Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 583–589

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Quantification of hydrophilic ethoxylates in polysorbate surfactants using diffusion ^{1}H NMR spectroscopy

Maarten Verbrugghe[a,*], Ellen Cocquyt[a], Pieter Saveyn[a], Paolo Sabatino[a,b], Davy Sinnaeve[b], José C. Martins[b], Paul Van der Meeren[a]

[a] Ghent University, Faculty of Bioscience Engineering, Particle and Interfacial Technology Group, Coupure Links 653, B-9000 Gent, Belgium
[b] Ghent University, Faculty of Sciences, NMR and Structure Analysis Unit, Krijgslaan 281 S4, B-9000 Gent, Belgium

ARTICLE INFO

Article history:
Received 20 July 2009
Received in revised form
15 September 2009
Accepted 19 September 2009
Available online 27 September 2009

Keywords:
Polysorbate
Ethoxylates
Amphiphilic
Hydrophilic
Diffusion ^{1}H NMR
Micelle

ABSTRACT

Polysorbate surfactants (commercially available as Tween) are widely used in pharmaceutical, cosmetic and food products. They are generally considered as esters of ethoxylated sorbitan with fatty acids. Diffusion ^{1}H NMR spectroscopy on a solution of polysorbate 20 in D_2O revealed that only one diffusion coefficient was found for the fatty acyl part. Using the Stokes–Einstein equation, it became obvious that this diffusion behavior was caused by micelles. On the other hand, two significantly different diffusion coefficients were found for the methylene groups of ethylene oxide (EO). This indicates the presence of two distinct EO containing species in solution. Since the slowest diffusing EO species has the same diffusion coefficient as the fatty acyl part, it corresponds to the micellar (i.e. fatty acyl bound) ethoxylates. The diffusion coefficient of the fastest diffusing EO species was a factor of four larger than that of the slowly diffusing species and was attributed to water-soluble non-esterified ethoxylates. A solution of polysorbate 20 in the presence of NaOD was prepared to investigate if hydrolysis of the sorbitan ester could be the reason for the occurence of these hydrophilic ethoxylates. It was found that alkaline hydrolysis does lead to an increasing fraction of non-esterified ethoxylates, but is not the cause of its presence in untreated polysorbate samples since these species were also found in solutions of polyethylene glycol oleyl ether (commercially available as Brij), which are not susceptible to hydrolysis. Fractionation of the EO species present in polysorbate 20 into an amphiphilic and a hydrophilic fraction was only partly obtained by activated carbon adsorption. On the other hand, sequential extraction of aqueous polysorbate solutions by ethyl acetate and chloroform enabled a nearly complete fractionation. ^{1}H NMR spectroscopy proved to be very useful since it allows *in situ* determination of the global composition of a surfactant sample, as well as quantification of both the amphiphilic and hydrophilic ethoxylate fractions via diffusion measurements.

1. Introduction

Surfactants, in particular polysorbates, are commonly utilized excipients in the chemical and pharmaceutical industry [1,2]. The common backbone structure of polysorbate surfactant molecules is a sorbitan ring with ethylene oxide oligomers of variable length attached to each of the four different hydroxyl groups. While the number of ethylene oxide subunits varies in each oligomeric chain, the total number n is constant for each polysorbate molecule (Fig. 1). In general, the hydrophilic head group is esterified to one or more long chain fatty acids. Thus, polysorbate 20 is an ethoxylated sorbitan monolaurate, whereas polysorbate 65 is an ethoxylated sorbitan tristearate. Due to their amphiphilic nature, polysorbate molecules are associated into micelles when the critical micelle concentration (CMC) is exceeded. The CMC value of polysorbate 20 surfactants was reported to be 90 μM at 25 °C [3].

Whereas Fig. 1 represents the main molecular species in polysorbate 20, it has been shown that a wide variety of molecular species is present. First of all, the polysorbates used in the formulation of biopharmaceuticals are mixtures of different fatty acid esters with the monolaurate (C_{12}) fraction of polysorbate 20 making up only 40–60% of the mixture [4]. In addition, besides sorbitol mono-anhydride (also known as sorbitan), sorbitol as well as sorbitol di-anhydride is present. Finally, not only sorbitan monoesters, but also di- and tri-esters may be formed. As the overall sorbitan to fatty acid ratio of polysorbate 20 equals one, the latter implies that some non-esterified ethoxylated sorbitans may be present. These hydrophilic species are highly water-soluble and hence do not take part into the micelles [5].

The knowledge of the precise composition of polysorbate formulations is a very important matter [6]. Thus, the presence of non-esterified ethoxylates implies that the degree of ethoxylation of the amphiphilic polysorbate molecules is in fact smaller than

* Corresponding author.
E-mail address: maarten.verbrugghe@ugent.be (M. Verbrugghe).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.09.025

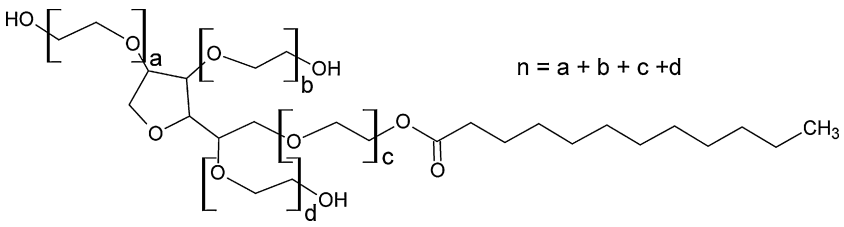

Fig. 1. Chemical structure of polyethylene glycol sorbitan monolaurate, the main component of polysorbate 20 ($n = a + b + c + d = 20$).

specified by the manufacturer, which may affect the surfactant's physicochemical behavior, such as its HLB (Hydrophilic–Lipophilic Balance) or CMC value. In addition, the presence of hydrophilic polymers, such as ethoxylated sorbitan, may induce depletion flocculation [7], which may undermine the desired stabilizing effect of these nonionic surfactants.

Several methods for the quantification of nonionic surfactants have been described in literature. Greff et al. presented a colorimetric method for the determination of nonionics in low concentration (0–20 ppm), based on the formation of a blue complex between ammonium cobaltothiocyanate and a polyethoxylated compound [8]. According to Khossravi et al., the disadvantage of this method is that no distinction can be made between intact polysorbate and degraded polysorbate [2]. Another indirect technique is based on the quantification of the fatty acid content upon hydrolysis [9]. Whereas the surface activity of di- and tri-esters is high, unesterified ethoxylated sorbitan is hardly surface active. Therefore, both these indirect methods yield misleading results for the residual nonionic concentration, following a process that discriminates according to the EO to fatty acid ratio such as adsorption. In fact, the non-adsorbed fraction is expected to have a higher EO to fatty acid ratio, whereas the adsorbed fraction will most likely have a lower EO to fatty acid ratio. Therefore, the determination of the residual fatty acid or EO content in the supernatant is not appropriate to determine the residual amount of polysorbate under these circumstances. Trathnigg et al. reported that the separation of non-esterified ethoxylates from polysorbate is possible using HPLC, but quantification was troublesome because of the lack of a standard of similar molecular weight distribution [10]. ^{1}H NMR enables a rapid analysis of the overall polysorbate composition. However, according to Khossravi et al., it results in similar proton peak areas for both degraded and non-degraded polysorbate 20 samples [2], which led these authors to conclude that a simple ^{1}H NMR experiment does not allow to determine the degree of degradation.

The main purpose of the current work was threefold. At first, a suitable NMR-based method was developed to quantify the presence of hydrophilic (i.e. not bound to fatty acid residues) ethoxylates in a commercial polysorbate preparation. Polysorbate 20 was used as a model surfactant. In order to investigate the effect of hydrolysis of polysorbates, a base-catalyzed hydrolysis experiment was conducted. In addition, polysorbate esters were compared to ethoxylated fatty alcohols (Brij 98), which are not susceptible to hydrolysis because of the absence of an ester bond. Finally, fractionation of the complex surfactant mixture was done, based on adsorption to activated carbon on the one hand and a sequential extraction procedure on the other hand, after which the resulting samples were monitored by ^{1}H NMR.

2. Materials and methods

2.1. Materials

Deuterium oxide (D_2O >99.8% atomD) was used as purchased from Armar Chemicals (Switzerland). Polysorbate (Tween) 20 (Sigma Ultra) was purchased from Sigma–Aldrich. Brij 96 and Brij 98 were obtained from Akzo Nobel (Amsterdam, the Netherlands). NaCl and $NaHCO_3$ (>99.5% purity) were purchased from Merck (Darmstadt, Germany), as well as $CHCl_3$ (99.0–99.4% purity). Ethyl acetate (reagent grade, min. 99.5% pure) was obtained from VWR International S.A.S. (Briare, France). The $NaOAc \cdot 3H_2O$ used was NormaPUR, obtained from VWR Prolabo. Granular activated carbon (type Organosorb 10) was obtained from Desotec (Roeselare, Belgium).

2.2. ^{1}H nuclear magnetic resonance (^{1}H NMR)

All NMR experiments were performed on a Bruker DRX spectrometer operating at a ^{1}H-frequency of 500.13 MHz. A 5 mm ^{1}H,^{13}C,^{15}N TXI-Z-gradient probe with a maximum gradient strength of 56.1 G cm^{-1} was used throughout. Temperature was controlled to within ±0.1 °C with a Eurotherm 2000 VT controller. Diffusion coefficients were measured by PFG-NMR with a convection compensated double-stimulated-echo experiment [11] using monopolar smoothened square shaped gradient pulses and a phase cycle modified according to [12]. Measurements were performed at 21 °C. The echo-decay of the resonance intensity obtained with the double-stimulated-echo sequences obeys Eq. (1), from which it is clear that the diffusion coefficient D is derived from the echo-decay as a function of the parameter k. A detailed description of the PFG-NMR method and the sequences mentioned above, is given in a review written by Johnson [13]:

$$I = I_0 \exp[-D(\gamma G \delta s)^2 \Delta'] \quad I = I_0 \exp[-D \cdot k] \tag{1}$$

where I = echo intensity with gradient; I_0 = echo intensity at zero gradient; D = diffusion coefficient; γ = gyromagnetic ratio; G = maximum gradient amplitude; s = gradient shape factor (here 0.9); δ = duration of the gradient pulse; Δ' = diffusion delay corrected for the finite gradient pulse duration; ($\Delta' = \Delta - 0.6021 \cdot \delta$).

The determination of the diffusion coefficient with corresponding 67% confidence interval was based on the fitting of a mono- or multi-exponential curve to the echo-decay of the peak integral of the selected resonances using the Monte Carlo procedure [14]. This fitting procedure was repeated 100 times for each experiment; according to Alper and Gelb [14], 60 fits are sufficient to produce a constant confidence interval.

2.3. Sequential extraction of unesterified ethoxylates

A polysorbate 20 solution was prepared in deionized water and subjected to a sequential extraction procedure as described by Szymanski and Lukaszewski [15]. Ethoxylated sorbitan esters were first extracted with ethyl acetate. In a second step, the non-esterified ethoxylate fraction was extracted using chloroform. In this way, the hydrophilic ethoxylate fraction could be studied separately from the amphiphilic ethoxylate surfactant molecules. The extracts were evaporated to dryness in a rotavapor and redissolved in D_2O. Both extracts were subjected to DOSY ^{1}H NMR experiments at a temperature of 21 °C.

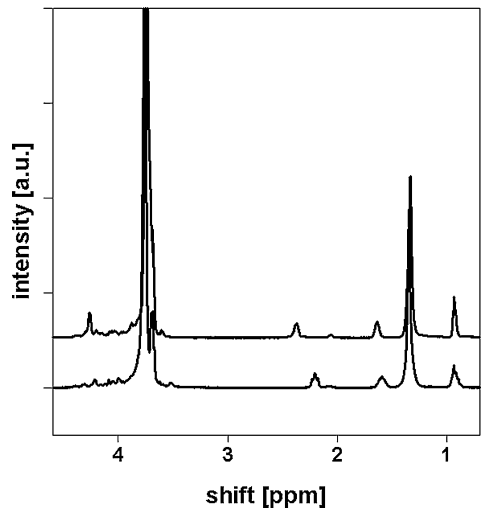

Fig. 2. ^{1}H-spectra of a 12 mg/ml polysorbate 20 in D_2O solution at 21 ° C in the absence (upper trace) and presence (lower trace) of 15 mM NaOD after 1.5 h of incubation. The peaks whose shift is more than 3 ppm belong to the ethoxylated sorbitan structure, whereas the peaks with a shift below 3 ppm originate from the fatty acyl part.

3. Results and discussion

3.1. Polysorbate 20

In Fig. 2 the ^{1}H-spectrum of polysorbate 20 in D_2O (12 mg/ml) at 21 ° C is represented by the upper trace. The largest peak at 3.7 ppm originates from the methylene (CH_2) groups of the ethylene oxide (EO) chains linked to the sorbitan ring. The broadening of the peak is due to a partly overlapping signal coming from the protons of the sorbitan moiety. Most of the aliphatic methylene groups in the fatty acyl part contribute to the resonance at 1.3 ppm, while the terminal methyl group of the fatty acyl chain resonates at 0.9 ppm. The α- and β-methylene groups proximal to the ester occur at 2.4 and 1.6 ppm, respectively. Finally, a relatively small resonance at 4.2 ppm originates from the methylene group of the ethylene oxide unit proximal to the ester bond.

In Table 1 the relative peak areas (calculated by integration) of the different resonances are given with reference to the terminal methyl signal of polysorbate 20 (Table 1, first two rows) together with the expected values based on the structural formula of polysorbate 20 (Table 1, third row). Doing so, one unit of intensity corresponds to one proton. The values on the first row represent the peak areas for a 12 mg/ml polysorbate 20 solution in D_2O as such, while the ones on the second row were determined upon 1.5 h of base-catalyzed hydrolysis (discussed in Section 3.2). In order to estimate the reproducibility of the NMR determinations, the sample *ante*-hydrolysis was measured in triplicate. Each measurement was processed by three different operators. Based on these data, the coefficient of variation of the relative peak areas ranged from 1.5 to 2.7%. The average number of EO subunits per fatty acyl residue can be determined by means of the relative peak areas. The total number of EO protons is found by the summation of the relative peak areas at 3.7 ppm (83.1) and 4.2 ppm (2.4), from which the number of sorbitan protons (8) contributing to these signals must be subtracted, leading to 77.5 units. As each EO subunit consists of 4 protons, the average number of EO subunits per fatty acyl residue is found to be 19.4. This is very close to 20, as specified by the manufacturer. Additionally, the presence of 21.1 methylene protons (17.1 + 2.0 + 2.0) and 1 methyl endgroup indicates that the fatty acyl residue is mainly lauric but some longer fatty acids also occur.

When diffusion ^{1}H NMR measurements (DOSY) were performed on the aqueous polysorbate 20 solution, a mono-exponential decay characterized by a single diffusion coefficient of $5.07 \pm 0.01 \times 10^{-11}$ m^2 s^{-1} was observed for the fatty acyl related ^{1}H NMR signals (Fig. 3). On the other hand, a bi-exponential decay of the echo intensity as a function of the parameter k was observed for the EO resonance. At 21 °C, the smallest diffusion coefficient amounted to $5.07 \pm 0.01 \times 10^{-11}$ m^2 s^{-1}, i.e. the same value as obtained for

Table 1
Peak areas of the polysorbate 20 resonances relative to the methyl acyl group at 21 °C. The values on the first two rows are based on the spectra depicted in Fig. 2 and reflect results obtained *ante*- and *post*-hydrolysis, whereas the values on the third row represent the theoretical values based on the molecular structure of polyethylene glycol (20) sorbitan monolaurate. The *ante*-hydrolysis values are given with standard deviation after integration was done on three different samples, each time in triplicate (3 different operators).

Resonance	CH_3	$(CH_2)_n$	β-CH_2	α-CH_2	$(CH_2CH_2O)_n$	CH_2OCO
Ante-hydrolysis	3.0	17.1±0.2	2.0 ±0.1	2.0 ±0.0	83.1 ±1.5	2.4 ±0.0
Post-hydrolysis	3.0	17.3	2.2	2.3	86.1	0.1
Expected	3	16	2	2	86	2

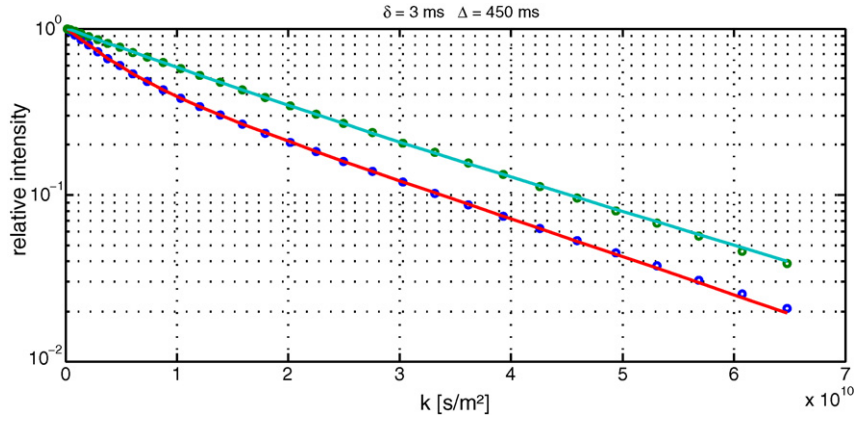

Fig. 3. Decay of the echo intensity as a function of the parameter k in a 12 mg/ml polysorbate 20 solution in D_2O at 21 °C. The echo-decay of the methylene groups $(CH_2)_n$ of the fatty acyl tail is mono-exponential and represented by the upper line, whereas the echo-decay of the EO subunits is bi-exponential and represented by the lower line.

Table 2
Diffusion coefficients of the EO methylene groups with their respective weight fractions found in specific extracts of a polysorbate 20 solution in D_2O by doing DOSY ^1H NMR measurements at 21 °C.

Extract	D_{slow} ($\times 10^{-11}$ m^2 s^{-1})	Fraction D_{slow} (%)	D_{fast} ($\times 10^{-11}$ m^2 s^{-1})
Untreated polysorbate	5.07 ± 0.01	63.24 ± 2.94	22.13 ± 0.85
Ethyl acetate	5.05 ± 0.07	100	–
Chloroform	2.97 ± 0.20	8.12 ± 0.48	23.01 ± 0.20

the fatty acyl chain. The larger diffusion coefficient was found to be equal to $22.13 \pm 0.83 \times 10^{-11}$ m^2 s^{-1}, indicating a faster diffusing EO species. Both values are similar to the diffusion coefficients found by Hillgren et al. [16] for polysorbate 80 solutions, which indicates that similar populations of molecules are dealt with: they found a diffusion coefficient of 5×10^{-11} m^2 s^{-1} for the methylene groups of ethylene oxide, as well as for the other groups present in polysorbate 80. Only for the EO methylene groups, a second diffusion coefficient of about 19.5×10^{-11} m^2 s^{-1}, indicative of a faster diffusing ethoxylated species was found. In fact, a bi-exponential decay of the attenuation of the ethylene oxide proton resonance was also observed by Foster et al. for Pluronic surfactants [17]. In all these cases a measurable amount of the total EO content is diffusing about 3–4 times faster than the rest. Whereas Foster et al. assign this bi-exponential decay to the presence of monomers and micelles, this explanation is very unlikely. First of all, Foster et al. themselves mention that their so-called fraction of monomers as derived from diffusion measurements is about 25 times larger than the CMC of the surfactant. In addition, micellisation is a highly dynamic process with a very rapid exchange between monomers and micelles on the ^1H NMR time scale. Hence, even if a significant fraction of monomers would be present, diffusion ^1H NMR will only provide an average diffusion coefficient of monomers and micelles instead of two separate diffusion coefficients. Taking into account the value of the critical micelle concentration (CMC) of polysorbate 20, which is about 90 μM at 25 ° C in H_2O [3], it can be stated that almost all of the polysorbate surfactant molecules will be associated into micelles and that the relative amount of monomolecular surfactant molecules can be neglected. As a consequence, the measured diffusion coefficients of the methyl and methylene groups of the fatty acyl part belong to polysorbate micelles.

As the diffusion coefficient of the slowest diffusing EO fraction is identical to that of the fatty acyl chain, it follows that this part of the EO pool must be present as micellar structures. As the diffusion coefficient for the other EO pool is about 4 times larger (Table 2), it must be attributed to a significant fraction of ethoxylate that is not inserted into the Tween micelles. Whereas the latter hydrophilic ethoxylate fraction may consist of free polyethylene oxide, we propose that non-esterified ethoxylated sorbitan molecules are more likely, based on the polysorbate production process [10]. Since the EO chains in Tween micelles are solvent exposed, the chemical shifts of the ethylene oxide subunits from free and micelle bound EO are identical. As a consequence, the ^1H NMR peak of EO contains contributions from two populations characterized by a different value of the diffusion coefficient. Using the Stokes–Einstein equation (Eq. (2)), the hydrodynamic diameter d_h of the Tween micelles can be evaluated to be about 8.3 nm from the diffusion coefficient D using 1.226 mPa s for the viscosity η of D_2O at 21 °C. This value fits perfectly in the range of 7–9 nm that was determined by dynamic light scattering [18]:

$$d_h = \frac{k_B T}{3\pi\eta D} \quad (2)$$

The bi-exponential decay profile indicates that only about 63% of the total amount of EO is associated into micelles, the remaining 37% belonging to the hydrophilic ethoxylate fraction. Momot et al. found that only one-third of the EO content of the nonionic surfactant Solutol HS15 is incorporated in micellar structures and two-thirds are free molecules [19]. Different explanations have been provided for the origin of the hydrophilic ethoxylate fraction and resulting bi-exponential decay profile. A first hypothesis is the hydrolysis of the ester bond. According to Moorthaemer [20] and Kerwin [4], the ester bond between the hydrophobic tail and the hydrophilic sorbitan ring is very sensitive to hydrolysis, but also to auto-oxidation and aerobic degradation. A second hypothesis is mentioned by Trathnigg et al. [10] and states that free ethoxylates are a byproduct of the manufacturing process of polysorbate. According to Momot et al., free ethoxylates are added to surfactant preparations to increase the manageability [19].

3.2. Relevance of hydrolysis

In order to check whether hydrolysis could be responsible for the emergence of hydrophilic ethoxylate species, a stimulated hydrolysis experiment was performed. A solution of 12 mg/ml polysorbate 20 in the presence of 15 mM NaOD in D_2O was prepared. ^1H NMR spectra were recorded every half an hour. The hydrolysis reaction causes amphiphilic ethoxylate molecules to be split into a hydrophilic ethoxylated sorbitan structure and a (dissociated) free fatty acid moiety. As a result, the CH_2 group next to the ester function (at about 4.2 ppm) is transformed into a $-CH_2OH$ group and its signal should become incorporated in the remaining ethoxylated sorbitan signal around 3.7 ppm. Fig. 2 indeed indicates that the peak at 4.2 ppm has disappeared upon alkaline hydrolysis. Peak integration revealed that the relative contribution of the EO methylene group next to the ester bond, as compared to the terminal methyl group, decreased from 2.4 before hydrolysis (Table 1) to 0.2 and 0.1 (Table 1) after 0.5 and 1.5 h of hydrolysis, respectively. After completion of the hydrolysis reaction, a mono-exponential decay was found for both the EO signal and the methylene signal $(CH_2)_n$ of the fatty acid. The diffusion coefficient amounted to $20.0 \pm 0.2 \times 10^{-11}$ and $15.7 \pm 0.2 \times 10^{-11}$ m^2 s^{-1} for ethylene oxide and fatty acid, respectively. According to Di Profio et al., the CMC of sodium laurate is 24 mM at ambient pressure and temperature [21]. Consequently, the fatty acid fraction that was released upon alkaline hydrolysis was not associated into micelles. Hence, contrary to the conclusions made by Khossravi et al. [2], these data show that hydrolysis may be evaluated from ^1H NMR spectra: the pronounced decrease of the relative peak area of the EO methylene group next to the ester bond indicates that at least 90% hydrolysis was obtained within the first 30 min. However, accurate quantification is hindered by the presence of some interfering minor components that result in an unstable baseline in the region from 4 to 4.5 ppm. Nevertheless, the conclusion can be drawn that the relative peak areas in the original sample (without NaOD) do not sustain the hypothesis that hydrolysis of the ester bond is responsible for the appearance of hydrophilic ethoxylates.

In a further test on the relevance of hydrolysis in the emergence of a bi-exponential decay for the EO ^1H NMR signal, the behavior of ethoxylated fatty acids was compared to that of ethoxylated fatty alcohols. Solutions of polyethylene glycol oleyl ether with 10 (Brij 96) and 20 ethylene oxide units (Brij 98) in D_2O were subjected to diffusion ^1H NMR experiments. In contradiction to polysorbate surfactants, the hydrophilic EO group of Brij surfactants is bound to the hydrophobic tail via an ether bond, instead of an ester bond. Whereas the former is not susceptible to hydrolysis, still, the decay profile of the EO groups of both Brij type solutions was bi-exponential: both a 'slow' micellar diffusion coefficient of about 2.9×10^{-11} m^2 s^{-1} and a 'fast' diffusion coefficient of about 19.5×10^{-11} m^2 s^{-1} were found at 21 ° C with almost the same contribution.

These results are a further strong indication that hydrolysis of the ester bond is not the cause of the appearance of hydrophilic ethoxylates in polysorbate 20. Hence, the commercial sample is thought to consist of both non-esterified (hydrophilic) and fatty acyl linked (amphiphilic) ethoxylated sorbitan whose molar fraction may be represented by X_{hydro} and X_{amphi}, respectively. Assuming that both fractions contain 20 ethylene oxide units per sorbitan and that the overall lauric acid to sorbitan ratio is equal to 1, it follows that the molar formula of the two fractions corresponds to $C_{46}H_{92}O_{25}$ and $C_{46+12x}H_{92+22x}O_{25+x}$ with x the number of fatty acid residues per sorbitan in the amphiphilic fraction. As the overall formula of polyoxyethylene (20) sorbitan monolaurate corresponds to $C_{58}H_{114}O_{26}$, it follows (e.g. from the number of carbons in each compound) that x corresponds to $(1 - X_{hydro})^{-1}$. As the hydrophilic ethoxylate component contains 88 protons contributing to the ^{1}H NMR signal at 3.7 ppm, whereas the amphiphilic ethoxylate has $88 - 2x$ protons at this position, it follows that the fraction of fast diffusing protons (*ffdp*) corresponds to:

$$ffdp = (X_{hydro} \cdot 88) / \left[X_{hydro} \cdot 88 + (1 - X_{hydro}) \cdot (88 - 2x)\right]$$
$$ffdp = X_{hydro} \cdot 88/86$$

As the fraction of fast decaying protons is 37%, the above equations reveal that the molar fraction of hydrophilic ethoxylate (X_{hydro}) equals 36%. Based on this information, the average number of fatty acyl residues per sorbitan in the amphiphilic fraction (x) equals 1.57. Assuming that all sorbitan moieties contain 20 EO groups, it follows that the effective degree of ethoxylation per fatty acid (in the amphiphilic fraction) is only 12.7.

3.3. Fractionation of polysorbate 20

Based on the different hydrophobicities of the esterified and non-esterified ethoxylated sorbitan fractions, two fractionation schemes were tried. In a first trial, activated carbon was selected as a hydrophobic adsorbent to selectively remove the fatty acyl bound ethoxylated sorbitans. Increasing concentrations (0, 10, 20, 40 and 100 mg/ml) of activated carbon (AC) were added to a 15 mg/ml Tween 20 solution in D_2O that contained 10 mM NaOAc. After overnight equilibration, the solid fraction was separated from the solution via centrifugation and the supernatant was subjected to ^{1}H NMR measurements. Using NaOAc as internal standard, the decrease in peak area of the different moieties in polysorbate 20 could be evaluated, which was ascribed to sorption to the activated carbon. Thus, Fig. 4 shows a steady increase in the adsorption of the fatty acyl groups, which was calculated from the decrease of the fatty acyl methylene signal. Fig. 4 shows that the amphiphilic (esterified) ethoxylates can indeed be nearly completely removed by adsorption to a hydrophobic sorbent.

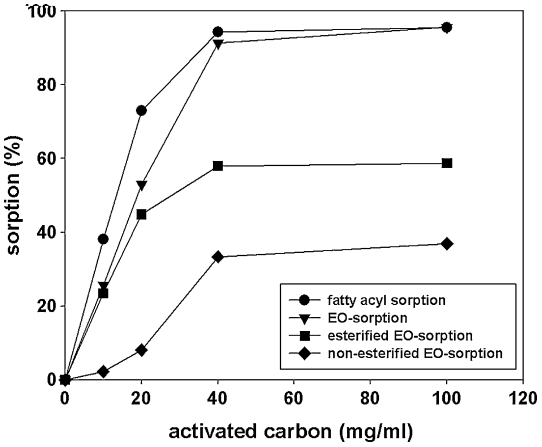

Fig. 4. Relative decrease in ^{1}H NMR peak areas of a 15 mg/ml polysorbate 20 solution in D_2O as a function of the amount of added activated carbon. NaOAc (10 mM) was used as internal standard.

The EO-sorption percentage was calculated in the same way, but using the relative decrease of the EO signal. Contrary to our initial expectations, the EO signal in the NMR spectrum also disappeared nearly completely at high AC concentrations, which seemed to indicate that not only the amphiphilic, but also the hydrophilic ethoxylate fraction was adsorbing. Assuming that only 63% of the EO NMR signal was due to amphiphilic EO, the percentage of esterified ethoxylates adsorbed was estimated by taking 63% of the percentage of fatty acyl sorption. In a final step, the non-esterified EO-sorption was estimated as the difference between the EO-sorption and the bound EO-sorption. Fig. 4 clearly points out that two ethoxylate populations of different hydrophobicity occur in polysorbates: whereas a preferential sorption of the esterified ethoxylates is observed at low AC concentrations, the non-esterified ethoxylates also start adsorbing at higher adsorbent concentrations. This finding was supported by literature data on adsorption of polyethylene glycol to activated carbon [22,23] and confirmed by TOC measurements at high activated carbon concentrations. The residual TOC in the supernatant of a 6 mg/ml polysorbate 20 solution obtained upon removal of the activated carbon by centrifugation was 95, 89, 81, 48, 21 and 4% (relative to the TOC content in the absence of activated carbon) upon addition of 2, 4, 10, 20, 40 and 100 mg/ml activated carbon. These data reveal that not only the amphiphilic ethoxylates, but also the hydrophilic ethoxylates are being adsorbed to activated carbon.

Preferential sorption of amphiphilic species was confirmed by diffusion measurements. The fast diffusing fraction of EO increased from 38.5 over 55.4 to 67.3% at 0, 10 and 20 mg/ml of activated carbon, respectively. This clearly shows that the sorbed molecular species are enriched in fatty acyl moieties, whereas the residual species become enriched in hydrophilic ethoxylated sorbitan moieties. This is completely in line with the hypothesis that only part of the polyethoxylated sorbitan molecules are fatty acyl bound. It should be noted that reliable decay curves (decaying to only some percents of the initial value) were only obtained for AC concentrations up to 20 mg/ml; at higher concentrations, the residual polysorbate concentration became too low to enable reliable diffusion experiments.

Since activated carbon adsorption does not allow a complete fractionation, an alternative procedure based on a sequential extraction was tried as originally proposed by Szymanski and Lukaszewski [15]. By following this method, two different extracts of a polysorbate 20 solution in D_2O with strongly different relative amounts of hydrophilic ethoxylates were prepared. The effect of the extraction procedure becomes clear from Fig. 5, which shows the stacked plot of the ^{1}H NMR spectra of the three different samples. For each of the three samples, the peak of the EO subunits at 3.7 ppm is very intense compared to the fatty acyl peaks below 2 ppm. The reference profile in the middle originates from an untreated polysorbate 20 solution. Fig. 5 indicates clearly that the fatty acyl peak areas in the chloroform extract are small compared to the peak area at 3.7 ppm. From this observation, it can be concluded that the chloroform extract contains a large portion of hydrophilic ethoxylates and a minor portion of amphiphilic ethoxylates.

In Fig. 6, which is based on the EO resonance signal at 3.7 ppm, the diffusion induced decay curve of the untreated sample is positioned between the decay curves of both extracts, which is a logical consequence of the fact that the main ingredients of both extracts are combined. The ethyl acetate extract contains almost solely ethoxylated sorbitan esters, which are rather slowly moving (micellar) structures characterized by a small diffusion coefficient, whereas the chloroform extract is rich in fast diffusing and freely moving ethoxylated sorbitans. The values of the different diffusion

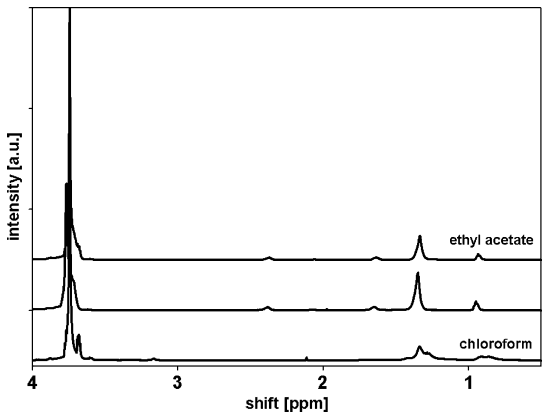

Fig. 5. ^{1}H NMR spectra of an untreated polysorbate 20 (reference) solution, as well as the chloroform and ethyl acetate extracts obtained via the procedure of Szymanski and Lukaszewski [15]. The large peak at 3.7 ppm belongs to the EO groups while the smaller peaks below 1.5 ppm belong to the fatty acid tail. All spectra were recorded at 21 °C.

coefficients found in every sample accompanied by the respective weight factors are given in Table 2. A bi-exponential fit proved to be the most accurate for the chloroform extract as well as for the untreated polysorbate solution, whereas a mono-exponential fit produced the smallest error for the ethyl acetate extract. This indicates that the former two samples both contained ethoxylated sorbitan esters as well as non-esterified ethoxylates (with a significant difference in diffusion coefficient), whereas the latter contained almost no hydrophilic ethoxylates. The results of this sequential extraction experiment prove that the fast decaying signal can only be accredited to hydrophilic ethoxylates which are present in the product as it is commercially distributed by the manufacturer.

The ratio between the signal intensities of the EO peak at 3.7 ppm and the terminal methyl peak at 0.9 ppm was found to be 53 to 3 for the ethyl acetate extract. Based on the average number of fatty acid residues per sorbitan in this fraction (x), the expected value for this ratio is $(88 - 2x)$ to $3x$. Based on the experimental value (53/3), x is calculated to be 1.60 from which the fraction of fast decaying protons should be 38%. This result nicely fits to the bi-exponential decay data in Table 2. Hence, the diffusion data on the mixture of the hydrophilic and amphiphilic species are corroborated by the spectral peak integration of the extracted amphiphilic compounds.

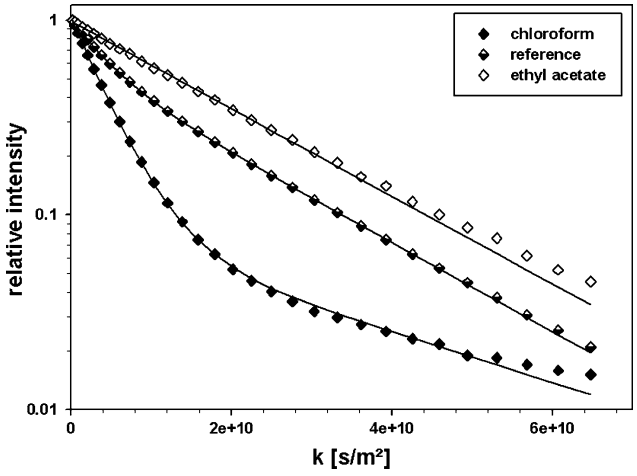

Fig. 6. Decay of the echo intensity of the EO groups as a function of the parameter k for the ethyl acetate extract (containing the amphiphilic ethoxylates), the chloroform extract (containing the hydrophilic ethoxylates) and the untreated polysorbate 20 (reference) solution. The spectra were taken at a temperature of 21 °C. The solid lines represent the best fit to the decay curves, which was mono-exponential for the ethyl acetate extract and bi-exponential for the chloroform extract and the unfractionated reference solution of polysorbate 20.

4. Concluding remarks

Whereas ^{1}H NMR is a powerful tool to investigate the overall composition of a sample, it was clearly shown that diffusion ^{1}H NMR measurements are required to allow the discrimination between (water-soluble) non-esterified and (micellar) esterified ethoxylates. Activated carbon adsorption as well as sequential extraction confirmed that commercial polysorbate (and ethoxylated fatty alcohol) preparations may contain a significant amount of hydrophilic ethoxylates. For the polysorbate sample used, the latter fraction amounted to almost 40% of the total EO content. This finding has far reaching consequences. First of all, the effective degree of ethoxylation of the surfactant molecules is significantly lower than specified. In addition, a significant part of the formulation does not have its desired wetting, stabilizing or surface tension decreasing effect. In fact, the latter fraction may even have detrimental effects since water-soluble polymers are known to induce depletion flocculation. As the real degree of ethoxylation of the fatty acids depends on the fraction of hydrophilic ethoxylates, the latter will also affect the HLB and CMC value, which will both increase as the fraction of hydrophilic ethoxylates decreases. This may badly affect the reproducibility of the physico-chemical properties of polysorbates from batch to batch and from manufacturer to manufacturer. In addition, quantification of nonionics by simple indirect methods such as the EO content or fatty acid content (upon hydrolysis) will give erroneous results whenever the EO to fatty acyl ratio has been changed, as will be the case in sorption studies. In fact, a fundamental study of sorption is largely hampered by the presence of widely different molecular species.

Acknowledgments

The authors thank the Fund for Scientific Research - Flanders (FWO-Vlaanderen) for a Ph.D. fellowship to Pieter Saveyn and Paolo Sabatino, as well as for various research and equipment grants (G.0365.03; G.0064.07; G.0678.08; G.0102.08).

References

[1] T. Kamiusuki, T. Monde, K. Omae, K. Morioka, T. Konakahara, Simultaneous separation of nonionic surfactants and polyethylene glycols by reversed phase high performance liquid chromatography, Chromatographia 51 (2000) 390–396.
[2] M. Khossravi, Y.-H. Kao, R.J. Mrsny, T.D. Sweeney, Analysis methods of polysorbate 20: a new method to assess the stability of polysorbate 20 and established methods that may overlook degraded polysorbate 20, Pharm. Res. 19 (2002) 634–639.
[3] R. Mahajan, J. Chawla, M. Bakshi, Effects of monomeric and polymeric glycol additives on micellar properties of Tween non-ionic surfactants as studied by cyclic voltammetry, Colloids Surf. A: Phys. Eng. Aspects 237 (2004) 119–124.
[4] B.A. Kerwin, Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways, J. Pharm. Sci. 97 (2008) 2924–2935.
[5] N. Sugimoto, R. Koike, N. Furusho, M. Tanno, C. Yomota, K. Sato, T. Yamazaki, K. Tanamoto, Quantitative nuclear magnetic resonance spectroscopic determination of the oxyethylene group content of polysorbates, Food Addit. Contam. 24 (2007) 799–806.
[6] L.P. Turner, D. McCullough, A. Jackewitz, Quantitative determination of polyethylene glycols in nonionic surfactants by high-pressure liquid chromatography, J. Am. Oil Chem. Soc. 53 (1976) 691–694.
[7] A.A. Zaman, Effect of polyethylene oxide on the viscosity of dispersions of charged silica particles: interplay between rheology, adsorption, and surface charge, Colloid Polym. Sci. 278 (2000) 1187–1197.
[8] R.A. Greff, E.A. Setzkorn, W.D. Leslie, A colorimetric method for determination of parts/million of nonionic surfactants, J. Am. Oil Chem. Soc. 42 (1965) 180–185.
[9] M. Hu, M. Niculescu, X.M. Zhang, A. Hui, High-performance liquid chromatographic determination of polysorbate 80 in pharmaceutical suspensions, J. Chromatogr. A 984 (2003) 233–236.

[10] B. Trathnigg, C. Rappel, R. Hodl, S. Fraydl, Molecular characterization of ethoxylates by complementary chromatographic techniques. Evaluation of efficiency and reliability, Tenside Surfact. Deterg. 30 (2003) 1–7.
[11] A. Jerschow, N. Muller, Suppression of convection artifacts in stimulated-echo diffusion experiments. Double-stimulated-echo experiments, J. Magn. Reson. 125 (1997) 372–375.
[12] M.A. Connell, P.J. Bowyer, P.A. Bone, A.L. Davis, A.G. Swanson, M. Nilsson, G.A. Morris, Improving the accuracy of pulsed field gradient NMR diffusion experiments: correction for gradient non-uniformity, J. Magn. Reson. 198 (2009) 121–131.
[13] C.S. Johnson, Diffusion ordered nuclear magnetic resonance spectroscopy: principles and applications, Prog. Nucl. Magn. Reson. Spectrosc. 34 (1999) 203–256.
[14] J.S. Alper, R.I. Gelb, Standard errors and confidence-intervals in non-linear regression—comparison of Monte Carlo and parametric statistics, J. Phys. Chem. 94 (1990) 4747–4751.
[15] A. Szymanski, Z. Lukaszewski, Determination of poly(ethylene glycol)s in environmental samples by the indirect tensammetric method, Analyst 121 (1996) 1897–1901.
[16] A. Hillgren, H. Evertsson, M. Alden, Interaction between lactate dehydrogenase and Tween 80 in aqueous solution, Pharm. Res. 19 (2002) 504–510.
[17] B. Foster, T. Cosgrove, B. Hammouda, Pluronic triblock copolymer systems and their interactions with ibuprofen, Langmuir 25 (2009) 6760–6766.
[18] E.S. Basheva, P.A. Kralchevsky, K.D. Danov, K.P. Ananthapadmanabhanb, A. Lipsb, The colloid structural forces as a tool for particle characterization and control of dispersion stability, Phys. Chem. Chem. Phys. 9 (2007) 5183–5198.
[19] K.I. Momot, P.W. Kuchel, B.E. Chapman, NMR study of the association of propofol with nonionic surfactants, Langmuir 19 (2003) 2088–2095.
[20] B. Moorthaemer, J. Sprakel, Improving the stability of a suspension, Pharm. Technol. Eur. 18 (2006) 30–34.
[21] P. Di Profio, S. Arca, R. Germani, G. Savelli, Surfactant promoting effects on clathrate hydrate formation: are micelles really involved? Chem. Eng. Sci. 60 (2005) 4141–4145.
[22] H.S. Wu, C.W. Yen, Ethylene oxide removal from simulated wastewater, J. Chin. Inst. Chem. Eng. 33 (2002) 243–251.
[23] C.F. Chang, C.Y. Chang, W. Holl, M. Ulmer, Y.H. Chen, H.J. Gross, Adsorption kinetics of polyethylene glycol from aqueous solution onto activated carbon, Water Res. 38 (2004) 2559–2570.