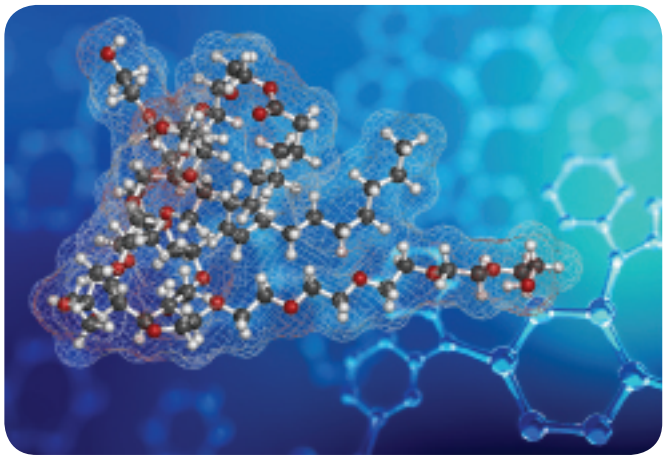

Analysis of Shelf-Life of Polysorbates by Electron Paramagnetic Resonance (EPR) Spectroscopy in 60 min – Impact in Biologics' Development

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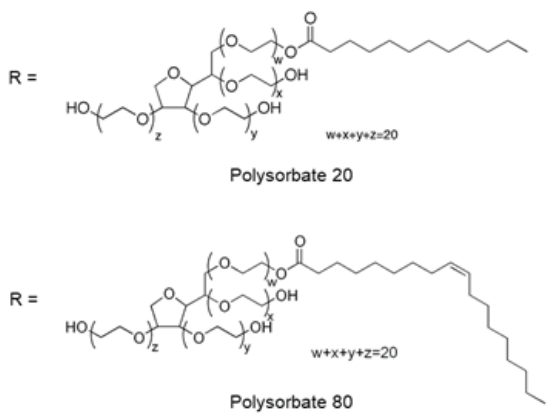
Polysorbates are an important class of amphipathic, nonionic surfactants that are widely used in the pharmaceutical industry. They are used both clinically and preclinically, due to their effectiveness at low concentrations and relatively low toxicities (1-2). In the formulation of proteins, they are used as surfactants to prevent surface absorption, limiting physical damage during purification, filtration, transportation, freeze-drying, storage and delivery (3). Polysorbate 20 (PS 20 – polyoxyethylene sorbitan monolaurate) and Polysorbate 80 (PS 80 – polyoxyethylene sorbitan monooleate) are the most common polysorbates used in the formulation of protein biopharmaceuticals. They are composed of diverse mixtures of different fatty acid esters and the solutions sold by the manufacturers are labeled either as polysorbate 20 or 80 or under the trade names Tween® 20 and Tween® 80 (Figure 1A).

Figure 1



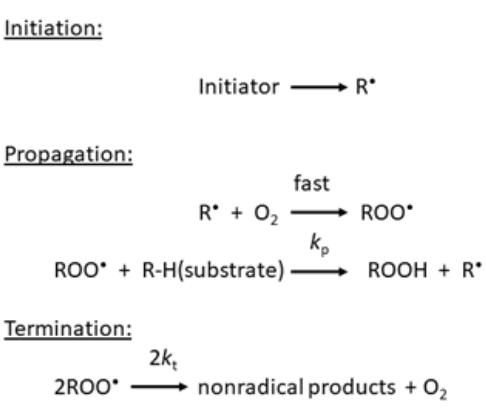


Figure 1A. Chemical structure of polysorbate 20 and polysorbate 80.

Figure 1B. General autoxidation mechanism.

It is known that polysorbates are prone to degradation by autoxidation (5-8). Figure 1B provides an example of a free radical chain reaction that leads to polysorbate degradation. It starts with the oxidation of fatty acid esters, either by metal-, temperature- or light-induced processes to form various fatty acid free radicals. These free radicals are usually carbon centered and react rapidly with oxygen to form alkylperoxyl radicals. Alkylperoxyl radicals promote further free radical formation by abstracting hydrogen atoms from other fatty acid ester molecules. The alkylperoxyl radicals are converted to hydroperoxides that either undergo thermolysis or react with metals to form alkoxyl, alkylperoxyl and carbon centered free radicals. This free radical chain reaction proceeds until it is terminated.

Stability testing at typical storage conditions is often too slow for practical use in quality control, and as a result such tests are often performed under "forced" conditions (e.g., using elevated temperature, excessive exposure to light, or in the presence of traces of transition metals) to initiate the degradation and the free radical process (4). EPR (electron paramagnetic resonance, a.k.a ESR, electron spin resonance) is a spectroscopic technique that specifically and directly measures samples containing free radicals. At the same time the technique is completely "blind" to components within the samples that do not contain free radicals (or unpaired electrons), and so is very specific. Free radicals are "short-lived", so to increase our ability to detect them, it is required to add a compound known as a spin trap. The spin trap reacts with the free radical to form a "radical adduct". Radical adducts are also free radicals, but they are much more stable than the original (half-lives as long as days, compared to milliseconds). One of the most popular spin traps is 5,5-dimethyl-1-pyrroline N-oxide (DMPO), which has been cited in Medline more than 1,000 times. DMPO has significant advantages over other nitrone spin traps – it is redox inactive and the EPR spectra of the radical adducts are easy to distinguish.

The purpose of this white paper is to show how to apply the EPR technique to evaluate the level of degradation in polysorbates that have occurred via autoxidation under typical laboratory storage (4 and 25°C) or inappropriate transportation (40°C) conditions, in order to provide compelling evidence for the suitability of EPR as a rapid method for the determination of the acceptable shelf-life of polysorbates. The forced degradation assay temperature was chosen to be 37°C since this is the physiological temperature used during development and testing of biological products.

Material and Methods

Tween® 20 and Tween® 80 were purchased from three different vendors. The spin trap DMPO was obtained from Dojindo Laboratories (Kumamoto, Japan). Glass capillaries and Critoseal® were purchased from VWR International.

EPR spectra at all the time points were obtained with a bench top EPR Bruker EMXnano spectrometer equipped with a variable temperature unit (VTU) with accessible temperature range of 100 - 425 K:

(https://www.bruker.com/products/mr/epr/emxnano/accessories/sample-temperature.html)

The formation of the spin trap radical adducts and their time evolution was monitored by a 2D experiment (field sweep vs. time) configured in Bruker's Xenon software. After the experimental data were acquired, the radical concentration was determined using double integration and the SpinCount module from the software.

Experimental protocol

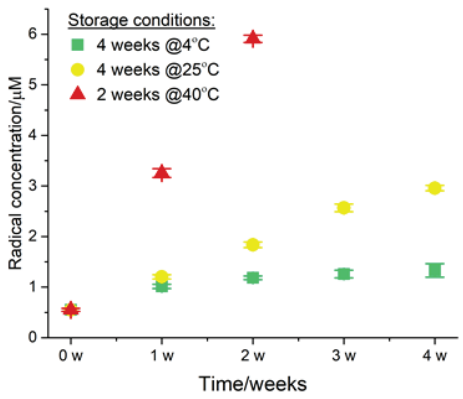
1. Warm up the spin trap DMPO to 35 – 36°C for 5-10 min so that it is liquid. The stock concentration is 9 M.
2. Add 300 µl of polysorbate to an Eppendorf tube and then add 3 µl of the stock DMPO. The final concentration of the spin trap DMPO is approximately 100 mM.
3. Vortex the tube and transfer the solution to a 100 µl capillary. Close the capillary with Critoseal®.
4. Insert the capillary with the sample into the resonator and tune the spectrometer.
5. Using variable temperature unit (VTU) set the temperature to 37°C and acquire a 2D_Field_Delay experiment for 60 min.

Results

Effect of temperature on the radical formation in Tween® 20

Two batches of Tween® 20 were stored for 1 month under two standard storage conditions (refrigerated storage at 4°C and room temperature storage at 25°C). A third batch was held at a slightly elevated temperature (40°C) for 2 weeks in order to accelerate the degradation. EPR spin trapping experiments were performed using the experimental protocol described above.

Figure 2

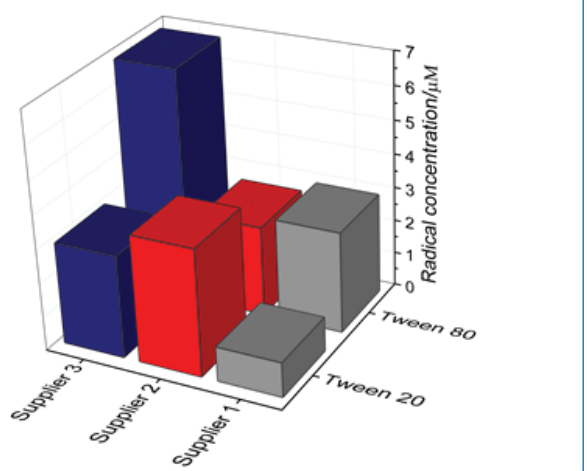


The formation of the radical adducts and their time evolution was monitored for 60 min at the physiological temperature of 37°C and the values at the 60 min time points are shown here. The EPR assay was then performed once a week, for a total period of 4 weeks. After the experimental data were acquired, quantitative EPR analysis using the SpinCount module from the Bruker Xenon software was performed to calculate the radical concentration at the end point (t_{60}). The data was exported and the t_{60} molarity was plotted versus storage time (Figure 2). This experiment showed that Tween® 20 contains 0.5 µM of free radicals as a baseline at t_0. The sample stored at 4°C for 1 month showed an increase by a factor of 2, and the final radical concentration was 1 µM at the end-point t_{60} (green squares). The yellow circles represent the radical evolution collected from the sample stored at room temperature (25°C) for 1 month. The final concentration at the end of the experimental time period was approximately 3 µM which is an increase of a factor of more than 6 relative to t_0. The sample stored at 40°C for 2 weeks had a radical yield of 6 µM which is more than a 30-fold increase compared to the baseline (red triangles). In general, the radical yield correlates directly with the level of degradation and directly with the shelf-life. Overall, the results clearly show how EPR can be used to determine the level of degradation and to predict long-term stability of polysorbates during the most common storage and accelerated ageing conditions.

Effect of different suppliers on the radical formation in Tween® 20 and Tween® 80

Tween® 20 and Tween® 80 from three different suppliers were tested as received (i.e. at t_0) under the same experimental conditions as described above with the objective of evaluating the relative oxidative status of the polysorbates received from the vendors (Figure 3). In general, the degree of variation between the polysorbate type and vendor was surprisingly high (showing a 7-fold variation) and this clearly indicates that careful evaluation and selection of vendor and product type is a very important step during formulation development. With EPR, it is now possible to routinely and easily measure Tween® 20 and Tween® 80 samples from multiple vendors for QA/QC of incoming raw materials.

Figure 3



Radical concentration in Tween® 20 and Tween® 80 from three different suppliers. The molarity was determined at the end-point of the assay (t_{60}).

Conclusion

Polysorbates are present in a large number of biopharmaceutical drugs and it is well known in the literature that caution must be used when storing drug products containing polysorbates. The EPR data described in this paper shows that degradation occurs and is detected under a variety of storage conditions leading to formation of free radicals The primary concern being that these radicals can readily oxidize and degrade proteins, leading to potentially severe and undesired affects in patients. We also show that there is significant and unexpected variation between polysorbates purchased from different vendors thereby showing that selection of both the polysorbate type and also the vendor are highly important steps. EPR spectroscopy is the only technique for direct and non-invasive detection of free radicals in polysorbates. By analyzing an EPR signal, one can identify, quantify and monitor the temporal behavior of the free radicals involved in the degradation of polysorbates.

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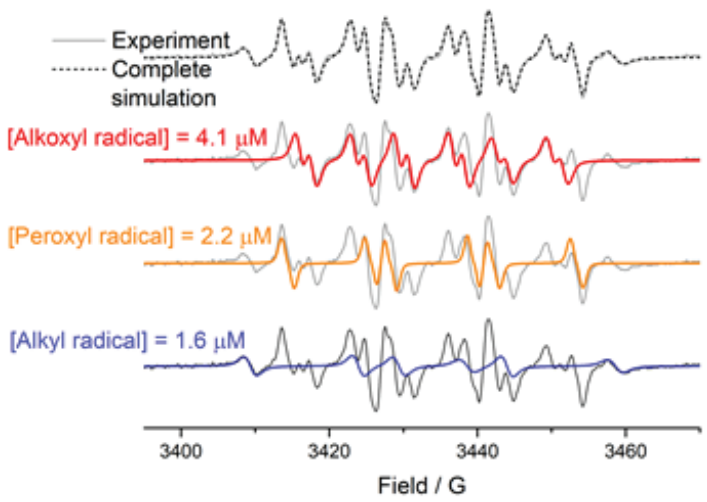
Appendix

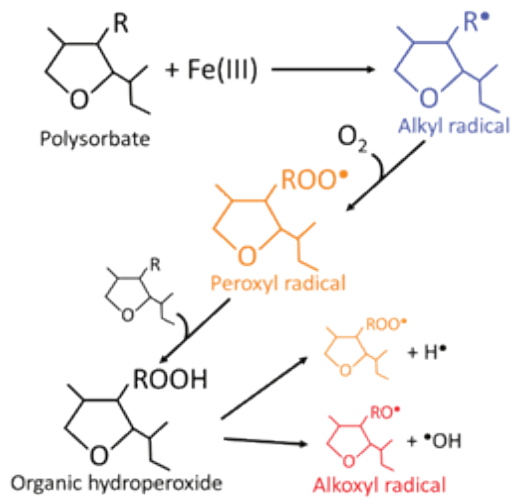
Identification and quantification of detected free radicals in polysorbates is a key step in understanding the mechanism of their autoxidation. With the EPR quantification package (SpinCount and SpinFit) implemented in Bruker Xenon software, the task of identifying and quantifying the polysorbate radicals is both straightforward and precise.

Figure A



An EPR spectrum of DMPO-radical adducts detected in polysorbates and the complete simulation are presented on the top in grey. From the simulated spectrum (the dotted trace) three different radicals were identified using SpinFit module – alkoxyl (simulated in red), peroxyl (simulated in orange), and alkyl (simulated in blue) radicals. The concentration of each radical species was determined using SpinCount module.

Figure B



The proposed mechanism of autoxidation in polysorbates strongly correlates with the EPR data. Reaction scheme was adapted from Ref. [3].

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