	Fluorescence probe method with DPH* at 25°C	(Hioka, et al. 2002)

The physical forms of the products are given by P = paste; F = solid form. The first digit of the trade name (two digits in a three-digit number) indicates the approximate molecular weight of the hydrophobe (PO) when multiplied by 300. The last digit indicates the approximate ethylene oxide (EO) content (% w/w) when multiplied by 10 (BASF 2010). The method refers to determination of the CMC. * DPH = 1, 6-Diphenyl-1, 3, 5-hexatriene

the activity of drugs towards multidrug-resistant cancer cells and drug-resistant bacterial strains (Kabanov and Alakhov 2002). Therefore, the Pluronics seem suitable for making formulations of curcumin to be used in topical PDT and aPDT.

Formulations with curcumin and Pluronic F127 or F68 have recently been investigated with respect to *in vitro* cytotoxicity (Sahu et al. 2011). The highest encapsulation of curcumin ($95.57 \pm 1.65\%$) was achieved in formulations containing F127. Curcumin dissolved in F68 has also been investigated with respect to *in vivo* inhibition of tumor growth and vasculogenic mimicry (Chen et al. 2011). In other studies, various Pluronics have been used to prepare formulations for delivery of curcumin. F127 was used as a coating agent, with β-cyclodextrin, on nanoparticles consisting of iron oxide (Yallapu et al. 2011), and to increase encapsulation of curcumin into nanoparticles consisting of alginate and chitosan (Das et al. 2010). F68 has been used to prepare solid lipid microparticles (Yadav et al. 2009). However, to our knowledge there are no reports on how the formulation parameters influence the solubility of curcumin in samples containing Pluronics.

The aim of this study was to investigate the solubility of curcumin in formulations with three different Pluronics (F127, P123 and P85), the influence of cosolvents and ionic strength on the solubilization of curcumin by Pluronics, and to compare two commonly used methods of sample preparation. CD is used as a reference solubilizer, and the solubilization of curcumin by Pluronics is compared to the solubilization of curcumin by CD.

2. Investigations and results

2.1. Solubility of curcumin at different curcumin: solubilizer molar ratios

The samples were prepared by the solvent evaporation method (SEM) as described in the literature (Aliabadi et al. 2006). The concentration of curcumin determined at 20, 188 and 356 h after reconstitution in the buffer was plotted against curcumin: solubilizer molar ratio originally present in the organic solvent (Fig. 2). The concentration of curcumin increased linearly with curcumin: Pluronic molar ratio up to a certain point. This point would represent the maximum solubilizing capacity of the Pluronics. By increasing the molar ratio above this point a precipitation of curcumin was observed after 356 h. The molar ratio leading to the maximum solubilizing capacity of the Pluronics under the given experimental conditions was between 0.3–1.0 for F127, 0.5–1.0 for P123 and 0.03–0.05 for P85 (Fig. 2).

The concentration of dissolved curcumin in samples with molar ratios ≤ 0.3 for F127, ≤ 0.5 for P123 and ≤ 0.03 for P85 remained constant over 356 h (Fig. 2). No precipitation was observed in these samples, and no degradation products of curcumin were observed in any of the HPLC-chromatograms. This showed that the Pluronics protect curcumin against hydrolytic degradation, and that the change in concentration in samples with molar ratios exceeding the maximum solubilization of each Pluronic is solely due to precipitation and not degradation.

The rate and amount of precipitation in samples with a molar ratio ≥ 1.0 for F127 and P123, and ≥ 0.05 for P85, varied between the 6 replicates during storage. This caused a variation in the concentration of dissolved curcumin between the replicates, represented by the error bars in the figures (Fig. 2). Therefore, large error bars in the figures are consistent with an incomplete precipitation process, while data points with minor error bars, at molar ratios exceeding the maximum solubilizing capacity of the Pluronics, are consistent with either a supersaturated solution before the precipitation is initiated or a sample with a complete precipitation (Fig. 2).

In samples where curcumin was solubilized by F127, precipitation was observed at 20 h at a curcumin:F127 molar ratio of 2.0. The precipitation process seemed complete for samples with molar ratio 2.0 and 1.5 at 188 hours. In samples with a molar ratio of 1.0 the precipitation process started between 20–188 h, and seemed to be complete at 356 h (Fig. 2).

In samples where curcumin was solubilized by P123, precipitation was observed at 20 h at a curcumin:P123 molar ratio of 2.0 and 1.5. The precipitation process seemed complete at 356 h in samples with a molar ratio of 2.0, while still incomplete at a molar ratio of 1.5. The precipitation process in samples with a curcumin:P123 molar ratio of 1.0 started between 20–188 h, and seemed complete at 356 h (Fig. 2).

Precipitation was observed at 20 h in samples with a curcumin:P85 molar ratio of 0.20, 0.10 or 0.05. The precipitation process was still not complete at 356 h independent of molar ratio (Fig. 2).

The concentration of curcumin increased linearly with the curcumin: CD molar ratio up to a maximum (0.8 in the case of 64.0 mM CD and 0.6 in the case of 5.2 mM CD), as was observed for the Pluronics (Fig. 2). Curcumin precipitated immediately upon addition of buffer to the dry film consisting of CD and curcumin in samples with molar ratios ≥ 1.0 (64.0 mM CD) and ≥ 0.8 (5.2 mM CD). The molar ratio giving the maximum solubilizing capacity of the CD was therefore between 0.8–1.0 in the case of 64 mM CD, and 0.6–0.8 in the case of 5.2 mM CD (Fig. 2). The concentration of dissolved curcumin in the samples exceeding the molar ratio at the maximum solubilizing capacity was substantially lower than the concentration at the maximum, and independent on molar ratio of curcumin: CD (Fig. 2).

When solubilized by HPγCD, the concentration of curcumin remained unchanged over a period of 356 h at each molar ratio (Fig. 2). No degradation products were observed in the HPLC-chromatograms. HPγCD has apparently a stabilizing effect on curcumin against hydrolytic degradation under the given conditions.

2.2. The influence of cosolvents and ionic strength on the solubilization of curcumin by Pluronics

Further experiments were performed to investigate if commonly applied pharmaceutical cosolvents, such as polyethylene glycol 400 (PEG 400) and ethanol, or an increase in ionic strength

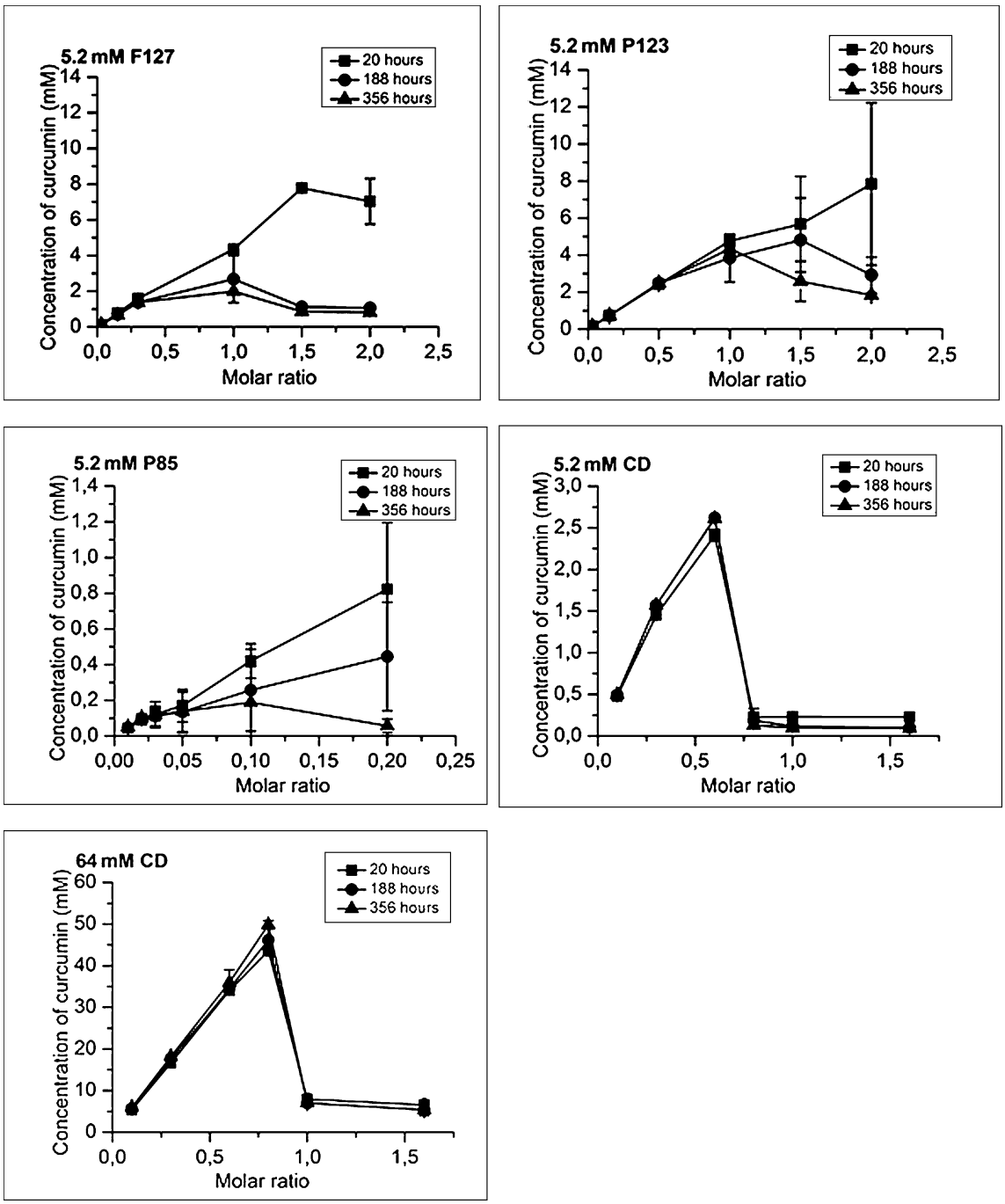


Fig. 2: Concentration of curcumin as a function of the molar ratio of curcumin: solubilizer (Pluronic or CD) originally present in the organic phase prior to evaporation (SEM). The concentration of curcumin at 20, 188 and 356 h after reconstitution in the buffer to the samples are plotted. The concentration is the average of 6 replicates, and the standard deviations are plotted as error bars

from 0.085 M to 0.17 M and 0.34 M, could stabilize the samples at the highest molar ratio of curcumin: solubilizer investigated, i.e., molar ratio 2.0 for F127 and P123, 0.20 for P85 and 1.6 for both 64.0 mM and 5.2 mM CD.

Fig. 3 shows the effect of cosolvents on solubilization of curcumin prepared at the respective molar ratios of curcumin: solubilizer.

The trends for F127 and P123 were similar. Precipitation of curcumin was observed at 20 h in samples containing PEG 400. The precipitation seemed incomplete at 356 h. The average concentration of curcumin in samples containing PEG 400 was larger at 356 h than the average concentration of curcumin in samples with ethanol and samples without cosolvent, at the same storage time (Fig. 3).

Further, ethanol induced rapid precipitation and rapid equilibration of the concentration of curcumin, compared to samples containing PEG 400 and samples without cosolvent, as there was almost no variation between the replicates at all three time points, and there was no large change in the concentration of curcumin after 356 h storage (Fig. 3).

PEG 400 seemed to stabilize samples containing P85 towards precipitation of curcumin. Further, no precipitation was observed in samples containing ethanol, at 20 h. However, at 188 h precipitation of curcumin in samples with ethanol was observed. The average concentration of curcumin in the samples with ethanol at this time point was 16.3 and 7.3 times lower than samples containing PEG 400 or without cosolvent, respectively, at the same time point. At 356 h, the concentration of curcumin in samples containing ethanol was about equal to the concentration observed at 188 h (Fig. 3).

The trends for samples containing 64.0 mM CD and 5.2 mM CD were also similar. As observed for F127 and P123, ethanol

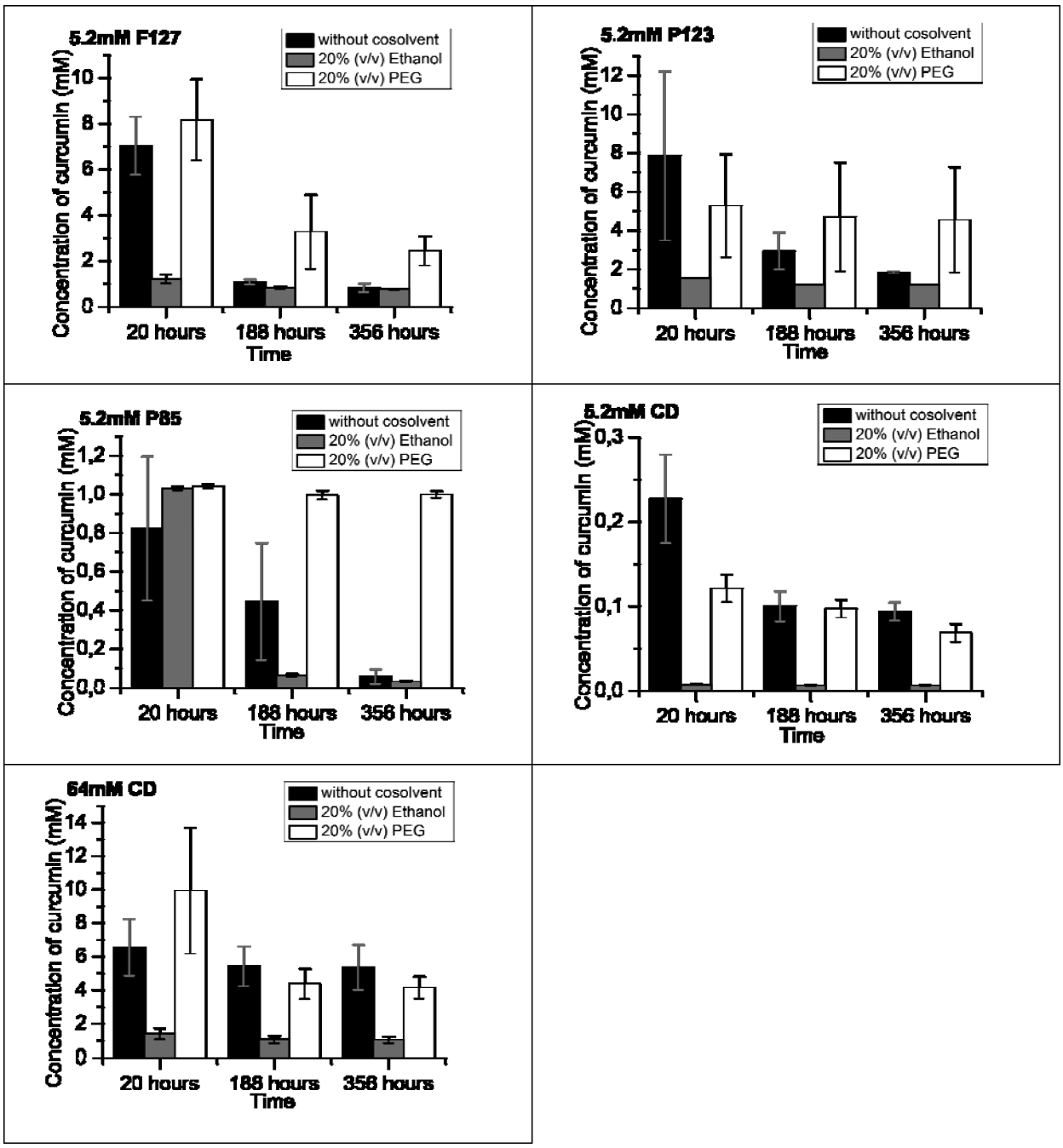


Fig. 3: Concentration of curcumin, solubilized by Pluronics or CD at the highest molar ratio investigated, in samples with or without cosolvents (PEG 400 or ethanol) at 20, 188 and 356 h after addition of buffer to samples prepared by SEM. The concentration is the average of 6 replicates, and the standard deviation is plotted as error bars

induced a rapid precipitation of curcumin and rapid equilibration conditions compared to samples containing PEG 400 and samples without cosolvent. Further, it was difficult to observe a difference in the solubilization of curcumin in samples containing PEG 400 compared to samples without cosolvents, as the average concentration of curcumin and the variation between the replicates were approximately the same in the two systems at 188 h and 356 h, respectively. The average concentration of curcumin at 20 h, in samples containing 5.2 mM CD without cosolvent, was 1.9 and 32.5 times higher than samples containing PEG 400 or ethanol, respectively. In samples containing 64.0 mM CD, the addition of PEG 400 seemed to have a slight synergistic effect on the curcumin solubility at 20 h (Fig. 3).

Fig. 4 shows the effect of ionic strength on the solubilization of curcumin as a function of time at a given molar ratio of curcumin: solubilizer (i.e., 2.0 for F127 and P123, 0.20 for P85 and 1.6 for both 64.0 mM and 5.2 mM CD). The trends are similar for both Pluronics and CDs. The large variation between the replicates at 20 h (all solubilizers) and at 188 h (P123 and P85) made it difficult to see any difference in the concentration of curcumin with an increase in ionic strength. However, at 188 h in samples containing F127 or CD, and at 356 (all solubilizers), the concentration of dissolved curcumin remained unchanged by an increase in ionic strength (Fig. 4).

Fig. 5 shows the effect of cosolvents on the solubilization of curcumin by F127, P123 and P85 when the samples were prepared by the shake-flask method (SFM). The trends are the same for all three solubilizers. The concentration of curcumin was highest in samples containing PEG 400, and it was higher in buffers containing ethanol than in buffers without cosolvents (Fig. 5).

The solubility of curcumin in the buffers without solubilizers, i.e., phosphate buffer with 20% (v/v) ethanol, phosphate buffer with 20% (v/v) PEG 400 and phosphate buffer without cosol-

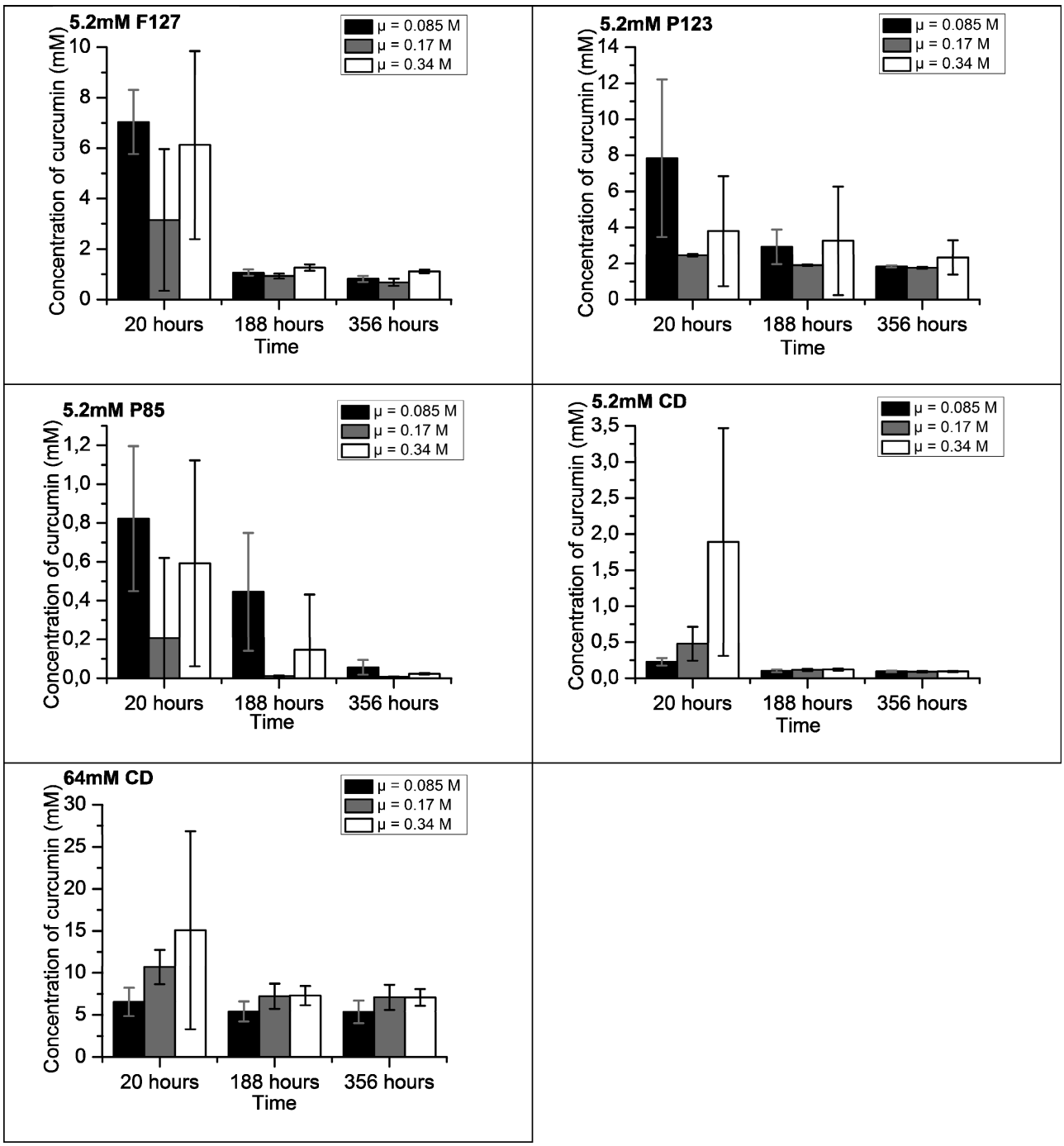


Fig. 4: Concentration of curcumin in samples with ionic strength (μ) 0.085 M, 0.17 M and 0.34 M containing Pluronics or CD at the highest molar ratio investigated (SEM). The concentration of curcumin at 20, 188 and 356 h after addition of buffer to samples is presented. The concentration is the average of 6 replicates, and the standard deviation is plotted as error bars

vent, was also investigated. The results are given in Table 2. For samples prepared with PEG 400 by SEM, degradation was observed by the occurrence of two new peaks at 7.5 and 11.2 min respectively in the HPLC-chromatograms of samples stored for 188 and 356 h. The measured concentration of curcumin in these samples at 188 and 356 h was 12.7 ± 0.9 and $15.0\ \mu M \pm 1.3$ respectively. A small peak in the chromatogram, corresponding to curcumin, was detected in the samples containing ethanol. The area was, however, below the quantification limit of the method (i.e. 0.5 μM), and the solubility could not be determined. Curcumin was not detected in the chromatograms of samples without cosolvents.

Table 2: Solubility of curcumin in buffers without Pluronics or CDs, in samples prepared by SEM (20 h after addition of the buffer) or SFM

	Phosphate buffer	20% (v/v) ethanol in phosphate buffer	20% (v/v) PEG in phosphate buffer
SFM	< LOQ	< 0.9 μM	$16.7\ \mu M \pm 0.4$
SEM	< LOQ	< 0.9 μM	$18.7\ \mu M \pm 2.9$

The concentration is reported in μM ± standard deviation, n = 6

2.3. The influence of the method of preparation on solubilization of curcumin by Pluronics

The solubilization of curcumin in samples prepared by SEM, measured at 20 h after addition of phosphate buffer, was compared to the solubilization of curcumin in samples prepared

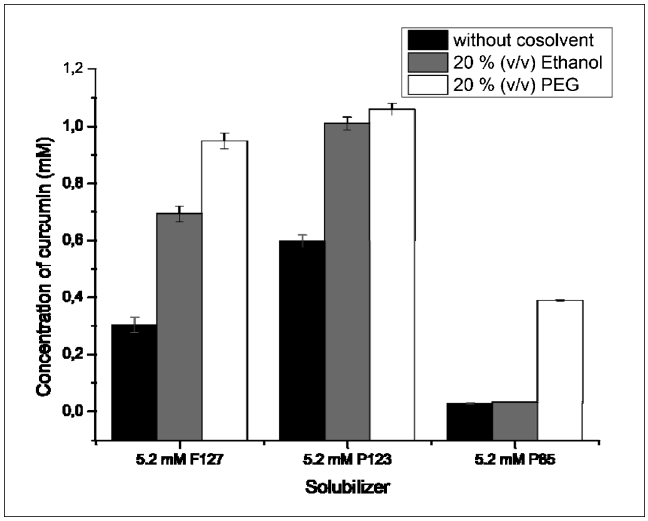


Fig. 5: Concentration of curcumin in samples with Pluronics, and with or without cosolvents (PEG 400 or ethanol), when prepared by SFM. The concentration is the average of 6 replicates

by SFM (Fig. 6). The concentration of curcumin in samples prepared by SEM was higher for all solubilizers compared to samples prepared by SFM (Fig. 6). The average ratio between solubilized curcumin in samples prepared by SEM compared to SFM was 25.6, 13.1, 30.6, 16.4, and 31.1 for F127, P123, P85, 64.0 mM CD and 5.2 mM CD respectively.

2.4. Characterization of the film formed after the evaporation process in samples prepared by SEM

The film formed after the evaporation process in samples prepared by SEM was, after being scraped off the inner surface of the round bottle, characterized by visual observation, scanning electron microscopy and differential scanning calorimetry (DSC). The films were prepared at curcumin:Pluronic molar ratios that gave the maximum solubilizing capacity of curcumin when dissolved in buffer. Curcumin changed from orange to dark red in the presence of solubilizer.

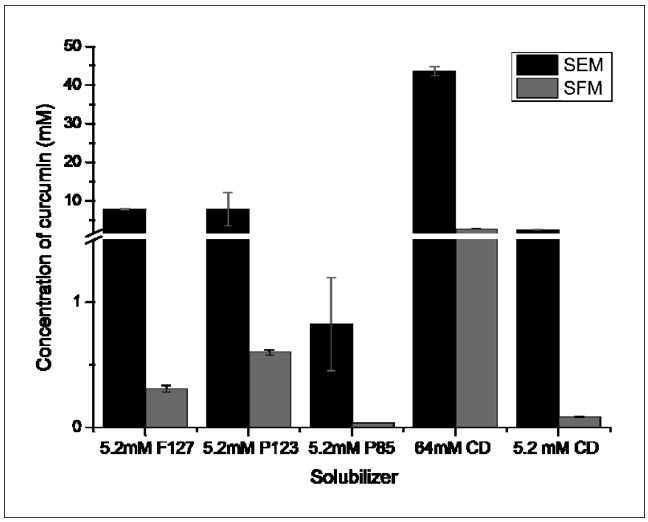


Fig. 6: Solubility of curcumin in samples prepared by SEM compared to samples prepared by SFM. The highest concentration of curcumin, 20 h after addition of buffer to the samples prepared by SEM, is plotted. Breaks are inserted at 1.0 mM – 1.5 mM curcumin, and the prebreak scale is with 0.5 mM increment, while the postbreak scale is with 10 mM increment. The concentration is the average of 6 replicates, and the standard deviation is plotted as error bars

Scanning electron microscopy was performed to investigate the shape and surface morphology of the pure substances and the combination products after the evaporation process. Micrographs of curcumin before the evaporation process were also recorded. These micrographs showed crystalline particles (approximately 2 μm wide) with a needle-like morphology (Fig. 7A). After the evaporation process all products were oblong and fragmented. In addition, curcumin had a smoother surface (Fig. 7B), F127 had a surface in layers (Fig 7C), while the combination of curcumin and F127 showed a rougher surface morphology (Fig. 7E). CD showed a very smooth surface morphology (Fig. 7D). The combination of CD and curcumin resulted in a smooth and porous product (Fig. 7F). Scanning electron microscopy was also performed on the combination of P123 and curcumin and the combination of P85 and curcumin. However, these combination products were gel-like, and upon sputter coating in vacuum they started to boil.

DSC was performed to investigate the thermal characteristics of pure curcumin before and after the evaporation process, and in combination with the solubilizers (P85, F127, P123 and CD) after the evaporation process. Curcumin before the evaporation process had a sharp endothermic peak at 183.3 °C (182.4–183.9 °C) corresponding to melting of crystalline curcumin. The endothermic peak of curcumin after the evaporation process was smaller, broader and there was a slight change in both peak 180.4 °C (180.1–180.8 °C) and onset 175.1 °C (174.9 – 175.2), compared to 181.8 °C (181.1–182.4 °C) for curcumin before the evaporation process.

The melting of curcumin was not detected in the combination product with F127 (Fig. 8A) and P123 (data not shown). The melting point of F127 was seen as a sharp endothermic peak at approximately 60 °C (Fig. 8A) (BASF 2011). P123 does not have a melting point as it is not crystalline, while the melting point of CD is 250 °C (BASF 2011;GuideChem 2011).

The thermogram of the combination product of CD and curcumin shows a broad endothermic peak at 186.9 °C (186.2–188.2), with an onset at 180.6 °C (174.6–182.4 °C) and a change in baseline. Further, the thermograms of CD, or in combination with curcumin, show a peak at 90–95 °C, which may be adsorbed water (Fig. 8B).

3. Discussion

3.1. Selection of Pluronic type and concentration

Several factors were considered when selecting the appropriate Pluronics for the current investigations. Pluronics with 30% (w/w) or more of the poly EO - group is required to obtain good water solubility of the solubilizer. The length of the poly PO-group is important for high drug loading of hydrophobic drugs. An increase in PO-chain length will also decrease the critical micelle concentration (CMC), which will reduce the amount of block copolymer needed to solubilize the compound of interest. Therefore, the length of the hydrophilic and hydrophobic chains should be of optimal size, since a large poly PO chain might also inhibit drug release. It has been reported that optimal length of the poly PO and poly EO-groups can enhance drug absorption into cancer cells (Kabanov and Alakhov 2002). Therefore, the influence of chain length on the solubilization of curcumin was investigated in the present study (Table 1). Assuming that curcumin would interact with the poly PO part of the Pluronics, one might assume that F127 and P123 would result in an efficient solubilization of curcumin due to the long PO-chain length. In addition, F127 is commonly applied in topical formulations (FDA 2009; Kabanov and Alakhov 2002). P85 has been shown to have an optimal PO-chain length with regards to enhanced drug absorption into cancer cell (Kabanov and Alakhov 2002).

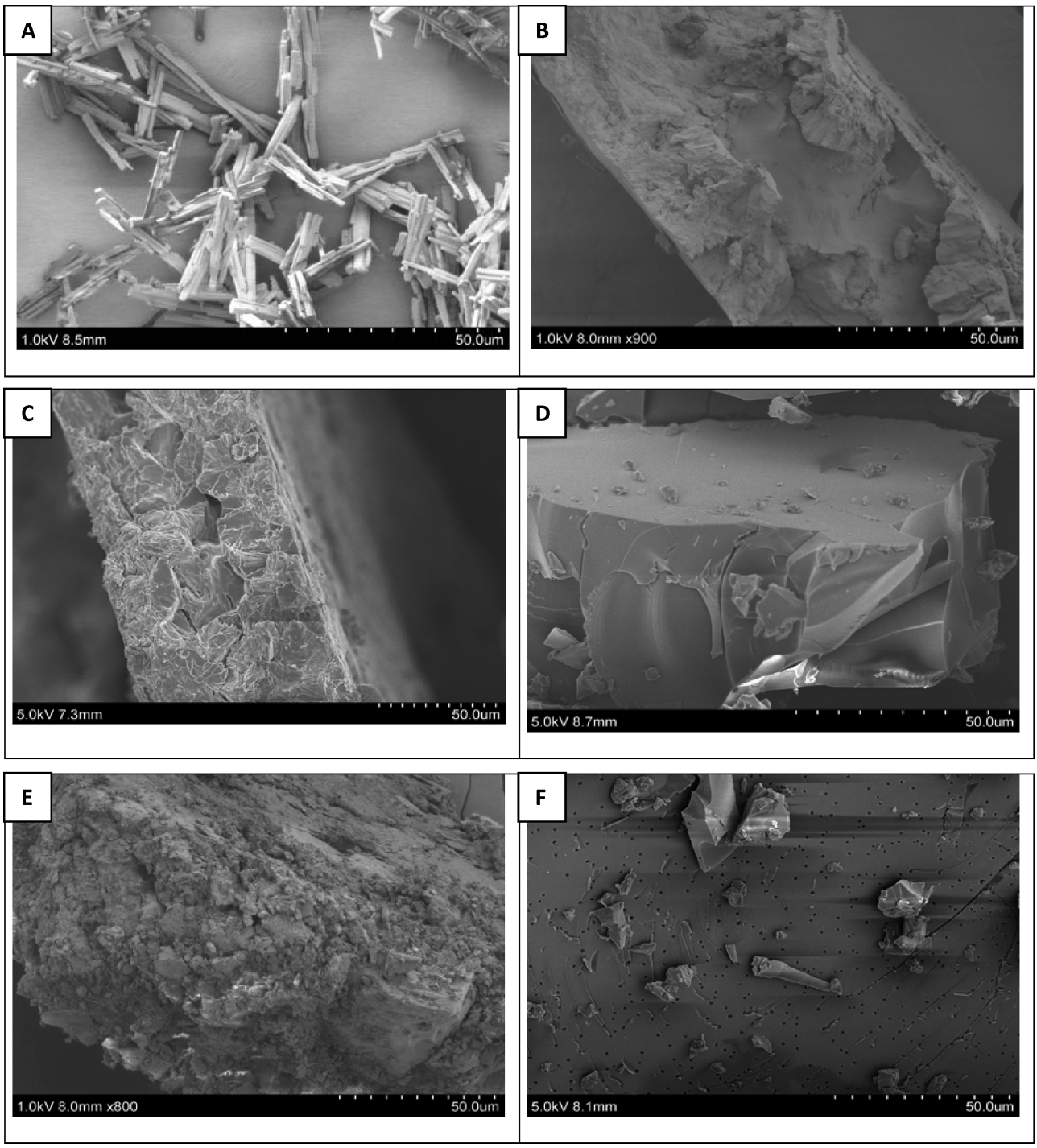


Fig. 7: Scanning electron micrographs of curcumin before the evaporation process (A), and the following samples after the evaporation process: curcumin (B), F127 (C), HPγCD (D), the combination of curcumin and F127 (molar ratio 0.3) (E) and the combination of curcumin and HPγCD (molar ratio 0.6) (F). The first two numbers in each micrograph are respectively the condition (kV) and working distance (mm). The magnifications for A-F are 1100, 900, 600, 1100, 800 and 900 respectively

Therefore, the Pluronics F127, P123 and P85 were chosen for the present studies.

F127 and P123 at concentrations above 5.2 mM were difficult to dissolve in phosphate buffer. Good solubility is especially important by use of SEM, where the film that is formed after evaporation is reconstituted in the buffer. Thus, a concentration of 5.2 mM was selected for all the Pluronics in the present work. This is well above the CMC of the compounds reported in the literature (Table 1).

3.2. The influence of the properties of the solubilizer on the solubilization of curcumin

The curcumin: Pluronic molar ratio that resulted in the maximum solubilizing capacity was almost the same in the case of F127 and P123, as was the highest concentration of curcumin detected in these samples after 20 h storage (Fig. 2). The curcumin:P85 molar ratio giving the maximum solubilizing capacity was approximately 10–20 times lower than for F127 and P123. Previous studies have shown that an increase in the length of the PO-block results in a decrease in CMC. Also, it is mainly the PO-core of the solubilizing structures that solubilizes hydrophobic compounds (Alexandridis et al. 1994). The present results can therefore be explained by looking at the number of PO-units in the Pluronic polymer. This number is approximately the same for F127 and P123 (65–70), while it is approximately 40% lower in P85. The reported CMC of F127 and P123 is also approximately the same, while the CMC of P85 is 3–5 times higher than for F127 and P123. The molecular weight

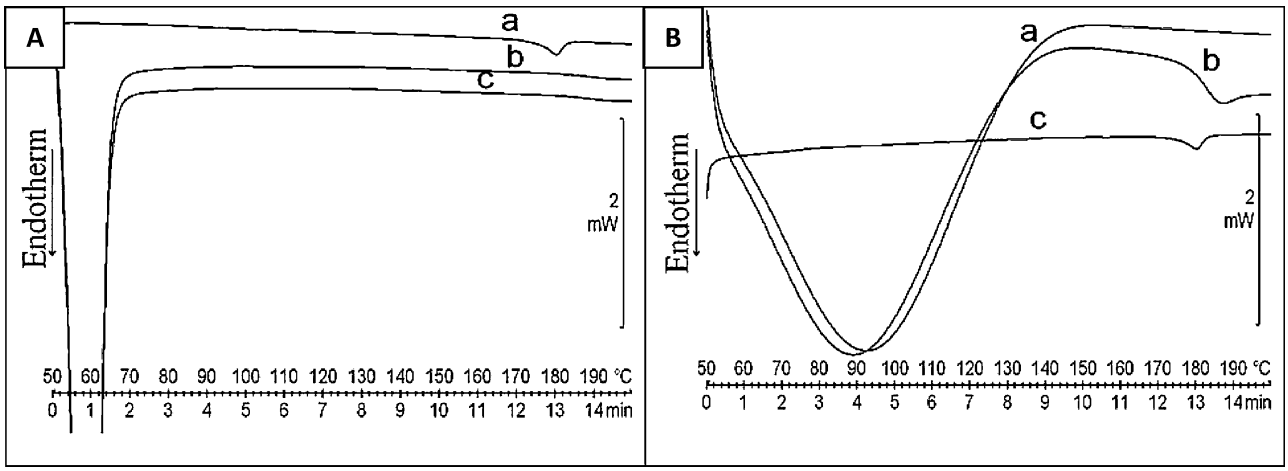


Fig. 8: A shows the DSC thermograms of curcumin after the evaporations process (a), the combination of curcumin and F127 (molar ratio 0.3) (b) and F127 after the evaporations process (c). B shows the DSC thermograms of HPγCD after the evaporation process (a), the combination of curcumin and HPγCD (molar ratio 0.6) (b) and curcumin after the evaporations process (c). Both temperature (°C) and time (minutes) are given on the x-axis

or the ratio of PO:EO units in the polymer does not seem to have the same influence on the solubilization of curcumin as the number of PO-units, as there is no correlation between these factors and the solubility of curcumin (Table 1). Solubilization of curcumin is therefore mainly determined by number of PO-units in the Pluronics as these units influence both the formation of solubilizing structures and the strength of interaction with curcumin.

Precipitation of curcumin was observed in samples prepared with a curcumin: Pluronic molar ratio above the maximum solubilizing capacity of the Pluronics. The molar ratio at the maximum solubilizing capacity of F127 and P123 was in the same range as the curcumin:CD molar ratio. However, in samples solubilized by CD, the precipitation occurred immediately after reconstitution in buffer (SEM). The slower precipitation rate in samples with F127 and P123 compared to samples with CD, at molar ratios ≥ 1, could be explained by the large Pluronic molecules (molecular weight of Pluronics is 3–8 times larger than CD) forming large solubilizing structures which inhibit the diffusion of curcumin molecules, or disturbs the precipitation process through other mechanisms (Brewster et al. 2008). Brewster et al. (2008) reported cyclodextrins to be better inhibitors of precipitation in supersaturated solutions than the surfactants Cremophor RH40, Polysorbate 20 and Tocopheryl Polyethylene Glycol Succinate. However, the present results show that Pluronic is a better precipitation inhibitor than CD. Guzman et al. have previously reported that Pluronics (F127) inhibited precipitation in supersaturated solutions of celecoxib (Guzman et al. 2007).

The molar ratio that exceeded the maximum solubilizing capacity of P85 was not comparable to CD (i.e., approximately 10–30 times lower than CD). However, the explanation for the slow precipitation of curcumin in samples with F127 and P123 would apply for P85 as well. The formation of supersaturated solutions of curcumin could be advantageous in topical delivery as it can increase the absorption into the diseased area.

The precipitation of curcumin was observed at a lower curcumin: CD molar ratio in samples containing 5.2 mM CD than in samples containing 64 mM CD. The solubility of curcumin in samples with curcumin: CD molar ratios exceeding the maximum solubilizing capacity of the CD was substantially lower than in samples at the maximum ratio (Fig. 2). Precipitation inhibition of supersaturated solutions by CDs is a non-inclusion based phenomenon and may occur through a number of mechanisms (Brouwers et al. 2009). E.g., CD can form aggregates at high concentrations, influencing solubilization (Jansook et al. 2010).

The precipitation rate also seemed to increase with increasing molar ratio of curcumin: Pluronic in the sample. This observation could be explained by the relationship between the rate of precipitation and the concentration of solute in the supersaturated solution; the larger the amount of curcumin initially solubilized above saturation, the faster it will precipitate from the solution (Florence et al. 1998).

3.3. The influence of cosolvents and ionic strength on the solubilization of curcumin by Pluronics

The results show that the precipitation process was either not initiated (P85) or slowed down (F127 and P123), in samples with Pluronics and PEG 400 (SEM) (Fig. 3). PEG 400 will reduce the dielectricity constant of the sample compared to samples without cosolvents, which will influence the aggregation of the Pluronic molecules and their formation of solubilizing structures (Myrdal 2002). PEG 200 is reported to increase the CMC by reducing the hydrophobic effect of the Pluronics (Zhang et al. 2008). Further, a cosolvent will increase the solubilizing capacity of the environment (thermodynamic process). The concentration of a compound in samples prepared by SFM represents the thermodynamic solubility of that compound in the given formulation (Brouwers et al. 2009). Therefore, a higher concentration of curcumin in samples prepared by SFM containing PEG 400, compared to samples without cosolvents, shows the thermodynamic effect of PEG 400 on the solubility of curcumin (Table 2). The combined thermodynamic effect of PEG 400 and Pluronic (P85, P123 and F127) increased the solubility of curcumin compared to samples without PEG 400. The large PEG 400 molecules might also, in combination with Pluronics, influence the diffusion and nucleation of curcumin, as the concentration of curcumin in samples prepared by SEM (at all three time points and all Pluronics) was between 2.6–8.6 times higher than the concentration of curcumin in samples prepared by SFM. Precipitation inhibition of the supersaturated solutions by PEG 400 and Pluronic (SEM) might therefore be ascribed to a combination of kinetic processes (e.g. inhibiting precipitation) and thermodynamic processes.

The addition of ethanol to the samples induced a decrease in the concentration of curcumin compared to samples without ethanol (SEM) (Fig. 3). Ethanol will also reduce the dielectrisity constant of the medium and has theoretically the same potential as PEG 400 to influence the solubilizing capacity of the environment. Ethanol has also been reported to increase the CMC of Pluronics (Armstrong et al. 1996). There was a slight increase in

solubility of curcumin in buffers with 20% (v/v) ethanol compared buffers without cosolvents. This thermodynamic effect was weaker than that of PEG 400 (Table 2). However, the combined thermodynamic effect of ethanol and Pluronic (F127 and P123) increased the solubility of curcumin compared to samples without cosolvents (Fig. 5). The rapid precipitation and lower concentration of curcumin in samples containing ethanol combined with Pluronics (SEM) might therefore be ascribed to a change in the solubilizing structures of the Pluronics. Further, as the molecular weight of ethanol is much lower than PEG 400, ethanol does not stabilize the supersaturated solutions through inhibition of the precipitation process (diffusion and nucleation). The solubility of curcumin in samples with 20% (v/v) ethanol (SEM) was at all time points approximately the same as the thermodynamic solubility of curcumin in the same formulation (F127 and P123). These combined results indicate that the supersaturated solutions of curcumin are mainly stabilized through kinetic processes (i.e. inhibition of precipitation).

Cosolvents can increase the bulk solubility and interfere with the complexation between CD and the compound (Singh et al. 2010). The results show that ethanol clearly interferes with the complexation, and thereby reduces the solubilization of curcumin compared to samples without cosolvents. On the other hand, PEG 400 does not seem to have any net influence on the solubilization of curcumin by HPγCD.

An increase of the ionic strength is reported to induce the formation of solubilizing structures by a salting out effect (Desai et al. 2001; Patel et al. 2010). However, the ionic strength did not influence the solubilization of curcumin in the systems investigated in the present work (Fig. 4).

3.4. Characterization of the film formed after the evaporation process in samples prepared by SEM

In SEM, a film on the inside of the round bottle consisting of curcumin and the respective solubilizer is dissolved by addition of buffer. While in SFM, curcumin is added as solid crystals to the formulation being investigated. Both the thermograms and micrographs show that the evaporation process induces a change in the solid state of pure curcumin. The change in the solid state of curcumin, from crystals (as were applied in SFM) to a non-crystalline solid state (as was applied in SEM), did however not significantly change the solubility of curcumin in phosphate buffer in the absence of Pluronic or CD (Table 2).

The difference in surface morphology, observed by scanning electron microscopy, between the pure compounds and the combination products may be the result of an interaction between the solubilizer and curcumin in the film formed after evaporation (Oh et al. 2011; Paradkar et al. 2004). Further, the absence of an endothermic peak corresponding to curcumin in the thermograms of the combination product of curcumin and Pluronic (P123 and F127), indicates that curcumin is in an amorphous state and forming interactions with the solubilizer (Nayak et al. 2010; Paradkar et al. 2004). The thermogram of the combination product of curcumin and CD shows that curcumin is not present in the same solid state as pure curcumin after the evaporation process, which indicates that curcumin is interacting with CD (Yallapu et al. 2010). Also, the red color of the combination products is a result of a change in electron distribution in the curcumin molecules, which may have been caused by the interactions between curcumin and the solubilizers. Further, P123 and P85 were gel-like, and no particles were observed upon visual characterization. The boiling in vacuum also indicate a non solid feature of these combination products.

Sahu et al. (2011) reported that curcumin is present in a semi-crystalline solid state in the film formed after evaporation of an organic solvent containing curcumin and F127. However, they did not report the curcumin: Pluronic molar ratio which was used in their investigation. The solid state of a compound in the film is dependent on the compound: solubilizer molar ratio (Ho et al. 2000; Paradkar et al. 2004).

Our results indicate that the film formed after evaporation is a solid dispersion of curcumin and the solubilizer. This is in accordance with the definition of solid dispersions given by Brouwers et al. (2011), i.e., amorphous particles of a poorly water-soluble compound dispersed in a hydrophilic carrier matrix.

3.5. The influence of the method of preparation on the solubilization of curcumin

The solid dispersion formed in samples prepared by SEM increased the solubilization of curcumin compared to the thermodynamic solubility (obtained by SFM) (Fig. 6). Solid dispersions induce supersaturated solutions when dissolved in buffer. This is caused by the solid state of the compound being solubilized (more or less amorphous), surface area enhancement and increased wetting (Brouwers et al. 2009; Oh et al. 2011; Paradkar et al. 2004). Thus, solid dispersion-induced supersaturation caused a higher solubilization in samples prepared by SEM compared to SFM. Further, inhibition of precipitation (i.e. kinetic processes) in samples prepared by SEM aided in maintaining high concentrations of dissolved curcumin.

There are no current reports on the use of curcumin and Pluronics to prepare solid dispersions. Paradkar et al. (2004) were the first to report the use of curcumin in a solid dispersion with polyvinylpyrrolidone (PVP), which improved the dissolution of curcumin compared to physical mixtures of curcumin and PVP or curcumin alone. Ho et al. (2002) were the first to report the use of Pluronics in solid dispersions to improve dissolution of a drug compound.

The maximum amount of curcumin solubilized (43.6 ± 1.3 mM) in the solid dispersion with CD (i.e., prepared by SEM) was > 6 times the highest concentration of solubilized curcumin previously reported in the literature (samples were prepared by a cosolvent evaporation method) (Gardikis et al. 2010), and increased the concentration of curcumin by a factor $> 10^6$ compared to the solubility in aqueous buffer (Tønnesen et al. 2002). Further, the concentration of CD needed was 12 times less by use of SEM compared to SFM in order to achieve approximately the same solubility of curcumin (Fig. 6). The large amount of CD that has to be added for solubilization of a small amount of curcumin by the conventional method of production (i.e. SFM), will be disadvantageous considering toxicology, formulation bulk and production costs (Loftsson et al. 1999). The use of SEM was an efficient way to increase the solubilization capacity and reduce the amount of solubilizer, both in the case of Pluronics and CD.

Although the samples prepared by SEM were supersaturated, the samples with curcumin: solubilizer molar ratio $\leq$ molar ratio at the maximum did not demonstrate precipitation of curcumin during the storage period (i.e., 356 h). An explanation could be that precipitation inhibition by the solubilizer increased the activation barrier for nucleation, the first step in the precipitation process, to a point were it could not be reached under the present conditions, thus forming a metastable, supersaturated solution (Brouwers et al. 2009).

However, the supersaturated samples which are physically unstable would be difficult to apply in a clinical setting unless they are prepared *ex tempore*. Therefore, further development is needed before these preparations could be used in a clinical setting. For example, further inhibition or delay of precipitation, or physical stabilization could be achieved by adding other

ingredients such as polymers like hydroxypropylmethylcellulose acetate succinate, which was proven to be one of the most efficient precipitation inhibitors among a series of excipients (Curatolo et al. 2009).

In conclusion, both Pluronics and CD are excipients which can induce the formation of supersaturated curcumin solutions through the formation of solid dispersions, when the samples are prepared by SEM. They can also be efficient precipitation inhibitors, although the Pluronics inhibit precipitation at higher curcumin: solubilizer molar ratio than CD. SEM is therefore a valuable method which can increase solubilization and bioavailability of the compound of interest. PEG also proved to be an efficient precipitation inhibitor in combination with Pluronics, while ethanol induced precipitation. Ionic strength did not interfere with the curcumin solubilization obtained by Pluronics or CD. P123 and F127 were the most efficient solubilizers and precipitation inhibitors of curcumin among the solubilizers investigated. Therefore, good solubilization of curcumin can be achieved with Pluronics possessing different physio-chemical properties, assuming that the PO-chain consists of a fair number of units (e.g. 60–70). This increases the selection of solubilizers which can be used to make an optimal formulation of curcumin for topical PDT. However, HPγCD is more water soluble than the Pluronics, and one can achieve a higher solubilization of curcumin assumed a high concentration of this CD. The bulk volume of the CD can be reduced by the application of SEM.

4. Experimental

4.1. Materials

Curcumin was synthesized according to the method given by Pabon (Pabon 1964). Pluronic P123 was purchased from Aldrich, Pluronic F127 from Sigma and Pluronic P85 was kindly donated by BASF Corporation. 2-hydroxypropyl-γ-cyclodextrin (CD) with a molar substitution of 0.5–0.7 (Cavasol® W8 HP, Mw ≈ 1576) was from Wacker Fine Chemicals. The moisture content of the CD was measured using Sartorius Moisture Analyzer MA30, and the amount of CD was corrected for the moisture content in the calculations. Ethanol 96% (v/v) was provided by Arcus. Polyethylene glycol 400 (PEG 400) was provided by Fluka. All other chemicals were commercially available substances of analytical grade or better. Water was distilled and deionized.

Phosphate buffer 0.05 M pH 5 was prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate. The ionic strength was adjusted to 0.085 M, 0.17 M or 0.34 M by addition of sodium chloride.

4.2. HPLC analysis

The mobile phase was composed of 38% (v/v) citric acid buffer 0.50% (w/v) pH 3 and 62% (v/v) methanol. The analyses were performed on a Waters Nova-Pak® C_{18}, 3. 9 x 150 mm, 4 μm particle size column by use of a Shimadzu Liquid Chromatography LC-9A pump, Shimadzu Auto Injector SIL-9A auto sampler, Shimadzu UV-Vis Spectrophotometric detector SPD-10A and a Shimadzu C-R5A integrator. The samples were detected at 350 nm.

The limit of quantification, defined as the concentration that gave a signal to noise ratio of 10, was 0.5 μM for all standards. Linearity of all standards (0.5 μM – 50 μM; n = 6) was 0.999 – 1. The precision (relative standard deviation), measured for all standards by 6 repeating injections at 0.5 μM, was 1.84–6.60%. The Pluronics were not detected at 350 nm when injected alone, and the retention time of curcumin (16.5 min) was not influenced by the presence of any of the Pluronics when compared to standards of curcumin in methanol diluted 1:1 with mobile phase.

4.3. Control of adsorption to the filter

Adsorption of curcumin to the filters was investigated by dissolving P85, P123 or F127 in 10 mL phosphate buffer in a 20 mL volumetric flask. To this solution was added 0.2 mL of a 1 mM solution of curcumin in methanol. The volume was finally adjusted to 20 mL with phosphate buffer, giving a solution with 5.2 mM Pluronic. The procedure was repeated with a 0.5 mM and 0.1 mM solution of curcumin in methanol. The final concentration of curcumin in the samples was then 10, 5 or 1 μM. One mL of each sample was filtered, and this was repeated 6 times. A new filter was used for each replicate. The concentration of curcumin before filtration and in the filtrate was determined by HPLC. The adsorption of curcumin to the filter was calculated as the % change in concentration of curcumin upon filtration.

The adsorption of curcumin in buffer with 20% (v/v) ethanol or 20% (v/v) PEG 400 and buffer without cosolvent was also investigated. 0.2 mL of a 0.1 mM solution of curcumin in methanol was added to each buffer. The final volume was 20 mL and the concentration of curcumin in each sample was 1 μM. One mL of each sample was filtered, and this was repeated 6 times. A new filter was used for each replicate. The concentration of curcumin before filtration and in the filtrate was determined by recording the UV-Vis absorption spectra (290–600 nm) on a Shimadzu UV-2401PC spectrophotometer. The adsorption of curcumin to the filter was calculated as the % change in absorption of curcumin at 420 nm upon filtration.

Filters with a membrane consisting of regenerated cellulose (Spartan 13/0.45 RC filters from Schleicher & Schull, Germany) and filters with a membrane consisting of polytetrafluorethylene (PTFE filters 13/0.45 from VWR International) were tested. Between these, the Spartan filters had less adsorption of curcumin (5.6% in samples with 5.2 mM P85, and no detectable adsorption of curcumin in samples with 5.2 mM P123 and samples with 5.2 mM F127) and was therefore applied in the solubility experiments. The adsorption of curcumin from samples containing P85 was corrected for in the solubility experiments by multiplying the concentration determined by HPLC with a factor of 1.056. The adsorption of curcumin in samples without Pluronic was 81%. The concentrations determined by HPLC in the solubility studies were therefore corrected for by multiplication of a factor 1.81.

4.4. Preparation of samples

Samples were prepared by the solvent-evaporation method and the shake-flask method. Samples that contained curcumin were protected from light throughout the preparation and storage by covering all containers containing curcumin by aluminum foil (except during the evaporation process, which was performed in the dark). All experiments were performed with 6 replicates. The experiments with Pluronics were performed at a temperature of 23 °C (± 3 °C).

4.4.1. The Solvent-evaporation method

To determine the solubility of curcumin at different molar ratios, curcumin was dissolved in acetone to give concentrations in the range 0.062–4.16 mM (experiments with F127 and P123), and 0.021–0.416 mM (experiments with P85). Each Pluronic (0.52 mM) was dissolved in acetone in separate volumetric flasks. To obtain an increased molar ratio of curcumin: Pluronic in the range 0.03–2.0 for F127 and P123 and 0.01–0.20 for P85, 10 mL of the appropriate solution of curcumin in acetone were added to 40 mL of the solution of Pluronic dissolved in acetone in a 100 mL round bottle. The organic solvent was then evaporated on a Büchi EL 131 Rotavapor equipped with a Büchi 461water bath at 40 °C for 6 min.

Four mL of the appropriate buffer was added immediately after evaporation of the organic phase, and the round bottle was placed on an Edmund Bühler agitation device at 125 motions/minute. The concentration of Pluronics in the final solution was 5.2 mM.

The sample (1 mL) was analyzed immediately after 20 h agitation. Two x 1 mL were withdrawn from the remaining solution and stored at room temperature for 188 and 356 h, respectively, protected from light by covering the containers with aluminum foil. The samples were filtered through a Spartan 13/0.45 RC filter (Schleicher & Schull, Germany). A selected volume of the filtrate was diluted with methanol and mobile phase prior to analysis by HPLC.

CD (6.4 and 0.52 mM) was dissolved in methanol, as it was more soluble in this organic solvent than in acetone. Curcumin was dissolved in acetone in a separate volumetric flask to give concentrations in the range 2.56–41.0 mM (experiments with 64 mM CD), and 0.21–3.33 mM (experiments with 5.2 mM CD). To obtain an increased molar ratio of curcumin: CD in the range 0.1–1.6, 10 mL of the appropriate solutions of curcumin in acetone were added to 40 mL of a solution of CD in methanol (6.4 and 0.52 mM) in a 100 mL round bottle. The samples were further prepared as described above, except for an increase in evaporation time up to 20 min.

The solubility of curcumin in the buffer without solubilizer was also measured. Curcumin (6.52 mM) was dissolved in acetone, 10 mL of the solution of curcumin was added to 40 mL of acetone in a 100 mL round bottle and further prepared as described above.

4.4.2. The Shake-Flask Method (SFM)

The Pluronics (5.2 mM) or CD (64.0 or 5.2 mM) were dissolved in the phosphate buffer. Two mL of each solution were transferred to 5 mL vials containing 6 mg of curcumin. The vials were sealed and agitated (Edmund Bühler agitator at 125 motions/minute) for 7 days. One mL of the samples was withdrawn from the vials and filtered through a Spartan 13/0.45 RC filter

(Schleicher & Schull, Germany). The filtrates were diluted with methanol and phosphate buffer prior to analysis by HPLC.
The solubility of curcumin in buffer in the absence of solubilizer was also measured. The samples were prepared as described above.

4.5. Characterization of the film formed after the evaporation process in samples prepared by SEM

4.5.1. Preparation of the samples

Each solubilizer was dissolved in organic solvent (Pluronics were dissolved in acetone and CD was dissolved in methanol) to a final concentration of 0.52 mM. Curcumin was dissolved in acetone to make solutions with concentrations of curcumin between 0.06–1.25 mM. To obtain curcumin: solubilizer molar ratios of 0.03 (curcumin: P85), 0.3 (curcumin: F127), 0.5 (curcumin: P123) and 0.6 (curcumin: CD), 10 mL of the appropriate concentration of curcumin in acetone was added to 40 mL of the solubilizer dissolved in organic solvent, in a 100 mL round bottle. Samples consisting of each solubilizer (P85, F127, P123 and CD) without curcumin were also prepared by adding 10 ml acetone to 40 ml of the organic solvent containing solubilizer. Finally, a sample consisting of curcumin without solubilizer was prepared by dissolving 10 mg curcumin in 50 mL acetone. The samples were further prepared as described in section 4.4, but instead of adding buffer, the dry film was scraped off the round bottle using a spatula. The samples were protected from light throughout the preparation by performing the evaporation process in the dark and covering all containers with aluminum foil.

4.5.2. Scanning electron microscopy

The samples were sputter coated with platinum before electron microscopy was performed by an S 4800 scanning electromicroscope from Hitachi.

4.5.3. Differential scanning calorimetry (DSC)

Mettler Toledo DSC822^e was applied to analyze 5 mg of each sample. The instrument was calibrated using indium. The samples were scanned in the temperature range 50–200 °C at 10 °C/minute. All samples were analyzed in triplicate. DSC was not performed on the combination of P85 and curcumin, as the amount of curcumin after the evaporation process (theoretically 2.6 μg) could not be detected by the calorimetric method. Only 40 μg of the film consisting of curcumin without solubilizer was analyzed, as this was equal to the smallest amount of curcumin that theoretically could be present in 5 mg of the samples containing a solubilizer.

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