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

Influence of vehicle properties and excipients on hydrolytic and photochemical stability of curcumin in preparations containing Pluronics: Studies of curcumin and curcuminoids XLVIII

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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The influence of vehicle properties and excipients on the hydrolytic and photochemical stability of curcumin in Pluronic preparations, and the interactions between curcumin and Pluronics was investigated. Curcumin was found to be degraded by general acid-base catalyzed hydrolytic degradation in alkaline preparations. The degradation rate of curcumin was higher in carbonate buffer than in phosphate buffer (pH 8.8), while it was higher in phosphate buffer than in citrate buffer (pH 7.8). At pH 8.0–8.8 the degradation rate of curcumin increased compared to preparations with pH < 8.0. The stabilizing effect of the Pluronics against hydrolytic degradation of curcumin was only detectable at pH 8.0–8.8, and it was highest for F127 and lowest for P85, in phosphate buffer pH 8.8. An increase in the ionic strength increased the stabilization against hydrolytic degradation of curcumin by all Pluronics. Addition of ethanol decreased the hydrolytic stability of curcumin in all Pluronics. Addition of PEG 400 decreased the hydrolytic stability in preparation with either P123 or F127 while the degradation in preparations with P85 remained the same as in P85 preparations without PEG 400. Vehicle properties and excipients did not to any large degree influence the spectroscopic properties or the photostability of curcumin in Pluronic preparations. Photochemical half life of curcumin was in the minutes range. Spectrophotometric data indicate that Pluronic aggregates most likely solubilize curcumin through hydrophobic interactions, although hydrogen-bonding may also be involved.

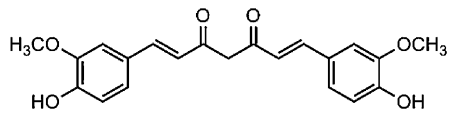
1. Introduction

Curcumin (Fig. 1A) can be used as a photosensitizer in topical photodynamic therapy; PDT (Dahl et al. 1994; Haukvik et al. 2009; Hegge et al. 2011; Tønnesen et al. 1987). However, curcumin is practically insoluble in aqueous solution (< 50 nM in phosphate buffer 0.05 M pH 5), and when dissolved, it is exposed to hydrolytic and photochemical degradation (Tønnesen and Karlsen 1985a; Tønnesen et al. 1986, 2002). The use of curcumin in PDT would therefore require a formulation which could both solubilize and stabilize the compound.

Pluronic® is a trademark for block copolymers which consists 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 (Fig. 1B). 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 2009a). Several Pluronics are approved by the FDA as pharmaceutical excipients for various administration routes (FDA 2009). We have previously investigated the influence of Pluronics on the solubilization of curcumin (Singh et al. 2012). Our results showed that Pluronics (F127, P123 or P85) were efficient solubilizers of curcumin. The thermodynamic solubility of curcumin in preparations containing Pluronics was increased > 100–1000 times compared to the solubility in aqueous solutions without solubilizers, and even higher concentrations of curcumin could be achieved by forming supersaturated solutions through the formation of solid dispersions of curcumin in Pluronic (Singh et al. 2012). We did not observe any degradation of curcumin when stored light protected for up to 356 h, at 23 ± 3 °C in preparations containing Pluronics (Singh et al. 2012). To our knowledge, there are no studies on the influence of Pluronics on the stability of aqueously dissolved curcumin.

The stability of freeze-dried formulations of curcumin and F127, and curcumin and F68, has recently been reported. The amount of curcumin remaining after 3 months storage at 25 °C was 95.8 % and 79.6 % for F127 and F68, respectively (Sahu et al. 2011). The stability of aqueous curcumin preparations containing Pluronics is however of interest in order to determine the shelf-life after reconstitution of e.g., a freeze dried product or to prepare a liquid curcumin formulation suitable for storage. Information on the influence of vehicle properties or excipients, such as pH, buffer system, buffer concentration, ionic strength, and cosolvents, on the stability of curcumin-Pluronic solutions could further be used to optimize the stability of the preparations. In the present work the influence of these formulation parameters on the stability of curcumin in preparations containing Pluronics was investigated. Pluronics P85, P123 and F127 were selected as they are efficient solubilizers of curcumin (Singh et al. 2012).

A



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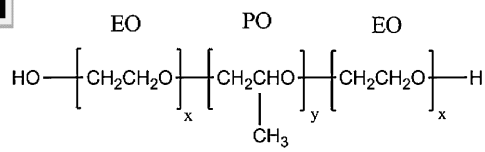


Pluronic	A-B-A structure	Mw	CMC (25°C)
P85	$EO_{26}-PO_{40}-EO_{26}$	4600	230 μM
F127	$EO_{100}-PO_{65}-EO_{100}$	12600	69 μM
P123	$EO_{20}-PO_{70}-EO_{20}$	5750	49 μM

*CMC was determined at 25°C by use of a fluorescence probe technique (Hioka et al., 2002;Sezgin et al., 2006)

Fig. 1: A; Chemical structure of curcumin. B; Chemical structures, molecular weight (Mw) and critical micelle concentration (CMC) at 25 °C of selected Pluronics. The physical form of the Pluronic is indicated 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 2009b)

2. Investigations and results

2.1. Absorption and fluorescence properties

The influence of ionic strength, phosphate buffer concentration, buffer system (phosphate buffer or citrate buffer) and cosolvents (20% (v/v) ethanol or PEG 400) on the interactions between curcumin and Pluronic was investigated by absorption and fluorescence emission measurements. The absorption- and fluorescence emission spectra were recorded and the curcumin fluorescence quantum yield was determined. The absorption and fluorescence emission spectra of curcumin were similar in all buffer systems (Fig. 2A and B). The absorption maximum was 428 nm, while the fluorescence maximum was 504–506 nm, except for preparations containing 20% (v/v) ethanol, where the maximum was 511 nm (Fig. 2C). The absorption and fluorescence maxima were in agreement with values previously reported (Sahu et al. 2011). The fluorescence quantum yield remained also constant in the different buffer systems (Fig. 2C).

2.2. Reaction kinetics

Verification of the reaction order kinetics was obtained from regression analysis of zero-, first-, and second-order kinetic plots. The best fit was obtained ($r^2 > 0.943$) by use of first-order kinetics in all the samples. The process was followed to at least 50% conversion. The observed first-order degradation rate constants and half-lifes were calculated.

2.3. The pH-rate profile

The hydrolytic pH-rate profile of curcumin (20 μM) was investigated in preparations containing 5.2 mM Pluronic F127, P123 and P85 at 25 ± 0.1 °C, presented in Fig. 3. General acid-base catalysis will influence the results, as further discussed. The observed first order degradation rate ($r^2 = 0.957–0.999$) of curcumin seemed to be independent on pH and type of Pluronic up to pH 5.7. A slight increase in degradation rate was observed in the pH range 5.7–8.0, followed by a large increase above pH 8.0–8.8 and showing a difference in stabilizing effect of the Pluronics (Fig. 3). The observed first order degradation rate of curcumin increased by factors 6.1, 5.8 and 3.6 in preparations containing P85, P123 and F127 respectively, when the pH was increased from 8.0 to 8.8. In comparison, the observed degradation rate increased only by a factor of 1.1–2.0 when the pH was increased from 6.8 to 8.0. The pH-rate profile also showed that the first order degradation rate was lowest at pH < 6 where the half life of curcumin was between 47–444 days depending on the type of Pluronic (Fig. 3).

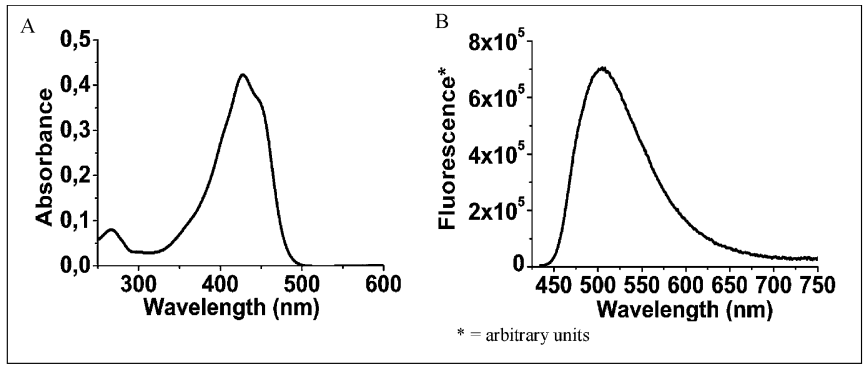
The relative influence of the three Pluronics (P85, P123 and F127) on the observed degradation rate of curcumin was only detectable at pH > 8.0 (Fig. 3). The degradation was followed up to pH 8.8, and at this pH the degradation rate of curcumin was ~ 1.4 times higher in preparations containing P123 compared to preparations containing F127. The degradation rate of curcumin in preparations containing P85 was ~ 1.7–2.8 times higher than in preparations containing P123 or F127 in the pH range 8.0–8.8 (Fig. 3).

2.4. General acid-base catalysis

The influence of the concentration of phosphate buffer (0.02–0.1 M) at pH 8.8 and 25 ± 0.1 °C, on the observed first order degradation rate ($r^2 = 0.943–0.999$) of curcumin was investigated in preparations containing Pluronic P85, P123 or F127. The results presented in Fig. 4 show a linear increase in degradation rate of curcumin with increasing concentration of phosphate buffer in all the samples ($r^2 = 0.999$, 0.995 and 0.974 for formulations containing P85, P123 and F127, respectively) (Fig. 4). The dependence of the degradation rate on buffer concentrations as well as pH indicates general acid-base catalysis. The slope of the linear curves were 0.37, 0.27 and 0.23 for preparations containing P85, P123 and F127 respectively, showing that P85 offered the least protection of curcumin towards hydrolytic degradation as a function of buffer concentration. The extrapolated values of the regression lines to zero buffer concentrations at pH 8.8 represent the sum of the specific base catalyzed and water catalyzed hydrolysis. As the extrapolated $k_{obs} = 0$ in the presence of all Pluronics in buffer free media pH 8.8, the presence of buffer salts seems to be a prerequisite for the hydrolysis to run.

2.5. Kinetic salt effects

The influence of ionic strength (μ = 0.15–0.30) at pH 8.8 and 25 ± 0.1 °C, on the observed first order hydrolytic degradation rate ($r^2 = 0.962–0.999$) of curcumin was investigated in aqueous preparations containing 0.05 M phosphate buffer and Pluronic P85, P123 or F127. The hydrolytic degradation rate decreased with increasing ionic strength (Fig. 5). The decrease in the degradation rate with increasing ionic strength was linear for preparations containing P85 ($r^2 = 0.999$) and P123 ($r^2 = 0.973$), while linearity was not that apparent for preparations containing F127 ($r^2 = 0.883$) (Fig. 5). Further, the influence of ionic strength on the degradation rate had a negative slope of −0.25, −0.11 and −0.10 for preparations containing P85, P123 and F127 respectively, showing that the increase in ionic strength had a stronger effect on hydrolytic stabilization of curcumin in preparations containing P85 than in preparations containing P123 or F127. Still the degradation rate of curcumin in preparations containing P85 was generally ~ 2.0–2.2 times higher than the degradation rate of curcumin in preparations containing P123, while it was



C

Buffersystem	Absorbance (nm)	Emission (nm)	$\phi_{Fluorescence}$
Phosphate buffer 0.05M, μ = 0.30, pH 4.80	428 nm	505 nm	0.042 (0.041-0.043)
Phosphate buffer 0.05M, **μ = 0.15**, pH 4.80	428 nm	506 nm	0.042 (0.041-0.042)
Phosphate buffer **0.1M**, μ = 0.30, pH 4.80	428 nm	506 nm	0.041 (0.039-0.043)
Citrate buffer 0.05M, μ = 0.30, pH 4.80	428 nm	504 nm	0.041 (0.040-0.042)
Phosphate buffer 0.05M, μ = 0.30, pH 4.80, **20% Ethanol**	428 nm	511 nm	0.042 (0.041-0.043)
Phosphate buffer 0.05M, μ = 0.30, pH 4.80, **20% PEG 400**	428 nm	505 nm	0.040 (0.039-0.040)

Fig. 2: Absorption (A) and fluorescence emission (B) spectra of curcumin (7 μM) in phosphate buffer 0.05 M, μ = 0.30, pH 4.8 containing 5.2 mM Pluronic P123 at 25 ± 0.1 °C. C; Absorption and fluorescence maximum (nm) and curcumin (7 μM) fluorescence quantum yield ($\Phi_{Fluorescence}$) in different buffer systems containing 5.2 mM P123 at 25 ± 0.1 °C. The minimum and maximum quantum yields are presented in brackets (n = 3)

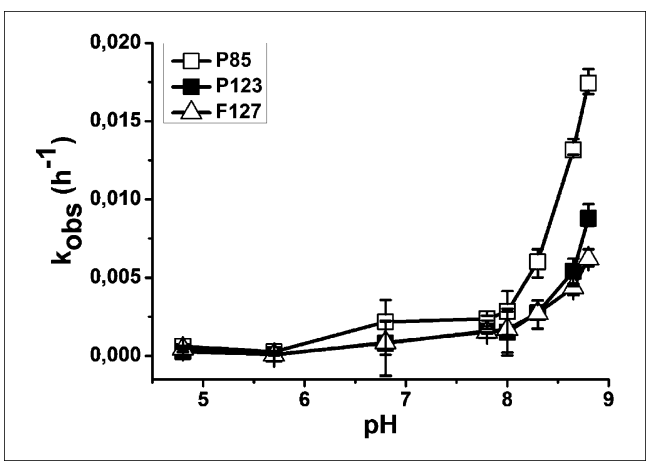


Fig. 3: The pH-rate profile for the observed first order hydrolytic degradation rate of curcumin (k_{obs}) in phosphate buffer (0.05 M), μ = 0.30, pH 4.8–8.8, containing 5.2 mM Pluronic F127, P123 or P85. The error bars represent the extreme values (n = 3)

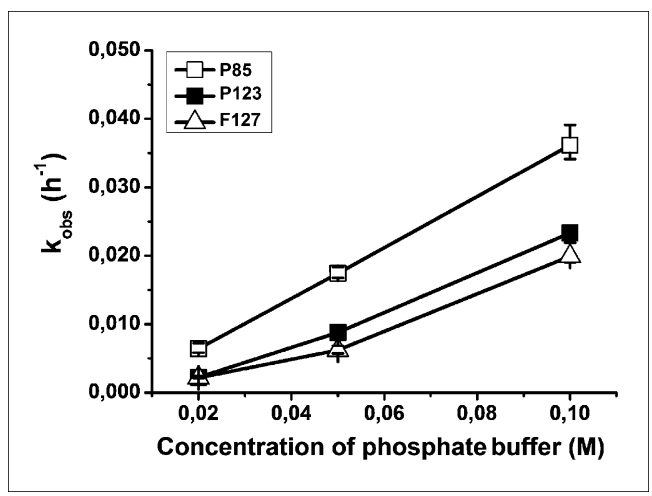


Fig. 4: Observed first order hydrolytic degradation rate of curcumin (k_{obs}) as a function of phosphate buffer concentration (0.02–0.1 M), μ = 0.30, pH 8.8, containing 5.2 mM Pluronic F127, P123 or P85. The error bars represent the extreme values (n = 3)

~ 2.5–3.7 times higher than the degradation rate of curcumin in preparations containing F127.

2.6. The influence of buffer system on general acid-base catalysis

The observed hydrolytic degradation rate of curcumin in preparations containing Pluronics (P85, P123 or F127) was investigated in three different buffer systems; phosphate buffer (pH 7.8 and 8.8), citrate buffer (pH 7.8) and carbonate buffer (pH 8.8) at 25 ± 0.1 °C. In addition, the hydrolytic stability of curcumin was also investigated in two relevant physiological buffers at 37 ± 0.1 °C, i.e., phosphate buffer saline (PBS) at pH 7.4 containing 0.01 M HEPES and PBS at pH 6.1, respectively. The degradation rate of curcumin in phosphate buffer was compared to the degradation rate of curcumin in citric acid buffer or carbonate buffer at both pH 7.8 and pH 8.8, respectively, in order to avoid exceeding the buffer capapcity of these buffers. Altough the buffer capacity was quite low (~ 0.02 M per pH unit in phosphate buffer pH 7.8, while it was ~ 0.003–0.004 M for the other three buffer systems) the pH of the buffer system remained constant during the experiments.

The observed first order degradation rate constant of curcumin (r^2 = 0.974–0.999) was 9.4; 17.1; and 16.9-fold higher in prepa-

Table 1: Observed first order hydrolytic degradation rate of curcumin in preparations containing 5.2 mM Pluronic P85, P123 or F127 dissolved in PBS (BioWhittakers, Lonza) at pH 6.10 or Dulbeccos PBS (PAA) at pH 7.4 containing 0.01 M HEPES, at 37 ± 0.1 °C

	PBS (BioWhittakers, Lonza) pH 6.10		Dulbeccos PBS (PAA) pH 7.40 containing 0.01 M HEPES	
Pluronic	k_{obs} (h^{-1}) (37 °C)	$t_{1/2}$ (h)	k_{obs} (h^{-1}) (37 °C)	$t_{1/2}$ (h)
P85	0.00054 (0.00057–0.00052)	53.7	0.004 (0.0046–0.0037)	7.2
P123	0.0003 (0.00035–0.00027)	96.7	0.0022 (0.0024–0.0019)	13.1
F127	0.00047 (0.00051–0.00040)	61.6	0.003 (0.0038–0.0026)	9.6

The degradation rate of curcumin is presented as the observed first order rate constants (hours^{-1}), with minimum and maximum values in brackets (n = 3), and the half-lifes ($t_{1/2}$ (hours).

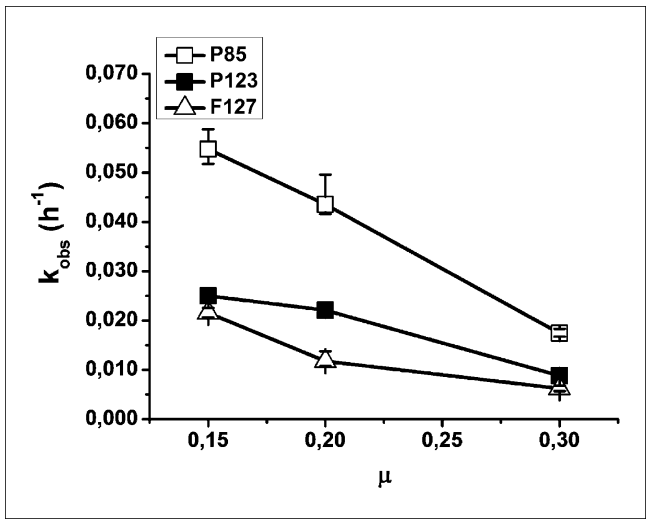


Fig. 5: Observed first order hydrolytic degradation rate of curcumin (k_{obs}) in phosphate buffer (0.05 M, pH 8.8) as a function of ionic strength (μ = 0.15–0.30), containing 5.2 mM Pluronic F127, P123 or P85. The error bars represent the extreme values (n = 3)

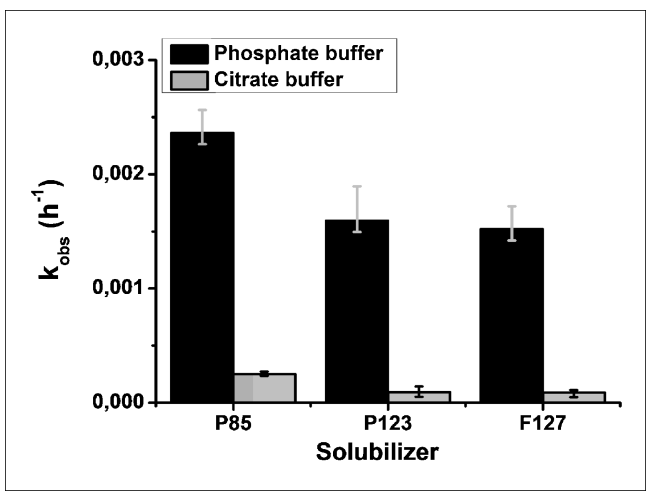


Fig. 6: Observed first order hydrolytic degradation rate of curcumin (k_{obs}) in preparations containing 5.2 mM Pluronic P85, P123 or F127 and two different buffer systems; phosphate buffer (0.05 M), μ = 0.30, pH 7.8 and citrate buffer (0.05 M), μ = 0.30, pH 7.8. The error bars represent the extreme values (n = 3)

rations containing phosphate buffer pH 7.8 and P85, P123 or F127, respectively, compared to preparations containing citrate buffer pH 7.8 and P85, P123 or F127 (Fig. 6). The observed first order degradation rate constant of curcumin (r^2 = 0.969–0.999) was 1.9-, 1.6- and 3.2-fold higher in preparations containing carbonate buffer pH 8.8 and P85, P123 or F127, respectively, compared to preparations containing phosphate buffer pH 8.8 and P85, P123 or F127 (Fig. 7). The observed first order

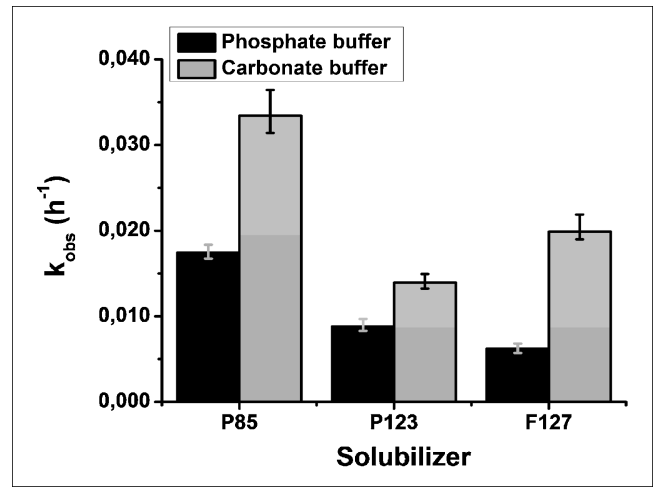


Fig. 7: Observed first order hydrolytic degradation rate of curcumin (k_{obs}) in preparations containing 5.2 mM Pluronic P85, P123 or F127 and two different buffer systems; phosphate buffer (0.05 M), μ = 0.30, pH 8.8 and carbonate buffer (0.05 M), μ = 0.30, pH 8.8. The error bars represent the extreme values (n = 3)

degradation rate constant of curcumin (r^2 = 0.945−0.999) at 37 ± 0.1 °C in preparations containing Pluronics and PBS at pH 7.4 containing 0.01 M HEPES, or Pluronics and PBS at pH 6.1 are presented in Table 1.

2.7. The influence of cosolvents on hydrolytic stability

The influence of cosolvents (20% (v/v) ethanol or PEG 400) on the hydrolytic degradation rate of curcumin was investigated at 25 ± 0.1 °C in buffered preparations (phosphate buffer 0.05 M, pH 8.8) containing Pluronics. The results, presented in Fig. 8, show that ethanol increased the degradation rate of curcumin by a factor ∼ 2–3 compared to preparations without cosolvent in all samples where the Pluronics were present. Further, PEG 400 caused an increase in the degradation rate of curcumin in preparations containing Pluronics compared to the preparations without cosolvent, except for preparations containing P85 where the degradation rate of curcumin remained unchanged. The degradation rate of curcumin in preparations containing PEG 400 and Pluronic was quite similar and independent on the type of Pluronic.

2.8. Photostability

The influence of concentration of buffer (pH 4.8), ionic strength, cosolvents and various buffer systems (phosphate buffer pH 4.8, citrate buffer pH 4.8, PBS at pH 6.1 and PBS at pH 7.4 containing 0.01 M HEPES) on the photodegradation of curcumin were investigated in preparations containing Pluronics (F127, P123 or P85) (Table 2). Phosphate buffer (0.05 M) pH 4.8, μ = 0.3,

Table 2: Influence of the concentration of buffer, ionic strength, cosolvents and various buffer systems on the photochemical degradation of curcumin in preparations containing 5.2 mM Pluronic F127 (dark grey), P123 (white) or P85 (light grey)

	Buffer		k_{obs} (min^{-1})	$t_{1/2}$ (min)
REFERENCE BUFFER	Phosphate buffer 0.05M	F127	0.105 (0.110–0.101)	6.6
	$\mu = 0.30$, pH 4.80	P123	0.08 (0.103–0.060)	8.7
		P85	0.09 (0.113–0.074)	7.7
CONCENTRATIION OF BUFFER	Phosphate buffer 0.02M		0.112 (0.131–0.106)	6.2
	$\mu = 0.30$, pH 4.80		0.074 (0.092–0.065)	9.4
	Phosphate buffer 0.10M		0.107 (0.112–0.104)	6.5
	$\mu = 0.30$, pH 4.80		0.088 (0.094–0.081)	7.9
			0.075 (0.092–0.064)	9.2
			0.104 (0.121–0.092)	6.7
IONIC STRENGTH	Phosphate buffer (0.05M)		0.121 (0.124–0.118)	5.7
	$\mu = 0.15$, pH 4.80		0.093 (0.104–0.082)	7.5
	Phosphate buffer (0.05M)		0.130 (0.135–0.092)	5.3
	$\mu = 0.20$, pH 4.80		0.085 (0.090–0.078)	8.2
			0.092 (0.097–0.084)	7.6
			0.110 (0.129–0.094)	6.3
COSOLVENTS	Phosphate buffer 0.05M		0.061 (0.067–0.054)	11.3
	$\mu = 0.30$, pH 4.80		0.072 (0.079–0.064)	9.6
	20% (v/v) ethanol		0.051 (0.054–0.046)	13.6
	Phosphate buffer 0.05M		0.064 (0.067–0.060)	10.8
	$\mu = 0.30$, pH 4.80		0.058 (0.061–0.054)	12.0
	20% (v/v) PEG 400		0.064 (0.071–0.059)	10.8
BUFFERSYSTEM	PBS (BioWhittakers, Lonza) pH 6.10		0.138 (0.141–0.034)	5.0
			0.086 (0.092–0.081)	8.1
			0. 305 (0.311–0.294)	2.3
	Dulbeccos PBS (PAA) pH 7.40, 0.01 M HEPES		0.133 (0.138–0.127)	5.2
			0.122 (0.126–0.118)	5.7
			0.191 (0.201–0.177)	3.6
	Citrate buffer 0.05M $\mu = 0.30$, pH 4.80		0.075 (0.082–0.071)	9.3
			0.088 (0.094–0.081)	7.8
			0.113 (0.119–0.092)	6.1

The influence of the concentration of buffer, ionic strength and cosolvents on the photochemical stability of curcumin was only investigated in phosphate buffer at pH 4.8. The photochemical degradation rate of curcumin is presented as the observed first order photochemical rate constants (k_{obs} (min^{-1})), corrected for differences in absorbtivity. Minimum and maximum values are in brackets (n = 3), and the calculated half-lifes ($t_{1/2}$ (min)) are presented.

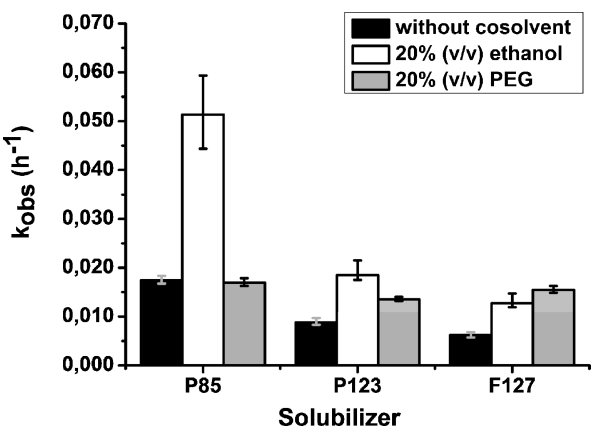


Fig. 8: Observed first order hydrolytic degradation rate of curcumin (k_{obs}) in preparations containing 5.2 mM Pluronic P85, P123 or F127 dissolved in phosphate buffer (0.05 M), $\mu = 0.3$, pH 8.8 with and without cosolvents; 20% (v/v) ethanol or PEG 400. The error bars represent the extreme values (n = 3)

was selected as a reference buffer system, because this buffer resulted in a high hydrolytic stability of curcumin. Curcumin is non-ionic at pH 4.8. Experiments were not performed with carbonate buffer as this buffer is not commonly applied at pH 4.8. The absorbance at the absorption maximum was 428 nm. The photodegradation apparently followed first order degradation kinetics ($r^2 = 0.992$–0.999). The observed half-lifes ($t_{1/2}$) were between 2.3–13.6 min. Dark reactions were not observed due to low pH and/or short experimentation time (each experiment was performed within 30 min). An increase in ionic strength or buffer concentration did not influence the photodegradation rate to any large extent. It was difficult to observe any clear difference in photodegradation rate between the three Pluronics. However, curcumin seems in most cases to have a slightly higher photostability in P123 compared to F127 and P85 (Table 2). The photostability of curcumin was slightly increased in preparations containing cosolvents compared to preparations without cosolvents (Table 2). The lowest photostability of curcumin was observed in PBS. There was apparently no difference between phosphate and citrate buffer regarding the observed photodegradation rate of curcumin.

3. Discussion

3.1. Interactions between curcumin and Pluronic and the influence of the buffersystems

Interpretation of the spectrophotometric data shows that the microenvironment around curcumin remained relatively constant although the buffer system was varied. This indicates that curcumin has a strong interaction with Pluronic and is thereby protected from the aqueous bulk. The most likely location would be the hydrophobic part of the Pluronic aggregates providing protection from the aqueous environment by the hydrophilic corona (Nagarajan 1999). The fluorescence emission spectra, fluorescence maximum and fluorescence quantum yield are reported to be more sensitive to the microrenvironment around curcumin than the absorption parameters (Nardo et al. 2008).

A small redshift in fluorescence maximum was observed in Pluronic preparations with phosphate buffer (0.05 M, $\mu = 0.3$, pH 4.8) and 20% (v/v) ethanol compared to Pluronic preparations with phosphate buffer and without ethanol. This shift may be caused by a change in the polarity of the medium resulting in a transfer of curcumin from the hydrophobic core of the Pluronic aggregates to the bulk solvent (where it can exist in free form or bound to Pluronic unimers) or the hydrophilic part of the Pluronic aggregates. Pluronic aggregates exist in a dynamic equilibrium with Pluronic monomers, which will be influenced by the external ethanol content. This equilibrium may also influence the distribution of curcumin between the hydrophobic core of the Pluronic aggregates and the bulk solvent or the hydrophilic part of the Pluronic aggregates. Hydrolytic degradation of curcumin will most likely occur outside the hydrophobic core of the Pluronic aggregates. Different buffer-salts or –concentrations or a change in ionic strength may induce changes in the position of the dynamic equilibrium between Pluronic aggregates and monomers and/or the distribution of curcumin throughout the sample, but such changes seemed too small to be detected by the spectroscopic measurements under the present experimental conditions. The presence of different populations of curcumin will be further investigated by time-resolved fluorescence spectroscopy. It should also be mentioned that the concentration of curcumin in the spectrophotometric investigations is lower than in the studies of hydrolytic stability, for practical reasons. A higher concentration of curcumin could increase the amount of curcumin being located outside the hydrophobic core of the Pluronic aggregates as the hydrophobic core could be saturated with curcumin. More curcumin will then be available to attack by nucleophiles in the aqueous bulk and/or in the hydrated PEO chains and thus more sensitive to changes in the buffer system. Also, as there is a dynamic exchange of curcumin between the core of the Pluronic aggregates and the aqueous bulk, degradation of curcumin in the bulk provides a concentration gradient resulting in continous diffusion of curcumin from the core of the Pluronic aggregates into the aqueous bulk. This degradation-dependent continuous diffusion will facilitate the detection of curcumin degradation and it could explain why the influence of vehicle properties and excipients on the hydrolytic degradation of curcumin could be detected while the influence of vehicle properties and excipients on the spectroscopic properties of curcumin could not (no continuous diffusion of curcumin from the core to the aqueous bulk).

Pluronics mainly solubilize compounds by hydrophobic interactions, although there are reports on the presence of hydrogen bonds between solubilizate and Pluronic, the latter acting as a hydrogen bond acceptor (Bugrin et al. 2007; Kozlov et al. 2000). Hydrogen bond accepting solvents e.g., dimethyl sulfoxide (DMSO) and dimethyl formamide (DMFA) are previously demonstrated to cause a red shift in absorption and fluorescence maximum of curcumin compared to non-polar solvents e.g., cyclohexane (Nardo et al. 2008). Hydrogen bond accepting solvents interacts with the enolic proton, perturbing the intramolecular hydrogen bond in the keto-enol moiety when in the cis-enol conformation of curcumin (which is predominant in solution) and causing a less rigid structure which is prone to out of plane vibration. This finally results in large Stokes shifts compared to solvents which do not perturb the intramolecular hydrogen bond (Nardo et al. 2008). The absorption maximum and fluorescence quantum yield of curcumin in Pluronics are relatively similar to the absorption maximum and fluorescence quantum yield of curcumin in the hydrogen bond accepting solvents previously investigated, e.g., DMSO and DMFA; the values being Abs. = 434 nm and 431 nm, and $\Phi_{Fl.} = 0.026$ and 0.041, respectively. This indicates that Pluronic to a certain extent causes perturbation of the intramolecular hydrogen bond in the keto-enol moiety of curcumin, and that intermolecular H-bond formation cannot be ruled out. The Stokes shift in case of curcumin in Pluronic (i.e., 3.8×10^4 cm^{-1} in ethanolic buffer and $3.5-3.6 \times 10^4$ cm^{-1} in the other buffers) is however, smaller than in the above solvents (i.e., 4.5×10^4 cm^{-1} and 4.8×10^4 cm^{-1} in DMFA and DMSO, respectively). This could indicate that curcumin is located in a microenvironment which restricts the vibrational flexibility of curcumin (i.e., the densely packed hydrophobic core of the Pluronic aggregates) as the Stokes shift is related to the flexibility for out of plane vibrations.

3.2. Influence of the properties of Pluronics on the stabilization of curcumin against hydrolytic degradation

The stabilizing effect of the Pluronics investigated against hydrolytic degradation of curcumin was F127 > P123 > P85 (in phosphate buffer or citrate buffer). It is mainly the size of the hydrophobic part of the Pluronic polymer that determines both the degree of aggregation (i.e., number of aggregated solubilizing structures) and the strength of the interaction between a drug molecule and Pluronic, two parameters which may increase protection against hydrolytic degradation (Alexandridis et al. 1994; Kozlov et al. 2000; Singh et al. 2012). Thus, curcumin could be expected to be more stable in the presence of F127 than P85 as observed, assumed that the concentration of the Pluronic is above the CMC. The hydrophilic corona also seems to influence the stability as the hydrophobic part (polypropylene block) of P123 and F127 is approximately of the same size, but F127 has a larger overall molecular weigth due to the large polyethylenoxide blocks (Fig. 1B). This may lead to an increase in microviscosity and thereby a decrease in diffusion rate of the reacting species resulting in a decrease in the degradation rate of curcumin (Kirkeminde et al. 2011; Nakashima et al. 2001).

The increased slope in the pH-rate profile at pH > 8.0 is most likely due to the ionization of curcumin. The first pKa of curcumin has previously been reported in the range 7.75–7.8, and it was observed that the ionization of curcumin resulted in and increased degradation rate (Tønnesen and Karlsen 1985a). However, according to Fig. 3 it seems like the pKa value of curcumin is slightly increased (pKa $\geq$ 8.0) in preparations containing Pluronics compared to the previously reported pKa value in preparations without solubilizers. This phenomenon has also been observed in preparations containing other amphiphilic solubilizers (Tønnesen 2002).

Previous studies performed at pH 8.0 have demonstrated that the hydrolytic stability of curcumin increased > 100 times in preparations containing hydroxy propyl-β-cyclodextrin, while it increased > 1000 times in preparations containing surfactants such as Triton X-100 or sodium dodecyl sulfate, when compared to the hydrolytic stability in preparations without solubilizers (Tomren et al. 2007; Tønnesen 2002; Tønnesen et al. 2002). The observed half-life of curcumin was however, found to be 14–26 and 1.4–2.6 times higher in samples containing Pluronics (P85, P123 or F127) compared to samples containing hydroxy propyl-β-cyclodextrin and Triton X-100, respectively, although the ionic strength was lower (μ= 0.085), the temperature higher (30 °C), and the concentrations of curcumin and solubilizers different from the present experiments. Pluronics are therefore considered efficient stabilizers of curcumin with respect to hydrolytic degradation also when compared to the solubilizers investigated so far.

3.3. Influence of phosphate buffer on the hydrolytic stability of curcumin in preparations containing Pluronics

The influence of phosphate buffer concentration on the degradation rate of curcumin at constant pH (i.e., 8.8) in preparations

containing Pluronics show that general acid-base catalysis plays a role in the hydrolytic degradation. An increase in buffer concentration from 0.02 to 0.10 M (i.e., 5 times) increased the observed degradation rate 6–11 times (depending on the type of Pluronic) when curcumin was ionized. The extrapolated values at pH 8.8 (ref. section 2.4) show that the specific base catalysed hydrolysis and the uncatalyzed processes are very low or nonexistent. Possibly, these processes can be masked by general acid-base catalysis which is dominating the degradation process. The pH-rate profile is highly influenced by the general acid-base catalysis. The ratio between the basic (HPO_4^{2-}) and acidic ($H_2PO_4^-$) form of phosphate ($pKa_2 = 7.2$) increased > 1500 times in the pH range between 4.8–8.0, but without resulting in an apparent increase of the degradation rate of unionized curcumin. From pH 8.0 to 8.8 the $HPO_4^{2-}/H_2PO_4^-$ ratio increased ~ 6 times at the same time as the observed degradation rate of curcumin increased ~ 4–6 times (depending on the type of Pluronic). These data show that under the given experimental conditions (i.e., in the presence of Pluronics) general acid-base catalysis seems to be of crucial importance to the degradation of curcumin when curcumin is ionized at pH ≥ 8.0.

The observed general acid-base catalyzed degradation of curcumin in preparations containing phosphate buffer does not correspond to the results reported in the literature (Tønnesen and Karlsen 1985a; Wang et al. 1997). However, previous studies were performed in aqueous phosphate buffer without solubilizers and the hydrolytic degradation occurred in the order of minutes, while the hydrolytic degradation occurred in the order of hours in preparations containing Pluronics. Therefore, retardation of the degradation rate of curcumin by the Pluronics may have facilitated detection of the general acid-base catalyzed reaction in these preparations.

3.4. Influence of ionic strength on the hydrolytic stability of curcumin in preparations containing Pluronics

According to the Brønsted-Bjerrum equation (the modified version was applied in the case of high ionic strength, i.e., $\mu \geq 0.3$), the decrease in degradation rate with increasing ionic strength would imply that an ionic form of the compound of interest is degraded by a species with an opposite charge due to the primary kinetic salt effect (Carstensen 1970; Florence et al. 2006). Plotting the data according to the Brønsted-Bjerrum equation resulted in linear plots with negative slopes ($r^2 = 0.984–0.999$) for all three Pluronics, indicating that negatively charged curcumin is degraded by a species with a positive charge. However, these observations do not correspond to the previously postulated degradation mechanism of curcumin as a nucleophilic attack by OH^- (in preparations without solubilizers) (Tønnesen and Karlsen 1985b). A change in ionic strength will also cause a change in the pKa of curcumin and the buffer system by the secondary kinetic salt effect. However, the small change in calculated pKa of curcumin from 7.62 to 7.66 due to a change in ionic strength from $\mu = 0.15$ to 0.30 would only result in minor changes in the calculated ratio of ionized:non ionized form of the molecule (from 13.8 to 15.1). The calculated change in pKa of the buffer system was from 7.35 ($\mu = 0.15$) to 7.39 ($\mu = 0.30$), and this change would result in ~ 10% more of the basic form (i.e., HPO_4^{2-}) of the buffer at ionic strength 0.15 compared to ionic strength 0.30, which is negligible compared to the observed reduction of degradation rate. Therefore, the most plausible explanation for the increased hydrolytic stabilization of curcumin with increasing ionic strength is the influence on the structure of the Pluronic aggregates. An increase in the ionic strength is reported to induce aggregation of Pluronics by a salting out effect (Desai et al. 2001; Patel et al. 2010). However, the interactions between curcumin and Pluronics did not seem to be influenced by an increase in ionic strength, as observed in the spectroscopic investigations. An increase in aggregation of the Pluronics will likely influence the distribution of curcumin throughout the sample leading to more curcumin in the hydrophobic core than in contact with nucelophiles in the aquous bulk and/or slow the diffusion rate of curcumin from the core to the aqueous bulk.

3.5. Influence of buffer system on the hydrolytic stability of curcumin in preparations containing Pluronics

Differences in the effect of general acid-base catalysis may explain the observed differences in hydrolytic degradation rate of curcumin in preparations containing phosphate buffer compared to preparations containing citrate buffer or carbonate buffer. A general acid-base catalyzed reaction is related to the proton donor or -acceptor properties of the buffer salts, thus the concentration and pKa-values of the buffer and the sample pH are essential factors. The probability of detecting general acid-base catalysis is highest when the pH is at the pKa value of the buffer (Ault 2007). The probability of detecting general acid-base catalysis is quite similar when comparing the degradation rate of curcumin in the phosphate buffer ($pKa2 = 7.2$) to the carbonate buffer ($pKa2 = 10.3$) at pH 8.8 (the difference between pH and pKa is 1.6 and 1.5 for phosphate buffer and carbonate buffer, respectively) while it is different when comparing the degradation rate of curcumin in the phosphate buffer to the citrate buffer ($pKa3 = 6.4$) at pH 7.8 (the difference between pH and pKa is 0.6 and 1.4 for phosphate buffer and citrate buffer, respectively). Thus, it seems like the general acid-base catalyzed hydrolysis of curcumin is more dominating in phosphate buffer than in citrate buffer under the current conditions. As the difference between pH and pKa is quite similar for carbonate buffer and phosphate buffer, the difference in the degradation rate of curcumin in these two buffers can be explained by the difference in base/acid ratio of the buffer salts (the total buffer concentration was kept constant), assuming that the catalytic mechanism is independent on the type of buffer. At pH 8.8 the calculated base/acid ratio of the carbonate buffer was 0.03, while it was 48 for phosphate buffer. This could also imply that the acidic buffer species may be more important in the catalysis of hydrolytic degradation of curcumin since the hydrolytic degradation rate was faster in carbonate buffer than in phosphate buffer at pH 8.8. Further, curcumin is reported to form a complex with citrate. This complex could stabilize curcumin against hydrolytic degradation (Tønnesen et al. 1985a). This complexation could not be detected by the spectroscopic investigations by e.g., a change in absorption maximum. However, the fraction of curcumin molecules present in the aqueous bulk, where the hydrolytic degradation is likely to occur, could be too low compared to the fraction of curcumin located in the hydrophobic Pluronic core to influence the absorption and fluorescence emission spectra.

The increased degradation rate of curcumin in preparations containing carbonate buffer compared to preparations containing phosphate buffer does not correspond with the results reported in the literature for preparations without solubilizers, where no difference between the two buffer systems was observed (Tønnesen and Karlsen 1985a). However, the half-life of curcumin in preparations containing carbonate buffer was only 2–3 min in the absence of solubilizer, while it was > 1244 min in preparations containing carbonate buffer and Pluronics (i.e., present study), at comparable pH. Therefore, the overall decreased degradation rate of curcumin in the presence of Pluronics may have facilitated the detection of any difference in the degradation rate between the two buffer systems.

3.6. Influence of cosolvents on the hydrolytic stability of curcumin in preparations containing Pluronics

Cosolvents will reduce the dielectricity constant of the preparations compared to preparations without cosolvents. This will influence both the aggregation of Pluronics and the bulk solubility of curcumin (Armstrong et al. 1996; Myrdal 2002; Zhang et al. 2008). We have previously observed that ethanol mainly interfered with the aggregation of Pluronics probably by disrupting the Pluronic aggregates, while PEG seemed to have a stronger influence on bulk solubility of curcumin than the aggregation of Pluronics (Singh et al. 2012). Therefore, ethanol would destabilize the solubilizing structures formed by the Pluronics, making more curcumin molecules available for hydrolytic degradation in the aqueous bulk phase. The destabilizing effect of ethanol on the aggregated state of the Pluronics may therefore be stronger for Pluronics with larger CMC values, and could explain the larger effect observed for preparations containing P85 compared to preparations containing P123 or F127. The degradation rate of curcumin in preparations containing PEG 400 and either of the three Pluronics was relatively similar to each other. Therefore, increased bulk solubility seems to attract more curcumin molecules from the solubilizing structures formed by the Pluronics to the hostile aqueous environment where degradation would be faster. However, cosolvents, such as PEG 400, stabilize curcumin towards hydrolytic degradation in the bulk phase, compared to preparations without cosolvents (Tønnesen et al. 2002).

3.7. Influence of various formulation parameters on the photostability of curcumin in preparations containing Pluronics

The photostability of curcumin in preparations containing Pluronics did not seem to be influenced to any large extent by the vehicle properties and excipients except for a small stabilizing effect that was observed in preparations containing Pluronics and cosolvents. As discussed above, cosolvents can destabilize the solubilizing structures formed by the Pluronics and increase the bulk solubility of the solute. Therefore, the slightly increased photostability in these preparations compared to preparations without cosolvents, indicate that curcumin obtains an increased photostability when forming interactions with ethanol or PEG 400 compared to Pluronics. This is consistent with previous observations, i.e., higher photostability is obtained in cosolvent-water mixtures than in preparations containing solubilizers such as cyclodextrins or Triton X-100 (Tønnesen 2002; Tønnesen et al. 2002). The results from the photodegradation studies corresponded also with the results from the photometric investigations, i.e., the interactions between curcumin and Pluronics were found to be relatively undisturbed by changes in vehicle properties and excipients, and thus the photochemical degradation rate remained relatively unchanged. The red shift in fluorescence maximum in buffers containing ethanol compared to buffers without ethanol could be related to increased vibrational relaxation as ethanol probably releases curcumin from the hydrophobic core.

In conclusion, Pluronics can be useful solubilizers and hydrolytic stabilizers of curcumin only if the formulation is protected from UV and visible radiation during preparation and storage, and during administration to a patient in a clinical setting. In preparations with phosphate buffer and citrate buffer both the PPO and the PEO part of the Pluronic seemed important with regards to hydrolytic stabilization of curcumin. Therefore curcumin was more stable in preparations with F127 than P123 or P85 in these buffers. General acid-base catalysis seems to be of crucial importance to the degradation of curcumin at pH > 8 in Pluronic preparations (i.e., when curcumin is ionized). Further, the hydrolytic stabilization of curcumin by Pluronics depends on vehicle properties and excipients such as pH, buffer concentration, ionic strength and the application of cosolvents. The distribution of curcumin between the hydrophobic core of Pluronic aggregates (hydrophobic interactions, although there are indications of hydrogen-bonding) and curcumin located in the aqueous bulk or the hydrophilic corona of the Pluronic aggregates is strongly favoured in the direction of curcumin localized in the hydrophobic core of the Pluronic aggregates. Hydrolytic degradation of curcumin is likely to occur mainly outside the hydrophobic core. The vehicle properties and type of excipients may affect the dynamic exchange of curcumin between the core and the aqueous bulk by influencing the aggregation of Pluronics and/or the formation of interactions between curcumin and the excipient in the bulk. The excipients and vehicle properties should be adjusted to optimize the hydrolytic stability of curcumin in pharmaceutical preparations.

4. Experimental

4.1. Materials

The following Pluronics were used: Pluronic F127 (Sigma), Pluronic P123 (Aldrich) and Pluronic P85 (BASF). Curcumin was synthesized according to the method given by Pabon (Pabon 1964). Ethanol 96% (v/v) was provided by Arcus (Oslo, Norway). Polyethylene glycol 400 (PEG 400) was provided by Fluka. Phosphate buffer saline (PBS) without calcium chloride and magnesium chloride, for buffers adjusted to pH 6.1, was provided by BioWhittaker, Lonza. Dulbeccos PBS without calcium chloride and magnesium chloride (PAA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were gifts from Kristian Bergs group at the Norwegian Radium Hospital. All other chemicals were commercially available substances of analytical grade or better. All chemicals were used as received. Water was distilled and deionized.

4.2. Preparation of the buffers

Phosphate buffer was prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate. The ionic strength was adjusted by addition of sodium chloride. The pH range was 4.8–8.8. The concentration of phosphate buffer, $\mu = 0.30$, at pH 8.8 was varied between 0.02 M–0.10 M. The ionic strength of phosphate buffer (0.05 M), at pH 8.8 was varied between 0.15–0.30.

Citric acid buffer (0.05 M) at pH 7.8 was prepared from disodium hydrogen citrate and trisodium citrate. Citric acid buffer (0.05 M), at pH 4.8 was prepared from sodium dihydrogen citrate and disodium hydrogen citrate. Carbonate buffer (0.05 M) at pH 8.8 was prepared from sodium hydrogen carbonate and disodium carbonate. The ionic strength in citrate buffers and carbonate buffers was adjusted to 0.3 by addition of sodium chloride. The reported pH values were measured after adjustment of the ionic strength. Buffers containing cosolvents were first made at twice the final buffer concentration and ionic strength, then the pH was measured, and finally the buffers were diluted 1:1 with the 40% (v/v) cosolvent-water mixture. The final concentration of cosolvent in the buffers would thus be 20% (v/v). PBS (BioWhittaker, Lonza) was adjusted to pH 6.1 with 0.1 M HCl, and Dulbeccos PBS (PAA) was adjusted to pH 7.4 with 10% (w/v) sodium hydroxide.

4.3. Quantitation of curcumin

The concentration of curcumin was quantified by reversed phase HPLC. The separation was performed on a Waters Nova-Pak® C_{18}, 3. 9 × 150 mm, 4 μm particle size column. The mobile phase was composed of 0.5% (w/v) citric acid adjusted to pH 3 with NaOH and methanol (38: 62). A flow rate of 0.8 ml min^{-1} was used. Curcumin was detected at 350 nm. The retention time of curcumin was ~18 min. The chromatographic system consisted of a Shimadzu LC-9A pump, a Shimadzu SP D-10A UV-VIS detector, a Shimadzu SIL-9A auto sampler and a Shimadzu C-R5A integrator.

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. The precision (relative standard deviation), measured for all standards by 6 repeating injections at 0.5 μM, was 2.21–4.45%. The Pluronics were not detected at 350 nm when injected seperately, and the retention time of curcumin (~18 mins) was not influ-

enced by the presence of any of the Pluronics when compared to standards of curcumin in methanol diluted 1:1 with the mobile phase.

4.4. Degradation studies

For hydrolytic degradation studies, 50 μL of a stock solution of curcumin in ethanol (4 mM) was added to a solution containing 5.2 mM Pluronic (F127, P123 or P85), dissolved in the appropriate buffer, to make a final volume of 10 mL (20 μM curcumin) (n = 3). The samples were kept in the dark at 25.0 ± 0.1 °C. The samples prepared in PBS (BioWhittaker, Lonza) at pH 6.1 or Dulbeccos PBS (PAA) at pH 7.4 were kept in the dark at 37 ± 0.1 °C. At specified time intervals 60 μL was withdrawn from the sample and diluted 1:5 with a solution consisting of 60% (v/v) mobile phase, 20% (v/v) methanol and 20% (v/v) 0.1 M HCl prior to HPLC-analysis. The measured pH in the samples was 2.12–4.04 (depending on the buffer system) after the dilution. The change in concentration of curcumin with time was monitored by HPLC until > 50% of the concentration of curcumin was degraded. The observed first-order rate constants (k_{obs}) for the degradation was obtained from linear regression analysis of the logarithm of the curcumin concentration plotted against time. All studies were carried out in triplicate.

For photolytic degradation studies 50 μL of a stock solution of curcumin in ethanol (1.4 mM) was added to the solution containing 5.2 mM Pluronic (F127, P123 or P85) dissolved in the appropriate buffer at 25.0 ± 0.1 °C to make a final volume of 10 mL (7 μM curcumin) (n = 3). The samples were irradiated in a SUNTEST CPS (Heraeus GmbH, Hanau, Germany) together with dark controls (i.e. samples wrapped in aluminum foil). The light source was a xenon lamp (1.8 kW) equipped with a glass filter transmitting light corresponding to exposure behind window glass (cut-off ~ 310 nm). The light intensity was measured to 3.2×10^4 lux and 4.0 W/m^2 in the visible and UV range respectively using a lux meter in combination with a UV-filter radiometer (Hagner EC1 Digital luxmeter, Hagner EC1 UV-A). The samples were exposed in a quartz cuvette under continuous stirring. The change in concentration of curcumin with exposure time was monitored by HPLC until > 50% of the concentration of curcumin was degraded. The observed first-order rate constants (k_{obs}) for the degradation were obtained from linear regression analysis of the logarithm of the curcumin concentration plotted against time. The photo degradation rate constants are directly proportional to the overlap integral between the emission spectrum of the irradiation source and absorption spectrum of the sample for the actual wavelength range. The calculated rate constants were therefore corrected for the difference in absorbtivity calculated as the integral of the area under the absorption curve and normalized to the sample with the highest absorbance. The absorbtivity was measured on a Shimadzu UV-2101 PC UV-VIS scanning spectrophotometer. All studies were carried out in triplicate.

4.5. Absorption and fluorescence spectroscopy

The interactions between curcumin and P123 in different buffer systems was investigated by absorption and fluorescence spectroscopy. The appropriate buffer system was prepared as decribed above to make a final concentration of 5.2 mM P123 and 7 μM curcumin, at 25.0 ± 0.1 °C. Absorption spectra were recorded on a Shimadzu UV-2401PC spectrophotometer with a quartz cuvette (light path 1 cm, accuracy ± 0.3 nm). Steady-state fluorescence emission measurements were carried out on a PTI modular Fluorescence System using Felix^{TM} for Windows software. The excitation source is a 75W xenon lamp and the monochromators are Model 101 with f/4 0.2-m Czerny-Turner configuration. The entrance and exit slits were adjusted to 2 nm. An excitation and emission correction was automatically performed. All measurements were performed at 25.0 ± 0.1 °C. The fluorescence quantum yields were determined by the relative comparison procedure, using quinine sulfate in 0.05 M H_2SO_4 as the reference (λ_{ex} = 344 nm, absorbance < 0.03; quantum yield = 0.51) (Velapoldi and Tønnesen 2004). In all the steady-state fluorescence emission measurements the samples were excited at their respective absorption maximum (i.e., 428 nm), obtained from the UV-VIS spectrum. The absorbance at the excitation wavelength was kept < 0.5. All experiments were performed in triplicates, and each fluorescence emission spectrum was recorded 3 times.

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