Degradation of high molar mass poly(ethylene glycol), poly(2-ethyl-2-oxazoline) and poly(vinyl pyrrolidone) by reactive oxygen species

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Abstract

During the last decade, polymeric biomaterials have become one of the hottest topics in drug delivery and tissue engineering. Nevertheless, for several regularly employed biopolymers, very little information regarding their stability and fate in a biological context can be found in literature. Some of these biomaterials are known to be prone to oxidative (bio)degradation when exposed to oxidative species, thus inducing gradual decomposition of the polymer backbone. Earlier studies revealed strong dependency of the degradation rate on the chain length of the respective polymer, albeit only relatively small molar masses were studied. Here we extend the investigations on the oxidative degradation of high molar mass poly(2-ethyl-2-oxazoline), poly(ethylene glycol) and poly(N-vinylpyrrolidone) (up to 500 kg/mol) and compare to the results obtained for lower molar mass. The previously observed trend of a more rapid degradation of polymers with higher degree of polymerizations could be corroborated for poly(2-ethyl-2-oxazoline) and poly(ethylene glycol), and was also observed for poly(N-vinylpyrrolidone).
Introduction

Increased levels of reactive oxygen or nitrogen species (ROS/RNS) in vivo are referred to as oxidative stress and commonly associated with pathophysiological processes\textsuperscript{[1]}. For instance, oxidative damage and its results are involved in the development of numerous serious diseases such as cancer,\textsuperscript{[2,3,4]} neurological disorders\textsuperscript{[5,6,7,8,9]} and others\textsuperscript{[10,11]}. According to some epidemiological studies, global health and nutrition are associated with the oxidative state, as specific diets containing foods rich of antioxidants such as vitamin C cause lower disease rates.\textsuperscript{[12]} However, earlier studies assuming ROS to be involved in aging and related degenerative diseases\textsuperscript{[12]} have been recently challenged.\textsuperscript{[13]} Positive influence on health due to antioxidant rich nutrition could not be proven,\textsuperscript{[14]} preventative antioxidant supplementation is supposed to be too non-specific, too little and too late\textsuperscript{[1]}. Irrespective of this discourse of the involvement of ROS in pathophysiological processes, oxidative species are known as an indispensable component of essential physiological mechanisms like respiration and host defense by macrophages and neutrophils. In particular, the latter is a process that must be considered to be of relevance for polymers applied in vivo, in particular in parenteral applications, where it is well known that a large portion of administered materials will become associated with the mononuclear phagocyte system (MPS).

In general, to assess the stability of polymers, many different factors and pathways should be taken into consideration. In a biological context, in addition to oxidative degradation, proteolytic and hydrolytic degradation must be considered most relevant. Stability of polymeric biomaterials used for e.g. implants or scaffolds against ROS has been studied in some detail\textsuperscript{[15,16]} Kohn et al. have investigated the effects of ROS on polymers and the behavior of cell in contact with the treated polymers. Interestingly, they observed a particular influence of the poly(ethylene glycol) (PEG) content in the investigated materials.\textsuperscript{[17]} On the other hand, stimulus responsive polymers have seen great interest\textsuperscript{[18]}, including oxidatively responsive drug delivery systems\textsuperscript{[19,20,21]}. 

It is well established that PEG, one of the most commonly used hydrophilic polymer for biomedical and pharmaceutical purposes, can form peroxides\textsuperscript{[22]} and is not stable against ROS.\textsuperscript{[23]} Previously, the stability of small molar mass PEG against degradation by ROS was compared to the stability of poly(2-ethyl-2-oxazoline) and poly(N-ethylglycine)\textsuperscript{[24]}, two members of polymer families discussed as potential alternatives of PEG.\textsuperscript{[25,26,27,28]} Interestingly, higher apparent degradation rates of POx compared to PEG was observed, although POx have been reported to not generate hydroperoxides, although no data were presented to support the claim.\textsuperscript{[27]} In all cases, a strong dependency of the apparent degradation rate with respect to the degree of polymerization (DP) and molecular weight (MW), respectively, was observed. Specifically, longer chains being degraded faster than shorter ones. Comparison of polymers with similar DP revealed higher apparent stability towards oxidative degradation of PEG than observed for the pseudo-polypeptides PETOx and PETGly. Even at relatively low concentrations of 0.5 mM hydrogen peroxide, significant degradation of the polymers within a few days was observed. Thus, although in general concentrations of hydrogen peroxide in the human body are very low (µM range) and discussed controversially,\textsuperscript{[29,30,31]} oxidative stress may influence the long term stability of biopolymers, in case they are not excreted but accumulate as has been reported for higher molar mass PEG.\textsuperscript{[32,33]} This should be particularly relevant in instances when administered polymers are taken up by immune cells producing ROS or accumulate at sites with increased ROS levels such as sites of (chronic) inflammation.

POx and polypeptoids are structural isomers that both comprise a tertiary amide in every repeating unit, the former as a pending motif with the amine being part of the polymer backbone, the latter comprising the amide fully within the backbone. Poly(N-vinylpyrrolidone) (PVP) can be viewed as another structural isomer, featuring a C-C backbone with the tertiary amide entirely in the side chain. PVP, frequently used as retarding agent in drug delivery and formerly as plasma expander as well as in skin-care products, is considered to be non-biodegradable.\textsuperscript{[34,35,36]} PVP with molecular weights
exceeding 20 kg/mol, such as PVP K30 (M_w = 40 kg/mol) and K90 (M_w = 360 kg/mol),
cannot be removed via renal excretion.[37] Instead, high molecular weight PVP is known to
be phagocytosed and permanently stored in organs of the RES by histiocytes (tissue
macrophages). Thus, patients who have received high molecular weight PVP are reported
to suffer from the so-called ‘PVP storage disease’. Prolonged administration might cause
bone destruction by infiltration of PVP storage cells in bone marrow as well as skin lesions,
anemia and pseudotumors. Therefore, PVP is considered not suitable for systemic
administration.[38,39]

Nonetheless, considering the structural similarities, we were interested in the degradation
of PVP upon incubation with ROS. In addition, we also wanted to study the degradation
profile of high molar mass PEtOx and PEG, as in a previous study only relatively low molar
mass samples were investigated.

Results and Discussion

Exposed to phagocytic cells, polymers might be prone to oxidative degradation by the released
ROS.[40] Traces of metal ions like Cu^{2+} and Fe^{3+} further enhance ROS reactivity in terms of
hydroxyl radical formation.[41, 42] A previous study demonstrated a strong correlation between
molar mass of a polymer and the apparent rate of oxidative degradation but the range of molar
mass investigated was small (2 kg/mol – 11 kg/mol). Therefore, we decided to investigate a
larger range of molar masses of up to 500 kg/mol. Furthermore, we also investigated the
degradation of PVP as a polymer comprising an arguably more stable C-C-backbone but a
tertiary amide side chain similar to POx. Hence, we compare here the oxidative degradation
of PEG (DP 45, 136, 230, 800 and 9100), POx (PEtOx DP 51, 108, 500 and 5000) and PVP
(DP 30, 90, 360 and 3250). Please note, that data presented for PEG (DP 45 and 136) and PEtOx (DP 51 and 108) have been taken from a previous study.\cite{24} In order to maintain constant and comparable conditions, we adhered to the H\textsubscript{2}O\textsubscript{2}/CuSO\textsubscript{4} system previously employed to produce hydroxyl radicals, applying a constant concentration of 50 µM CuSO\textsubscript{4} and 0.5, 5 and 50 mM H\textsubscript{2}O\textsubscript{2}, respectively. Polymers mass concentration was chosen at β = 1 g/L, incubated at a physiological temperature of 37 °C for the indicated time, frozen and subsequently freeze-dried.

**GPC-Analysis – Elugrams**

Freeze-dried samples were dissolved in HFIP and separated from insoluble components (salt and potentially insoluble degradation products) by filtration (0.2 µm). The remaining clear solutions were analyzed by gel permeation chromatography. An important limitation of the chosen experimental approach is the fact that we cannot rule out that fractions of the degraded polymer may be insoluble in HFIP and thus, be neglected in the analysis.

High molecular weight representatives of commercially available PEtOx and PEG were degraded very rapidly. At 50 mM H\textsubscript{2}O\textsubscript{2} concentration, within 30 minutes, the original elution peak is strongly shifted to higher elution volumes and broad bi- or multimodal distributions, respectively, were observed (Figure 1). It should be noted that the commercially available higher molar mass PEtOx are known to show a rather large dispersity.

Elevated baselines at low elution volumes, in particularly observed in the case of PEtOx\textsubscript{5000} are probably due to measurement artefacts due to the low signal-to-noise ratio after considerable degradation. For both PEtOx\textsubscript{500} (50 kg/mol) and PEtOx\textsubscript{5000} (500 kg/mol) strong elution peaks at the column volume of 34.5 mL are observed after prolonged periods of time, indicating low molar mass degradation compounds, whereas almost no high molecular weight components remain after 3 d. Interestingly, although smaller peaks at the column volume are observed for PEG\textsubscript{500} and PEG\textsubscript{9100} as well, two prominent elution peaks at 29 and 32.5 mL are apparent at all concentrations of H\textsubscript{2}O\textsubscript{2}, indicating the formation of rather stable intermediates. The corresponding apparent molar masses are 9000 g/mol (29 mL) and 1100 (32.5 mL),
respectively (PMMA calibration). Comparable elution patterns for each polymer are observed independently from the H$_2$O$_2$ concentration and also different starting molar masses (Figure 2). Thus, oxidative degradation of PEtOx and PEG appear to follow similar degradation patterns at different molar masses with only the degradation rate being dependent on the concentration of ROS. In contrast, the degradation patterns between the different polymers tested, as judged from the changes in the elution profiles depend strongly on the polymer investigated. In particular, the development of the elugram patterns of PVP strongly differ from those of PEtOx and PEG (Figure 3).

Figure 1: Exemplary gel permeation elugrams of high molecular weight PEtOx$_{500}$ (50 kg/mol) and PEtOx$_{5000}$ (500 kg/mol) (top) as well as PEG$_{800}$ (35 kg/mol) and PEG$_{9100}$ (400 kg/mol) after incubation with 50 µM CuSO$_4$ and 50 mM H$_2$O$_2$ for indicated periods of time.

Observable especially in case of high molecular PVP (DP 360 and 3250), the initial phase of oxidative degradation is characterized by a rapid shift of the elution peak to higher elution
volumes with hardly any broadening of the distribution. This might be ascribed to side chain cleavage predominantly occurring in the early phase of oxidative PVP degradation. The side chain cleavage conceivably leads to a significant change polymer solubility and coiling pattern which may express itself in a shift in elution volume in gel permeation chromatography.

Figure 2: Exemplary normalized gel permeation elugrams of poly(vinylpyrrolidone) (PVP), poly(2-ethyl-2-oxazoline) (PEtOx) and poly(ethylene glycol) (PEG) incubated at different concentrations of H$_2$O$_2$ (supplemented with 50 µM CuSO$_4$) at similar stages of degradation (residual apparent M$_w$ given in %).
Figure 3: Exemplary gel permeation elugrams of poly(vinylpyrrolidone) (PVP) with degree of polymerizations (DP) ranging from 30 (top left) to 3250 (bottom right) after incubation with 50 µM CuSO$_4$ and 50 mM H$_2$O$_2$ for different periods of time.

As the degradation advances, a significant low molar mass tailing is observed, which may be indicative of scission of the C-C-backbone. However, at around 40 % reduction with regard to $M_w$, the elugram of PVP$_{3250}$ still resembles a narrow distribution, whereas a significant low molar mass tailing is observed for PVP$_{360}$, PVP$_{90}$ and PVP$_{30}$ (Figure 4). However, it also should be noted that the lower molar mass PVP already show a pronounced low molar mass tailing before degradation, which makes interpretation difficult. In addition, since a great many different macromolecular species, with vastly differing solubility are expected to result, this analysis must be viewed with great caution. However, while the current analysis does not yield sufficient information on the degradation products, it is quite clear that the investigated polymers are degraded rather readily, depending on the ROS concentration.
Figure 4: Exemplary gel permeation elugrams of poly(vinylpyrrolidone) (PVP) samples of different initial degree of polymerization (DP = 30 to 3250) at a comparable remaining $M_w$ of 40%.

**GPC-Analysis – Development of $M_w$**

In order to perform a semi-quantitative analysis of the polymer degradation, we can plot the % residual apparent $M_w$ obtained from the GPC elugrams against the incubation time. However, we must point out once more, that the measured $M_w$ is only relative to the calibration standard and cannot be expected to reflect actual $M_w$. As expected from a glimpse at the elugrams, the degradation rate increases with increasing concentration of H$_2$O$_2$. An earlier study$^{[24]}$ reported strong correlations of the polymer chain length and degradation rate, which inspired the present study with strongly increased molar masses. The present data (Figure 5) confirms the increasing apparent degradation rate with increasing degree of polymerization. At the highest concentration of 50 mM H$_2$O$_2$, PEG$9000$ lost 93 % of its initial molar mass within only 30 minutes of incubation. Identical conditions resulted in even 97.5 % molar weight lost observed for the PEtO$5000$ sample.
Following the rapid initial weight loss, degradation is decelerated considerably. The apparent degradation rate of PEG230 closely resembles the one of PEG136. Notably, with the exception of PEG230, all of the presented degradation experiments were carried out with fresh H₂O₂ solution.
Therefore, the somewhat slower apparent degradation of PEG_{230} may be attributed to inactivation of \( \text{H}_2\text{O}_2 \) during prolonged storage.

Interestingly, the early phase of oxidative PVP degradation is characterized by a slight increase of \( M_w \) (Figure 6).

![Development of apparent molar mass of PVP samples plotted against the incubation time.](image)

Figure 6: Development of apparent molar mass of PVP samples plotted against the incubation time. Data are presented as means ± standard error of the mean (SEM) (\( n = 3 \)). Lines are intended as a guide for the eye only.

Although only evident for PVP_{30} and PVP_{90} at low concentrations of \( \text{H}_2\text{O}_2 \), we suppose this phase to be part of the other PVP degradation series as well, though not recognizable due to the fast, subsequent degradation. This might be attributed to a change in polymer coiling leading to higher apparent \( M_w \) at low degrees of degradation. As mentioned before, \( M_w \) is determined out of the GPC elugrams and GPC measurement is first and foremost based on the hydrodynamic radius of the polymers. If partial side chain cleavage results in enhanced
solubility in HFIP, higher values for $M_w$ are obtained regardless of the actual existing weight loss. Nevertheless, potential chain coupling reactions of hydroperoxides would also result in an increase of the hydrodynamic radius.

Determination of time to 50% loss of apparent $M_w$ ($t_{50\%}$) allows direct comparison of the degradation rates with respect to DP or molar mass (Figure 7). An exponential decrease of $t_{50\%}$ with DP/molar mass is observed. Considering degradation related to DP, more pronounced degradation of PEtOx compared to PEG is observed over the entire range of investigated chain lengths. PVP weight loss appears comparable, if somewhat slower to PEG at 0.5 and 5 mM $H_2O_2$ but markedly slower at 50 mM. Interestingly, while PEG and PEtOx degradation appear to become increasingly similar with increasing concentration of $H_2O_2$, the difference to PVP is increasing. This appears more apparent as the $t_{50\%}$ are plotted against molar mass. This can be explained by the respective molar masses of the repetition units. PVP and POx are comparable, PEG differs strongly in this point (PEtOx: 99 g/mol, PVP: 111 g/mol, PEG: 44 g/mol). Although still observable, influences of polymer chain length become less evident with increasing chain length. This is especially distinct for the lowest $H_2O_2$ concentration of 0.5 mM, where, for instance, $t_{50\%}$ of PEG$_{800}$ and PEG$_{9100}$ appear to not differ at all. Plotting $t_{50\%}$ against $1/H_2O_2$ (Figure 8) also reflects this observation, with almost overlaying linear fits for PEG$_{800}$ and PEG$_{9100}$. Despite the tenfold increased chain length, only minor differences between the degradation rates of PEtOx$_{500}$ and PEtOx$_{5000}$ are apparent as well. In case of PVP, this behavior is less pronounced though also noticeable within the investigated range of chain lengths. Plotting the slope of $t_{50\%}$ against $1/H_2O_2$ against the molar mass or the DP, respectively, we can further reduce the data (Figure 9). It becomes apparent that especially at very low DPs/molar masses (< 10 kg/mol), the susceptibility for oxidative degradation changes markedly with the DPs/molar masses, irrespective of the concentration of ROS, while at higher molar masses, the differences with respect to the oxidative degradation become less pronounced. This hints at an end-group effect, such that the end-groups may act as ROS scavengers that react with ROS without leading to pronounced polymer degradation. It is interesting that qualitatively, this effect is very similar for PEG, PEtOx and PVP, three
polymers that are polymerized by different methods and presumably contain different end-groups.

Figure 7: Dependence of $t_{50\%}$ plotted against degree of polymerization DP (left column) and against molar mass (right column), respectively. Error bars represent 95 % confidence intervals. * Data taken from ref. [28].
Conclusion

The oxidative degradation of hydrophilic biomaterials may be relevant after their parenteral administration. In case of PVP, the PVP-storage disease is well-documented in humans after parenteral administration of polymers with a molar mass exceeding 20 kg/mol. The results presented here suggest
that the PVP would be degraded/metabolized to some extent and it can be assumed that the insoluble degradation products may contribute to materials stored in macrophages. Similarly, our results suggest that PEG, in particular polymers of higher molar mass, may be (partially) metabolized/degraded in activated macrophages. However, we must point out that these projections toward a hypothetical situation in vivo should be considered with extreme caution. Future work may be directed at delineating potential end-group effects by testing samples of small molar mass with different terminal groups.

**Experimental**

**Materials**

All chemicals and solvents were purchased from Sigma Aldrich, Acros or Roth and used as received unless otherwise stated. PEG$_{230}$ ($M_n = 10$ kg/mol, $M_n^{GPC} = 60$ kg/mol, $\bar{D} = 1.09$, Roth #2634.1), PEG$_{800}$ ($M_n = 35$ kg/mol, $M_n^{GPC} = 100$ kg/mol, $\bar{D} = 1.1$, Sigma Aldrich #81310), PVP$_{30}$ ($M_n = 3.5$ kg/mol, $M_n^{GPC} = 1.8$ kg/mol, $\bar{D} = 4.6$, Acros #276142500), PVP$_{90}$ ($M_n = 10$ kg/mol, $M_n^{GPC} = 11$ kg/mol, $\bar{D} = 2.7$, Sigma Aldrich #PVP10), PVP$_{360}$ ($M_n = 40$ kg/mol, $M_n^{GPC} = 39$ kg/mol, $\bar{D} = 2.5$, Roth #4607.1), PVP$_{3250}$ ($M_n = 360$ kg/mol, $M_n^{GPC} = 276$ kg/mol, $\bar{D} = 2.2$, Roth #CP15.1), PEtOx$_{500}$ ($M_n = 50$ kg/mol, $M_n^{GPC} = 34$ kg/mol, $\bar{D} = 1.9$, Sigma Aldrich #372846) and PEtOx$_{500}$ ($M_n = 500$ kg/mol, $M_n^{GPC} = 131$ kg/mol, $\bar{D} = 3.5$, Kremer Pigmente #63905) did not contain radical inhibitors according to the providers and was used as received. PEG$_{9100}$ ($M_n = 400$ kg/mol, $M_n^{GPC} = 395$ kg/mol, $\bar{D} = 1.5$, Sigma Aldrich #372773) contained BHT which was removed via dialysis. Phosphate buffered saline (PBS) was prepared by dissolving sodium chloride (8.00 g, 137 mmol), potassium chloride (0.20 g, 2.68 mmol), disodium hydrogen phosphate (1.42 g, 10.0 mmol) and potassium dihydrogen phosphate (0.27 g, 1.98 mmol) in 1 L millipore water yielding isotonic PBS with a pH value of 7.4.

**Analytical Methods**

Gel Permeation Chromatography (GPC) was performed using a Polymer Standard Service (PSS, Mainz, Germany) system (MDS RI detector) with a 50 mm PFG precolumn and three 300 mm PFG columns
(pore size 7 mm) at 40 °C and HFIP (containing 5 mmol/L ammonium trifluoroacetate) as the eluent with a constant flow rate of 1 mL/min. Prior to each measurement, samples were filtered through 0.2 mm PTFE syringe filters (Roth, Karlsruhe, Germany). The system was calibrated using poly(methyl methacrylate) standards (PSS, Mainz, Germany) with molar masses ranging from 800 g/mol to 1600 kg/mol. Data were processed using WinGPC software.

Polymer Degradation Studies

Studies on the oxidative degradation of PEtOx, PEG and PVP were performed as described elsewhere[24]. Shortly, polymers were dissolved in PBS (1 g/L) and incubated over night at 37 °C. Subsequently, 50 µM CuSO₄ and H₂O₂ (0.5, 5 or 50 mM) were added and samples were shaken at 37 °C. In order to replenish degraded H₂O₂, addition of H₂O₂ was repeated every 24 h. Withdrawn aliquots of 5 mL were frozen and subsequently freeze-dried for subsequent GPC analysis.

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Supporting Info:

Tabulated version of the data presented in Figures 5–9 are presented are available in the supporting information.


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