Polysorbates 20 and 80 Used in the Formulation of Protein Biotherapeutics: Structure and Degradation Pathways

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ABSTRACT: Polysorbates 20 and 80 (Tween® 20 and Tween® 80) are used in the formulation of biotherapeutic products for both preventing surface adsorption and as stabilizers against protein aggregation. The polysorbates are amphipathic, nonionic surfactants composed of fatty acid esters of polyoxyethylene sorbitan being polyoxyethylene sorbitan monolaurate for polysorbate 20 and polyoxyethylene sorbitan monooleate for polysorbate 80. The polysorbates used in the formulation of biopharmaceuticals are mixtures of different fatty acid esters with the monolaurate fraction of polysorbate 20 making up only 40–60% of the mixture and the monooleate fraction of polysorbate 80 making up >58% of the mixture. The polysorbates undergo autooxidation, cleavage at the ethylene oxide subunits and hydrolysis of the fatty acid ester bond. Autooxidation results in hydroperoxide formation, side-chain cleavage and eventually formation of short chain acids such as formic acid all of which could influence the stability of a biopharmaceutical product. Oxidation of the fatty acid moiety while well described in the literature has not been specifically investigated for polysorbate. This review focuses on the chemical structure of the polysorbates, factors influencing micelle formation and factors and excipients influencing stability and degradation of the polyoxyethylene and fatty acid ester linkages. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:2924–2935, 2008

Keywords: polysorbate; Tween; surfactants; stability; degradation; micelle; proteins; protein formulation; excipients; degradation

INTRODUCTION

The proper formulation of recombinant proteins for pharmaceutical products is vital to their long-term stability and reproducible activity. To maintain biological activity, proteins generally must be maintained in a specific, three-dimensional conformation. This conformation is only marginally stable, and thus relatively minor perturbing forces can disrupt protein structure causing loss of biological activity or an immunological response. Such perturbations are commonly encountered as proteins are produced, stored, transported, and delivered to patients. For example, it is well known that during common industrial processes such as filtering,[1] storage,[2] agitation[3,4] freeze/thawing,[5–7] lyophilization,[8–10] nebulization,[11] and spray drying[1,12–17] proteins can suffer damage to their native conformation. Further, delivery of

Abbreviations: HSA, human serum albumin; CMC, critical micelle concentration; PEG, polyoxyethylene glycol.

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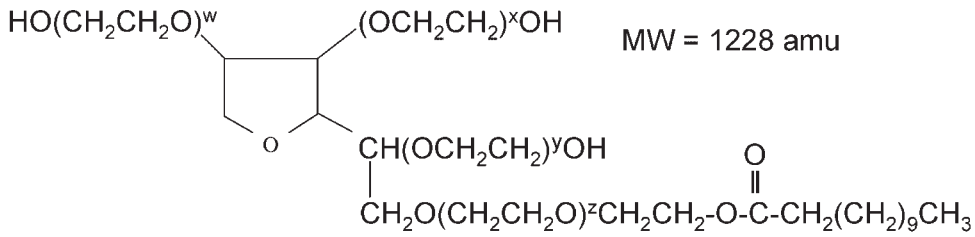
protein pharmaceuticals to patients may also provoke losses of conformational integrity *via* unfavorable interactions of proteins with surfaces (e.g., inner walls of catheter tubing or syringes).[18]

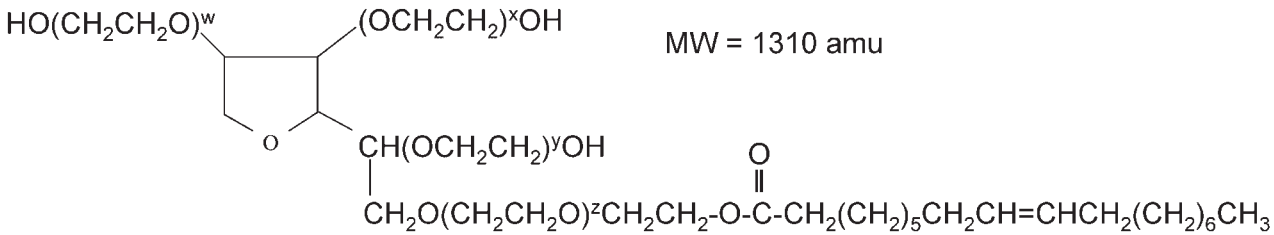
Surface-active agents, or surfactants, are often added to protein solutions to prevent physical damage during purification, filtration, transportation, freeze-drying, spray drying, storage, and delivery. Human serum albumin (HSA) was initially used in a number of protein formulations as a stabilizer to prevent surface adsorption. Recent concerns though about the presence of infectious agents (e.g., viruses, prions) in blood products has prompted many regulatory agencies to no longer allow its use forcing the exploration of other options. Towards that end polysorbates, nonionic surfactants, are increasingly being used as replacements for HSA. The polysorbates are both biocompatible and excellent stabilizers against surface adsorption. However, they must be used with caution since under some conditions there degradation can lead to chemical modification of proteins and interactions with some proteins may lead to changes in conformational structure with unknown consequences.[1,19] To prevent problems from occurring it is important to understand the issues associated with there use including their structure, stability, and mechanisms through which they may interact with proteins. This article reviews the structure and stability of polysorbates 20 and 80, the most commonly used nonionic surfactants in the biotechnology industry. For information on the interaction of polysorbate 20 and 80 with proteins the reader is referred to the review by Randolph and Jones.[20]

POLYSORBATE STRUCTURE

The polysorbates are amphipathic, nonionic surfactants composed of fatty acid esters of polyoxyethylene sorbitan.[21] Polysorbate 20 (polyoxyethylene sorbitan monolaurate) and Polysorbate 80 (polyoxyethylene sorbitan monooleate) are the most common polysorbates currently used in formulation of protein biopharmaceuticals.[22] Their structures are shown in Figure 1. The structures shown here are those of the chemically homogenous polysorbates. Solutions of polysorbate sold by manufacturers and labeled as polysorbate 20 or 80 are chemically diverse mixtures of different fatty acid esters[23–25] and will be discussed later. Both types of polysorbates have a common backbone and only differ between the structures of the fatty acid side chains. The hydrocarbon chains provide the



Polysorbate 20 (Tween 20): polyoxyethylene sorbitan monolaurate



Polysorbate 80 (Tween 80): polyoxyethylene sorbitan monooleate

(w + x + y + z) = 20

Figure 1. Chemical structure of polysorbates 20 and 80. $w+x+y+z$ refers to the total number of oxyethylene subunits on each surfactant molecule and may not exceed 20.

hydrophobic nature of the polysorbates while the hydrophilic nature is provided by the ethylene oxide subunits. The common backbone structure is a sorbitan ring with ethylene oxide polymers attached at three different hydroxyl positions. While the number of repeat ethylene oxide subunits varies at each position their total number $(w+x+y+z)$ equals 20 and is constant for each polysorbate. The fatty acid moieties are attached through an ester linkage to the ethylene oxide oxygen at the z position. The laurate moiety of polysorbate 20 is a straight chain hydrocarbon structure and the oleate moiety of polysorbate 80 contains a double-bond forming a kink in the hydrocarbon chain.

The polysorbates used in the manufacture of drugs, both biotechnology and pharmaceutical, are sold either as polysorbate 20 or 80 or under the trade names Tween® 20 and Tween® 80. In the literature the names polysorbate and Tween are used interchangeably and both should be searched for retrieving articles from Medline® or other indexing services. As stated earlier polysorbate solutions are generally sold as mixtures of the fatty acid esters.[23–25] The European Pharmacopoeia[26] defines the percentage of the different fatty acid esters of each polyoxyethylene sorbitan that must be present in each solution. The United States Pharmacopoeia does not define the composition of the fatty acid esters present in the solutions. The compositions of the mixtures and chemical structures are shown in Table 1. As can be seen the lauric acid containing component of polysorbate 20 is 40–60% of the total number of fatty acid species while the oleic acid containing component of polysorbate 80 is $\geq$58% of its total. The remaining fatty acids are a mixture of both saturated and unsaturated fatty acids with caproic, caprylic, capric, and lauric acids being present only in polysorbate 20 with all other fatty acid esters present in both polysorbate 20 and 80 solutions. Analysis of polysorbates from different vendors using reverse phase HPLC coupled with a charged aerosol detector has shown only minor differences in the fatty acid contents between mixtures from different suppliers (Sungae Park, personal communication). A supply of polysorbate 80 made of 99% pure oleic acid is now available from NOF Corporation albeit this has not made its way into manufactured products. The majority of polysorbates are produced using strictly plant sources and are the required polysorbates used in biotechnology products. The information regarding the source of the fatty acids used to manufacture the polysorbates can be obtained from the manufacturer.

SOLUTION AND SURFACE ACTIVE PROPERTIES OF POLYSORBATE

Because of their dual hydrophobic/hydrophilic nature, surfactants in solution tend to orient themselves so that the exposure of the hydrophobic portion of the surfactant to the aqueous solution is minimized.[20] In systems containing air/water interfaces, surfactants will tend to accumulate at

Table 1. Fatty Acid Contents of Polysorbate 20 and 80

	EU Specifications		
Acid	PS-20 (%)[a]	PS-80 (%)[b]	Structure
Caproic	$\leq$1		$CH_3(CH_2)_4COOH$
Caprylic	$\leq$10		$CH_3(CH_2)_6COOH$
Capric	$\leq$10		$CH_3(CH_2)_8COOH$
Lauric	40–60		$CH_3(CH_2)_{10}COOH$
Myristic	14–25	$\leq$5	$CH_3(CH_2)_{12}COOH$
Palmitic	7–15	$\leq$16	$CH_3(CH_2)_{14}COOH$
Palmitoleic		$\leq$8	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Stearic	$\leq$7	$\leq$6	$CH_3(CH_2)_{16}COOH$
Oleic	$\leq$11	$\geq$58	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Linoleic	$\leq$3	$\leq$18	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Linolenic		$\leq$4	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$

[a]European Directorate for the Quality of Medicines and Healthcare. European Pharmacopoeia On-line. 5th Edition 2007, 01/2005:0426.
[b]European Directorate for the Quality of Medicines and Healthcare. European Pharmacopoeia On-line. 5th Edition 2007, 04/2006:0428.

these interfaces, forming a surface layer of surfactant oriented in such a fashion that only their hydrophilic ends are exposed to water.[20,27,28] Such orientation and hydrophobic surface adsorption can also occur at solid/water interfaces such as those found in vials, syringes, and other glass and plastic containers.[20] Surfactant adsorption can be in direct competition with protein adsorption at the same interface possibly resulting in reduced binding of the protein at the interface or less damage to the protein adsorbed at the interface.[20] The driving force for the surface adsorption is the hydrophobic effect which in this case can be described by the Gibbs free energy equation for the transfer of the hydrocarbon chain from an organic solvent into water.[28–30]

$$\Delta G_t = \Delta H_t - T\Delta S_t$$

At temperatures near 25°C the entropic term, ΔS_t, for a hydrocarbon is large and negative becoming more unfavorable with an increase in the size of the hydrocarbon. The contribution from the entropic term greatly outweighs that from the enthalpic term, ΔH_t, which is zero at or near 25°C for many small hydrocarbons.[29,31,32] The temperature at which the $\Delta H_t = 0$ is known as the T_h, the temperature of minimum solubility. Below this temperature ΔH_t is negative while above T_h the ΔH_t is positive and increasing with temperature. Therefore, as ΔS_t becomes less negative with increasing temperature the ΔH_t will continue to increase and account for a greater proportion of the free energy change (ΔG_t).[29,31,32] Within the temperature range of 4–37°C used for most liquid protein formulation studies, the entropy term will be dominant.

Transfer of the long chain hydrocarbons of the polysorbates into water is generally assumed to accompany the ordering of water into cage like structures around the hydrocarbon chains resulting in a loss of solvent entropy. The hydrophobic effect which drives the adsorption process arises from the fact that water would rather hydrogen bond with itself rather than form cage-like waters around the hydrocarbon chains.[29,31,32] Adsorption of the polysorbate at the surface through the hydrocarbon chains results in removal of the hydrophobic surface from the water molecules accompanied by a more favorable entropy contribution to the solvent system. Additionally, at increasing polysorbate concentrations this allows for van der Waals contact between the hydrocarbon chains at the interface. Due to the hydrophobic effect surfactant molecules adsorb at interfaces even at low surfactant concentrations. Because of thermal motion there will be a balance of adsorption and desorption such that equilibrium interfacial conditions will take some time to establish.[28] An example of this for polysorbate 80 above and below the CMC is shown in Figure 4. The time to establish equilibrium at the interface may be an important factor regarding the ability of the polysorbate to prevent surface adsorption and aggregation of proteins during acute stresses such as shaking.

Surfactant micelles are spherical aggregates of the polysorbates with the ethylene oxide subunits pointing outwards in contact with the surrounding solution and the hydrocarbon tails in the center away from the water. The interior properties of the micelles are similar to those of liquid hydrocarbon.[28] As the surfactant concentration increases and the surfaces become saturated the monomers in solution associate into micelles. This normally occurs within a narrow concentration range and is referred to as the critical micelle concentration or CMC (Fig. 2). The number of polysorbate molecules per micelle is ~50–100 but this value can vary depending on the actual polysorbate concentration and solution conditions, that is, temperature, solutes, etc. Similar to adsorption, the main driving force behind

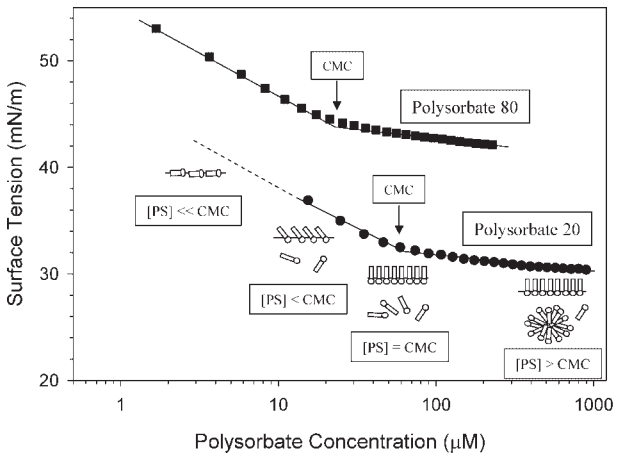


Figure 2. Surface tension measurements of polysorbate 20 and 80 and model depicting the relationship of the surfactant molecules coating the air/liquid interface. Surface tension was measured in both pure water and containing 140 mM sodium chloride using the ring method with a Kruss Processor Tensiometer K100. Model of surfactants coating the air/liquid interface was adopted from Randolph and Jones[20] and Porter.[27]

micelle formation is the tendency of the hydrocarbon chain of the surfactant to minimize water contact, the hydrophobic effect. The ethylene oxide chains hydrogen bond with water maintaining the solubility of the surfactant molecules. Polysorbate 80 which is composed of longer hydrocarbon chains than polysorbate 20 (see preceding discussion) has a more negative ΔS_t resulting in a lower CMC value than that for polysorbate 20. As the temperature increases ethylene oxide containing nonionic surfactants exhibit a monotonic lowering in CMC due to dehydration of the ethylene oxide chains.[33] Measurements of the CMC for either polysorbate 20 or 80 at temperatures above or below 25°C have not been reported.

Lowering of the CMC with increasing temperature results in phase separation and is known as the cloud point. At temperatures just below the cloud point, nonionic surfactant micelles exhibit a marked increase in size becoming visible even to the naked eye.[33] The clouded out phase also contains substantial levels of water, indicating that complete dehydration is not necessary for phase separation.[33] The cloud point temperature has been reported as 76°C for a 3% (w/v) solution of polysorbate 20 in 1 M NaCl and 65°C for a 3% (w/v) solution of polysorbate 80 in 1 M NaCl.[34] Recent experiments in our lab (Fig. 3) have confirmed the earlier result for polysorbate 80 but showed an increase in the cloud point for polysorbate 20 by approximately 10°C, possibly due to the increased quality of the polysorbates available in newer preparations or a difference in the overall hydrocarbon makeup of the surfactant. The early experiments by Donbrow et al.[34] in 1978 on the cloud point of polysorbate were done in 1 M NaCl and none have been reported since then. Therefore, we tested the effects of NaCl concentration and found that the cloud point was also related to the salt concentration in the aqueous solution. Other excipients such as sugars were not examined. As shown in Figure 3 the cloud point appears inversely proportional to the increasing NaCl concentration for both polysorbate 20 and 80, albeit we were unable to observe a cloud point at temperatures up to 100°C for polysorbate in water alone. These results are important as the effect of temperature on protein unfolding using techniques such as differential scanning calorimetry or circular dichroism may be influenced by this phenomenon. No mention of salt effects on the cloud point of polysorbates was found in any publications, although it is known that salt increases the hydrophobic effect.[30] Further experiments are required to determine if the effect on the cloud point is being exerted on the ethylene oxide or hydrocarbon chains. Ananthapadmanabhan[33] noted that an increase in the alkyl chain length of a given nonionic surfactant lowers the cloud point and would explain the difference in cloud points at a given NaCl concentration between polysorbate 20 and 80. This also suggests that the hydrophobic effect on the hydrocarbon moiety driving micelle formation is significant at the temperature at which clouding occurs.

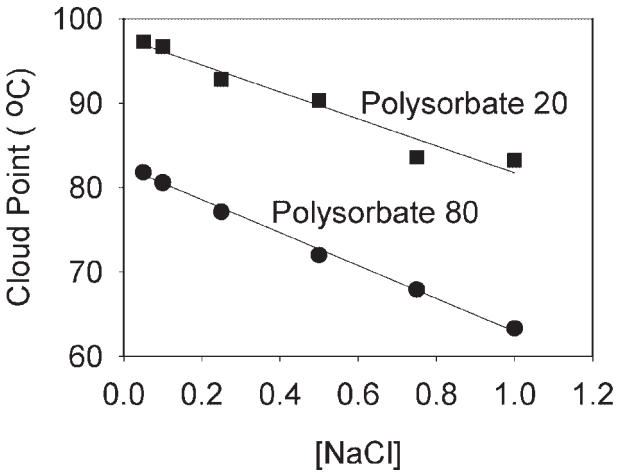


Figure 3. Influence of salt on the cloud point of polysorbate 20 and 80. Cloud point of polysorbate (2% w/v solution) in each salt concentration was measured using a Stanford Research Systems MPA100 automated capillary melting point apparatus. The cloud point was recorded at the initiation of clouding.

When the concentration of the surfactant is much lower than its CMC, surfactant molecules lay flat at the air/water and hydrophobic solid/water interfaces (Fig. 2).[20,27] As the concentration is increased, more molecules adsorb to these interfaces, such that the surface concentration remains linearly proportional to the bulk concentration.[30] This crowding forces the surfactant molecules to order themselves such that the hydrophilic groups are oriented towards the bulk water and the hydrocarbon chains are pointed towards the air or hydrophobic solid.[27,35] At sufficiently high surfactant concentrations (i.e., at or above the CMC), there is an oriented monolayer of surfactant molecules and maximum surfactant absorption, at the interface. At the air–liquid interface the surface saturation is responsible for the sharp slope change (to essentially zero) observed in experimental plots of surface

properties (surface tension, osmotic pressure) versus surfactant concentration.[27] Polysorbates at the hydrophobic solid/water interface have not been well characterized and the possibility remains that a well ordered monolayer as well as hemi-micellar structures are also formed. Both types of structures have been demonstrated for other nonionic surfactants.[36] Nonetheless, the surface active property of polysorbate at the air/liquid and liquid/surface interfaces is the reason they are used in protein formulations to prevent protein aggregation[37–41] and surface interaction.[42–45] Studies using protein films at the air/liquid interface have shown polysorbate 20 can displace bovine serum albumin[46] and β-casein/β-lactoglobulin mixtures.[47–50]

CMC values can be determined by a number of techniques including surface tension,[43,51,52] osmotic pressure,[53] fluorescence,[54–59] micellar electrokinetic chromatography,[60] calorimetry,[61–63] light scattering,[64–66] electron paramagnetic resonance,[67] and analytical ultracentrifugation.[68] For illustrative purposes surface tension measurements and dye partitioning will be discussed here. The surface tension method uses a surface tensiometer and measures the point at which the solution surface is saturated with surfactant. As the surfactant coats the air–liquid surface the surface tension drops until it reaches a minimum value then levels out. This occurs as a sharp transition and defines the value for the CMC[20,69] being approximately 0.007% (w/v) or 55 μM for polysorbate 20[52,70] and 0.0017% (w/v) or 13 μM for polysorbate 80.[70,71] This assay does not define the interaction of polysorbate free in solution but indicates the concentration of all surface-active species at the liquid surface interface. Patist et al.[70] found that polysorbates containing surface-active impurities in technical grades of surfactants gave anomalous CMC results when measured by the surface tension method. Additionally, since the assay is measuring an equilibrium process between polysorbate free in solution and at the varying interfaces the working CMC value of a polysorbate as it relates to a formulation in a specific container may vary depending on the container type and surface to volume ratio. For the dye-binding assay an increase in fluorescence is measured when a hydrophobic compound, such as 1,6-diphenyl 1,3,5-hexatriene partitions from the aqueous phase into the oily phase of the micelle interior.[56,70] Chattopadhyay and London[56] have optimized the use of this system including effects of probe concentration, incubation and light exposure and their work should be consulted prior to experimentation with this system. In contrast to the surface-tension measurement, dye partitioning provides a picture of the interactions in solution and shows micelle formation occurring over an extended concentration range of surfactant (Fig. 5). This occurs since the formation of micelles is an equilibrium phenomenon between monomers at the air–liquid interface, free in solution and in micelles.[20,27,69,71] By the dye-partitioning method initiation of micelle formation is observed at surfactant concentrations of 0.001% (w/v) for polysorbate 80 and 0.002% (w/v) for polysorbate 20. Since the polysorbates are nonionic in nature the presence of electrolytes such as sodium chloride or amino acids do not affect the CMC values of the polysorbates[72] as demonstrated by the overlap in the data for the solutions containing salts in both polysorbate 20 and 80 (Fig. 5). An additional factor that may also affect CMC values but has not been described in the literature is the affect of surface to volume ratio of a container.

CHEMICAL STABILITY OF POLYSORBATES

Chemical stability of the polysorbates is another important consideration for their use. The polysorbates are notorious for undergoing autooxidation[34,73–79] and cleavage at the ethylene oxide subunits[34,74] as well as hydrolysis of the fatty acid ester bond.[34,80] A generalized reaction scheme depicting these processes is shown in Figure 6. Autooxidation of the ethylene oxide

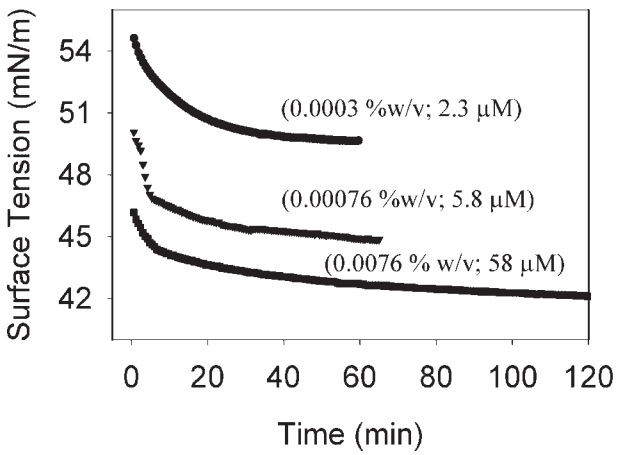


Figure 4. Surface tension measurements of polysorbate 80 over time at a fixed polysorbate concentrations. Polysorbate 80 in water was mixed for 1 min then the surface tension measured over time using the ring method with a Kruss Processor Tensiometer K100.

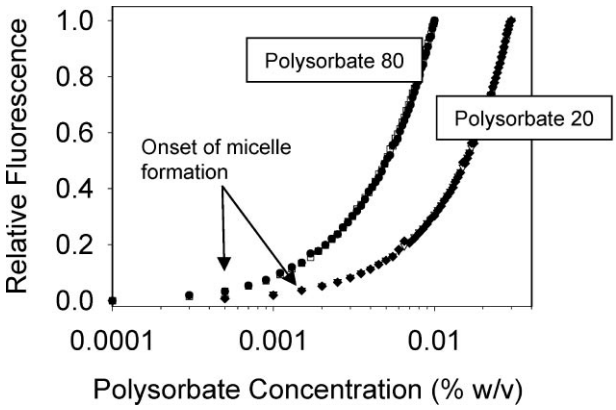


Figure 5. Dye partitioning of 1,6-diphenyl 1,3,5-hexatriene in solutions with increasing concentrations of polysorbate. Method was adopted from that of Chattopadhyay and London.[56] Open squares represent polysorbate 80 in water, filled circles represent polysorbate 80 in a salt solution, open triangles represent polysorbate 20 in water and filled diamonds represent polysorbate 20 in a salt solution.

results in hydroperoxide formation, side-chain cleavage and eventually formation of short chain acids such as formic acid.[73] Hydroperoxide formation by polysorbate is known to cause oxidation of proteins as was shown for the two therapeutic proteins recombinant human ciliary neurotrophic factor (rhCNTF) and recombinant human nerve growth factor (rhNGF),[81] The oxidation was prevented by addition of antioxidants such as cysteine, thioglycerol, and methionine.[81] Hydrolysis of the fatty acid ester bond results in formation of the long chain fatty acids linoeic for polysorbate 20 and oleic for polysorbate 80.[34,80,82] The reactions are dependent on the solution pH, presence of oxygen, peroxides, heat, UV light and metal ions such as copper. Storage of the polysorbates at room temperature (25°C) primarily results in hydrolysis of the fatty acid ester while storage at higher temperatures also favors autooxidation of the ethylene oxide subunits. Storing the polysorbate solutions away from light, in the presence of nitrogen and at lower temperatures helps prevent degradation from occurring. Degradation

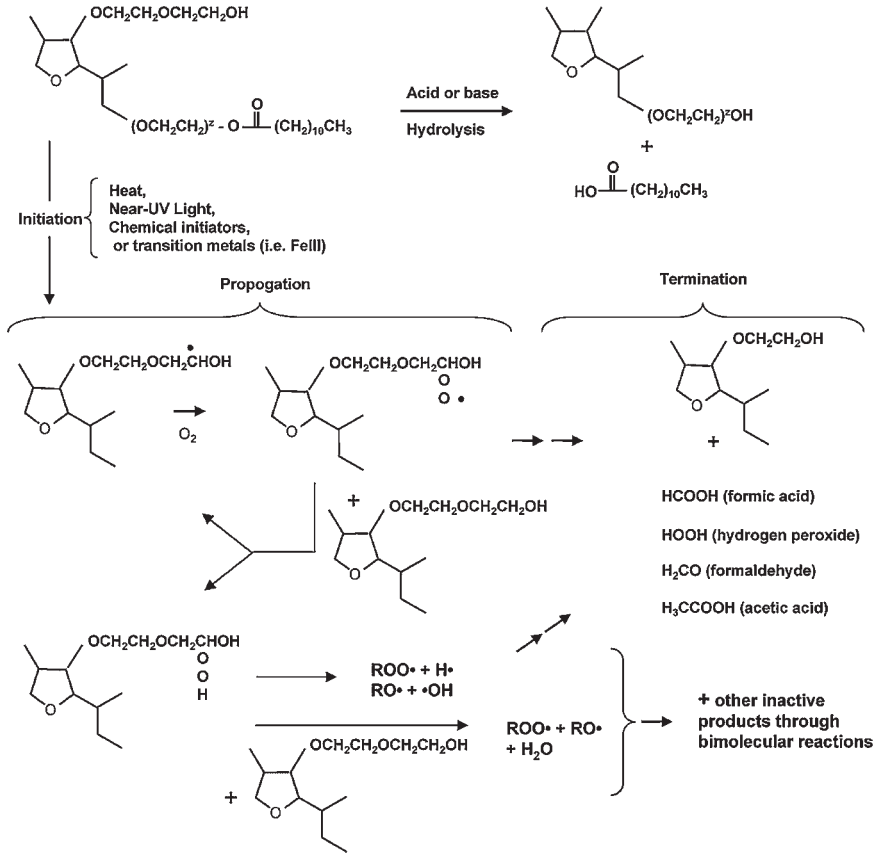


Figure 6. Reaction scheme depicting the hydrolysis of the fatty acid ester of polysorbate 20 and the autooxidation process of the ethylene oxide subunits. Reaction schemes were adapted from those described by Donbrow.[34,73,83]

can also be prevented by addition of low concentrations of the antioxidant butylated hydroxytoluene (BHT).[83]

The kinetics of hydrolysis for the fatty acid ester bond is affected by the pH and temperature of the solution. Hydrolysis of the ester bond is both acid and base catalyzed[80] and the mechanism of ester hydrolysis has been described previously for both acid catalyzed[84] and base catalyzed[85] reactions. Based on the similarity in structure of both polysorbates 20 and 80 around the ester bond the kinetics of the reaction were expected to be similar for the compounds. Bates et al.[80] found nearly identical rates of hydrolysis for polysorbates 40, 60, and 80. Donbrow et al.[34] used the activation energies and rates determined by Bates for polysorbate 80 to predict the hydrolysis rates for a solution of polysorbate 20 at 25 and 40°C and found good agreement between the theoretical and experimental results in a pH 3.95 solution. Studies of polysorbate 80 at 80°C showed that the reaction was catalyzed markedly below pH 3 and above pH 7.6. The lowest rates were obtained between pH 3–7.6 being essentially constant within that range. In addition to the pH dependence the rates of hydrolysis were also concentration dependent. When tested between values of 0.02 to 0.1% (w/v) at pH values of 1.1 and 10.3 and 80°C the initial rates of cleavage decreased with increasing polysorbate concentration. It was suggested that this was due to an increase in the fraction of monomers existing in micelles and or a marked alteration in the micellar structure making it more difficult for the protons or hydroxide anions to reach the ester bonds. Interestingly, different polysorbates with a fivefold change in their CMC values demonstrated similar rates of hydrolysis when examined under acidic conditions.

Autooxidation of the polysorbates occurs along the ethylene oxide moieties of both polysorbate 20 and polysorbate 80. Since the polyethylene oxide structures of the polysorbates are similar then the oxidative degradation processes are expected to be the same. The overall oxidation reactions (Fig. 6) which occur in distinct phases were initially described by Donbrow et al.[34,73,83] Phase I is initiation of radical formation by light or a catalyst forming a carbon based radical on either the α or β carbon. Phase II is reaction of the carbon radical with oxygen forming an organic peroxide followed by abstraction of a hydrogen to form an acid and a new carbon radical allowing propagation of the reaction. Finally the reaction is terminated by self-quenching of the radicals through bimolecular interactions.[34,73] The autooxidation process involves scission of both C–O and C–C bonds producing short chain acids such as formic acid and acetic acid, suggesting that the peroxide formation mainly occurs at the ethylene oxide moieties near the ends of the chains resulting in shortening of the polyoxyethylene chains. Low concentrations of formaldehyde have also been identified as contaminants in polysorbate solutions[86,87] consistent with formaldehyde identified by Donbrow et al.[73] resulting from the C–C fission of the hydroperoxides or free radicals. The chain shortening will change the hydrophilic to lipophilic ratio of the surfactants resulting in modification of their physical characteristics such as CMC value and cloud point formation.[34,51] Jaeger et al.[75] used catalase to demonstrate that 75% of the peroxide formed in polysorbate 80 is in the form of hydrogen peroxide with the remainder being other types of peroxides. The peroxide formation was prevented by storing the solutions under an atmosphere of nitrogen.[21]

Factors affecting the autooxidation process include temperature, light and the presence of transition state metals such as copper. Recently, Harmon et al.[88] have taken advantage of this property of polysorbate 80 together with iron III to generate peroxy radicals and developed an oxidant stressing system for studying degradation of small molecules. As stated above the autooxidation process results in formation of hydroperoxides and acids that can be assayed for using various analytical techniques.[21,34,75,76,78] An interesting feature of peroxide formation in polysorbate solutions is that it undergoes an initial lag period followed by a rapid rise in peroxide formation and eventually a decline in the peroxide concentration.[21,34] If peroxide content was the only measure used for estimating polysorbate quality then the degree of degradation in a sample could be greatly underestimated at later stages of the reaction. As the peroxides increase and eventually decline, the acid content of the polysorbate solutions increase leading to a decrease in the solution pH. Depending on the storage temperature the solution may fall to a pH value of 2–4 for a 3% (w/v) polysorbate solution, while nondegraded polysorbate solutions have a pH of ~6–7.[34] Lower temperatures of 25–40°C favor hydrolysis of the ester bond leading to long chain fatty acids with a pK_a of ~4.9 with higher temperatures also leading to autooxidation and short chain acids with pK_a values of ~3.6. Light also increases the rate of polysorbate

degradation in solution. Ha et al.[21] demonstrated that after 5 weeks at 40°C solutions of polysorbate 80 contained eightfold as much peroxide as solutions stored in the dark. Interestingly, removal of the air prevented peroxide formation even in the presence of light suggesting that the oxygen in the air was the likely culprit involved with the autooxidation process. The presence of transition state metals such as copper may also catalyze autooxidation of the polysorbate. Donbrow et al.[34] showed that addition of copper sulfate to solutions significantly increased the rates of polysorbate degradation.

Oxidation of unsaturated and polyunsaturated fatty acids is also a well-known process but has not been discussed in the literature in relation to polysorbate oxidation. As described in the preceding paragraphs oxidation of polysorbate is mainly associated with oxidation of the ethylene oxide moiety and not the unsaturated fatty acid moieties such as oleate and linoleate present in solutions of polysorbate 20 and 80 to varying degrees (Tab. 1). The oxidation of unsaturated fatty acids occurs in a manner similar to that for polyethylene oxide (PEG). The reaction mechanism that will be described here was derived from studies of unsaturated fatty acids in organic solvents such as benzene but will provide a basis for understanding of what may occur in the polysorbates as well. Similar to PEG autooxidation of the unsaturated fatty acids is a free radical chain process consisting of initiation, propagation and termination.[89–91] The key event in initiation is formation of a lipid radical, $R^{\cdot}$. This can occur by thermal or photochemical hemolytic cleavage of an RH bond or by hydrogen atom abstraction from R–H by an initiator free radical. The propagation step normally begins with addition of molecular dioxygen to $R^{\cdot}$ and the second step of propagation, the rate limiting step, is abstraction of a hydrogen atom from RH by a peroxyl radical $ROO^{\cdot}$ to generate ROOH and another $R^{\cdot}$ eventually resulting in reactions leading to non reactive species.[89–91] Because these reactions are propagated by reaction with molecular oxygen the removal of dioxygen would be expected to eliminate this process as well. As stated though this process has not been described in the literature as related to polysorbates and as such will be a useful area of research.

CONCLUDING REMARKS

Polysorbates are present in a large number of biopharmaceutical drugs listed in the 2006 Physicians Desk Reference. The concentrations used in the formulations range from 0.0003% (w/v) to 0.3% (w/v). Over the past decade the quality of polysorbate solutions has increased dramatically such that many manufacturers now offer highly purified, low peroxide, and low acid content solutions. While the solutions are initially low in reactive oxygen species care must still be used when storing drug products containing the polysorbates since, as described above, degradation can occur under a variety of conditions leading to formation of acids and peroxides that can readily degrade proteins. A good idea is to use the lowest amount of polysorbate possible in a formulation to minimize the risk of damage to a protein product. Additionally, it should also be kept in mind that polysorbate solutions are generally not a homogenous mixture of a single fatty acid ester but are usually a mixture of the fatty acids mentioned in Table 1. Pure oleic acid esters for polysorbate 80 can be obtained, albeit how this versus a mixture might affect protein stability has not been reported. Finally, data are now emerging that suggest polysorbates not only can bind to proteins (discussed extensively in Randolph and Jones[20]) but may also affect the structure of the protein in solution, an area of investigation that could provide a fruitful area for future research.

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