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Photo - oxidation Facilitating the Preservation of High Molecular Weight Dissolved Organic Nitrogen in the Ocean

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Abstract
The majority (90%) of dissolved organic matter (DOM) in the ocean is biologically refractory (average residence time ≥ 4000 yr). In particular, the high molecular weight (HMW) dissolved organic nitrogen (DON) can persist into the deep ocean providing a food source for benthic organisms. Most of the deep-sea and sedimentary N are preserved as proteinaceous materials, despite the parent source of native proteins being considered labile. How the proteinaceous materials become more refractory during the transformation processes and are largely preserved is not fully understood. Our recent ESI-FTICRMS results have shown that photo-oxidation of protein compounds causes both fragmentation and crosslinking, yet the latter resulted in an overall increase in the intensity-averaged MW (> 20%); heavily oxidized (e.g., by radical oxygen species, ROS) and cross-linked proteins become more prone to aggregate and resistant to biodegradation. Thus, investigations on how photo-oxidation processes can change the refractory nature of proteinaceous materials via crosslinking, and induce aggregation, resulting in the preservation of HMW DON, would be highly desirable.

Key Word and Phrases
Photo-oxidation, Radical Oxygen Species, Chemical Crosslinking of Proteins, Preservation of Organic Nitrogen, Ocean.

1. Preservation of Dissolved Organic Nitrogen

Nitrogen (N) is one of the major limiting nutrients for primary production, and plays a key role in biogeochemistry and cycles of many other elements in the ocean [1]. Dissolved organic nitrogen (DON) accounts for more than 99% of the organic nitrogen in the ocean. DON is mainly produced by biotic processes, such as the release from actively growing phytoplankton and bacteria, viral lysis or autolysis of microbes [2]. Modern techniques such as NMR have shown the freshly produced DON includes low molecular weight (LMW) (<1kDa) molecules such as free amino acids and urea [2], and high molecular weight (HMW) molecules with diverse structures, such as amino polysaccharides, proteins, and amines [3] (Fig. 1). The LMW amino acids and urea can be rapidly utilized by heterotrophic organisms and are thus considered to be labile [2]. About 30% of DON represents HMW DON that can be obtained by ultrafiltration [4]. HMW amines are labile because they can be taken up by micro-organisms through cell deaminases [5], and thus, are scarce in the water column[4]. In contrast, the amino polysaccharides and amides are relatively refractory across oceanic regions [6], [7], and make up the major HMW DON pools in the water column [4]. The N-acetyl amino polysaccharides (e.g. chitin and peptidoglycan) dominate and account for about half of the freshly produced HMW DON [4]. However, a large fraction of amino polysaccharides is degraded and removed while being transported into the deep ocean. As a consequence, most of the deep-sea HMW DON and sedimentary organic nitrogen is present as proteinaceous materials [4], [6]. The hydrolyzable proteins and nonhydrolyzable amides account for more than 80% of HMW DON in deep sea, indicating that proteinaceous materials can escape biodegradation.

The proteinaceous materials are originally from biotic sources, and the native proteins are considered to be labile and unlikely to survive [8], [9]. It remains controversial how the proteins are transformed to become resistant to biodegradation, and as a consequence, become the dominant fraction in the HMW DON of the deep ocean. Recognizable proteins by proteomics greatly decrease from the surface ocean to the deep sea [10]. Previous studies have suggested biophysical preservation pathways for dissolved organic matter including a) sorption to mineral surfaces or

sediment mesopores [11], [12], b) incorporation within networks of non-hydrolyzable materials, e.g., the algaenans and porin [13], [14], and c) non-covalent reactions and change of hydrophobicity [9]. However, most of these hypotheses rely on the protection of biomolecules through biophysical processes involving aggregation or absorption, and could not explain the preference in preserving proteinaceous materials over the other DON molecules. Abiotic transformation (aging and UV-oxidation) of proteins from labile to refractory has been observed, but the mechanisms have not been fully revealed [8]. One difficulty is that published research only rely on identifiable proteins in protein libraries, thus ignoring refractory proteins. Thus, further studies of the mechanisms that could explain how proteinaceous materials become refractory, leading to their preservation as the majority of HMW DON in the deep sea.

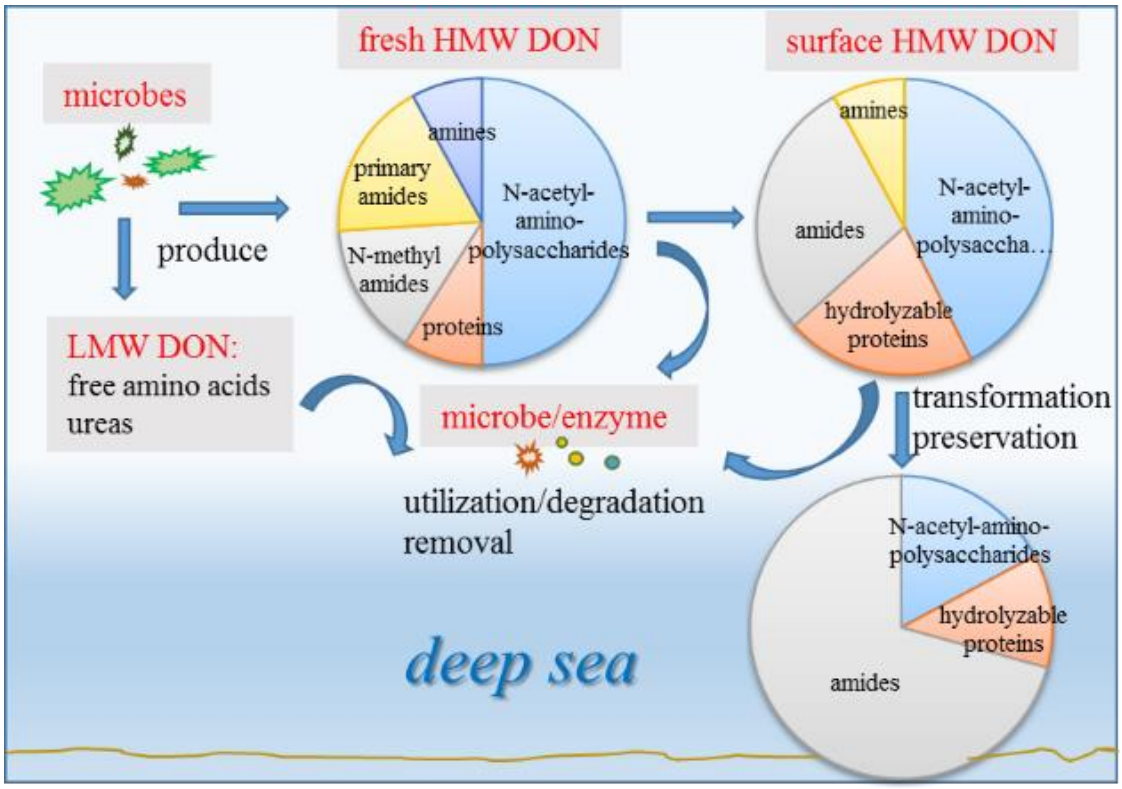

Fig. 1 Chemical composition and preservation of DON in the ocean. Proteinaceous materials (hydrolyzable protein and nonhydrolyzable amides) are preserved into deep ocean and sediments [3].

2. Chemical Cross-Linking of Proteins during Photo-oxidation

The biological degradation and microbial utilization of proteins is initiated by enzymatic peptide hydrolysis, i.e. by proteases, which are excreted from various organisms and are ubiquitously present in the ocean [15]-[18]. The transformation of proteins from labile to refractory in the ocean is rarely studied due to its challenging nature [8], [9], [19]. However, in biochemical and health science studies, it has demonstrated that heavily oxidized and covalently cross-linked proteins can become resistant to biodegradation [20]. The chemical cross-linking process is mostly mediated through reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) [21], hydroxyl radical (HO•) [22], [23], and hydrogen peroxide (H_2O_2) [24], via various biochemical and physiological pathways.

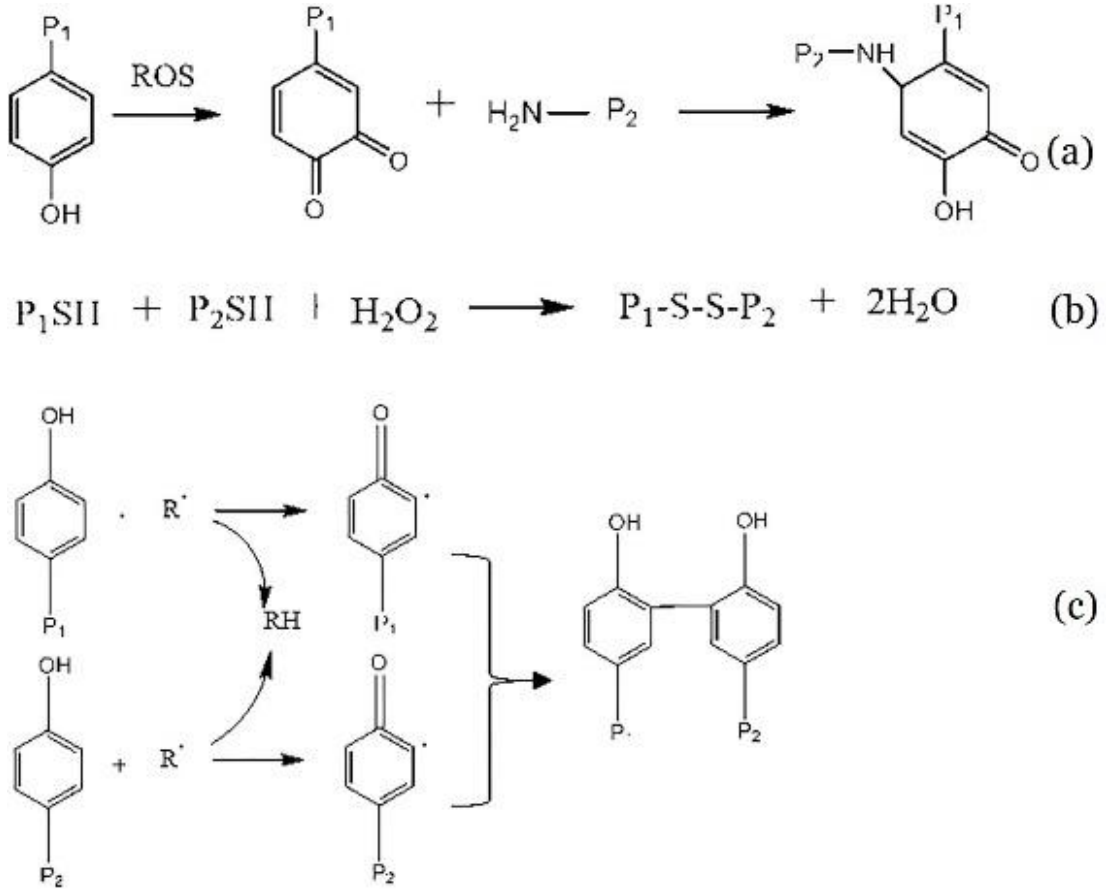

Fig. 2 The pathways of chemical cross-linking/polymerization of proteins. P refers to protein, and R· to radical [25].

These pathways include (Fig. 2): a) Michael addition of a N-amino group to a carbonyl group on proteins to form cross-linked derivatives, in which the carbonyl group (e.g. on 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid (DOCH)) is formed from oxidation of aromatic amino acids (tyrosine and phenylalanine), [25]; b) Oxidation of two cysteine residues by ROS to form intra- or inter-disulfide cross-linked derivatives; c) Radical-radical reactions between two tyrosine radicals that are formed via one-electron oxidation of the aromatic rings by HO• attack [26]. These pathways identified in health science likely also occur in oceanic systems, where ROS can be produced by sunlight and/or microbial enzymes.

In the surface ocean, oxidative conditions can be achieved by natural sunlight irradiation. The energy emitted by sunlight can excite the side chains of proteins, such as tryptophan, in the presence of molecular oxygen. Subsequently, ROS are produced via charge transfer from excited states of proteins to dissolved oxygen in the immediate environment of the proteins [27]. Besides proteins, the ROS can also be generated from other DOM compounds such as terrestrial DOM containing chromophores that enhance its absorption and photoreactivity. ROS are then produced from the reaction of excited state DOM compounds with oxygen [28], [29]. The H2O2 is relative stable, and its concentration in the surface ocean is reported as 0.1~0.2μM (3.8~7.6μg) [30], [31]. Other ROS such as HO•, can be produced from the irradiation of H_2O_2 and/or NO_3^-, and is the most oxidative ROS species. Our previous studies [28], [32] have shown that the H_2O_2 and HO• took part in the light-induced aggregation process of DOM (Fig 3), and thus these ROS are likely also involved in the transformation of HMW DON in the ocean.

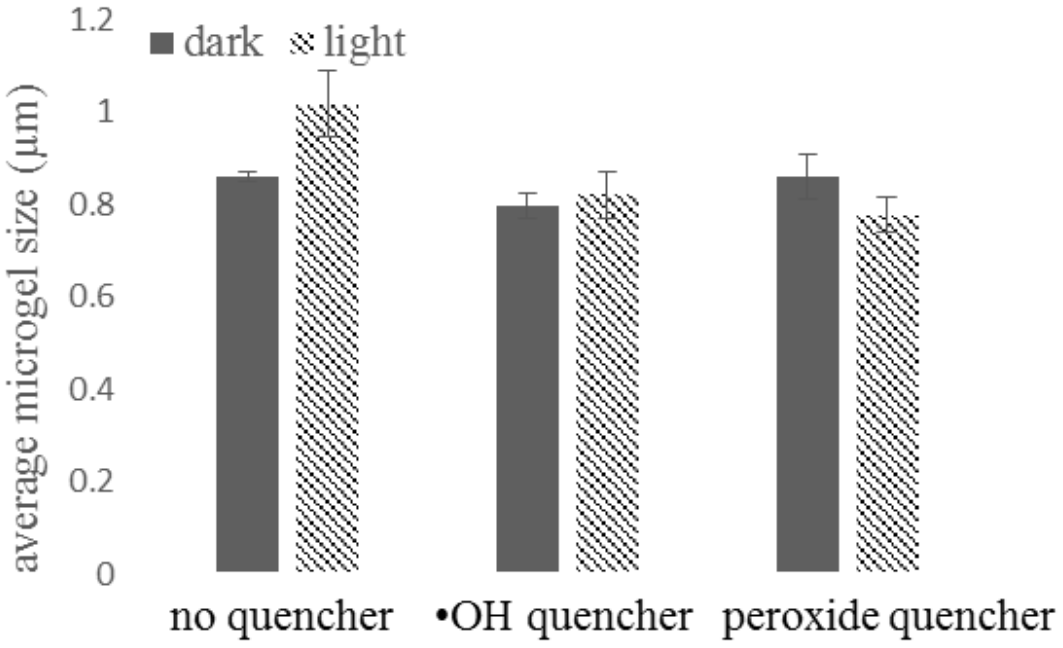

Fig. 3 Aggregation (microgel size increase) was observed in Gulf of Mexico seawater after irradiation ($p=0.02$). The addition of quenchers for hydroxyl radical (•HO) and H_2O_2 stopped the aggregation process [1].

Protein cross-linking includes internal cross-linking (within the single protein molecule) and intermolecular cross-linking (between protein and protein, or between protein and peptide fragment). Though both of which likely change the protein structural conformation and thus lead to aggregation [25], the proposed work is focused mainly on the intermolecular-cross-linking, with the MW increase to be determined by FTICR-MS [25], [33], and visualized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [22]. The FTICR-MS is a "top-down" approach, in that the proteins can be analyzed intact rather than being cleaved first and then injected into a regular MS. In addition, the introduction of the sample via electrospray, which is a 'soft' ionization method, has maintained the molecules mostly intact with minimal fragmentation. One of the advantages of ESI-FTICRMS over other types of MS is that it is capable of confidently determining the charge of the molecules with its high resolving power, and thus, it is possible to assign formulas for the multiply-charged molecules. In a few previous studies, ESI-FTICRMS has been successfully applied to detect the oxidation of model proteins such as insulin (5.7kDa) and ubiquitin (8.6kDa) by ROS via pathway 2 shown in Fig. 2, with the resulting cross-linked products [25], [33], [34]. By putting in the protein sequence, mass modification and amino acid specificity, a fragmentation library was calculated giving all possible cross-linking possibilities for the b-type, y-type and internal fragment ions [34]. The FTICR-MS has also been used for many N-containing natural samples [35]-[37], to demonstrate the oxidation of DOM by ROS [38].

Our preliminary results demonstrated that FTICR-MS (College of Science Major Instrumentation Cluster, Old Dominion University, Virginia; led by P.G., Hatcher) is fully capable of analyzing a 6.5kDa model protein aprotinin and its photo-oxidative products with no pre-digestion (Fig 4). When being analyzed with FTICT-MS, aprotinin of original state has m/z 1302, z=5. Under irradiation conditions, the major observations we have (see Fig. 4) are: 1) the spectrum of aprotinin first showed fewer peaks (at 20 min), compared to that in the dark condition, then the peak number consistently increased with time (at 2-hr and 6-hr) likely due to photodegradation; 2) the intensity-averaged MW, calculated by adding the product of individual MW and its corresponding intensity, and dividing the sum by the total intensity, increased by 34%, 24% and 21% at 20 min, 2-hr and 6-hr, respectively, suggesting cross-linking of this protein occurred ("red-

framed region", Fig. 4) simultaneously and the overall effect was that cross-linking exceeded fragmentation ("purple framed region").

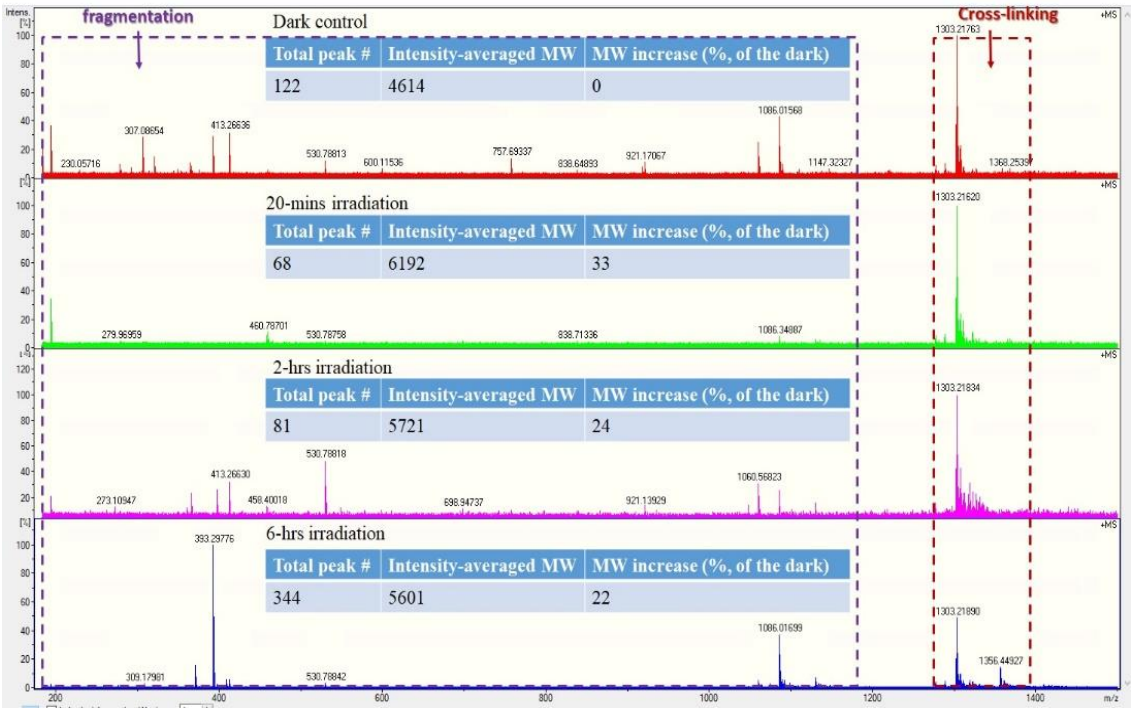

Fig. 4 ESI-FTICRMS spectra of aprotinin in the dark, and at 20-mins, 2-hrs, and 6-hrs irradiation (Peaks with S/N > 4 are used) (Xu, et al., unpublished results)

SDS denatures proteins by destroying all non-covalent bonds, and thus SDS-PAGE can be used to visualize the chemically cross-linked products of HMW without the proteolysis pretreatment (Fig. 5). For fetuin in dark control, the main bands are at 64kDa (monomer), 150kDa, and >250kDa (the bands ≥150kDa are probably formed from aging (24h) and stacking phase (Crow et al., 2001)) (Fig 5). During irradiation, these bands started to disappear, while a band on the upper detection limit appeared, indicating the formation of multimers with a very high molecular weight (>250kDa). Similarly, the main bands for lectin are at 30kDa and 45kDa in the dark condition. After irradiation, these two LMW bands disappeared, and a broad band appeared above 100kDa. For cytochrome c (monomer at 12kDa and dimer at 24kDa), a crosslinking was observed after long term irradiation (24-hr), and this cross-linking was more evident in the presence of H_2O_2. Furthermore, it is notable that the total organic carbon concentration (measured on a Shimadzu TOC-L analyzer) did not change significantly before and post-irradiation. In addition, protein concentration (measured by Lowry Protein Assay Kit (Pierce, 23240, USA)) changed by <30% within 6-hr [39]. The combined evidence of the disappearance of LMW bands, appearance of HWM band, as well as the slight change in TOC and protein concentration, all together corroborate the occurrence of intermolecular protein crosslinking [40].

The carbonyl group, an index of oxidative stress of proteins, can initiate chemical cross-linking reactions through Michael addition and/or radical-radical reactions (Fig. 2), thus their concentrations post-irradiation experiment serve as an indicator or proxy for the occurrence of cross-linking [41]. For the native proteins, the carbonyl content is 0–6 nmol/mg protein (Fig 6). After irradiation, the carbonyl content increased dramatically, and it reached 28 ± 3, 23 ± 1, and 34 ± 9 nmol/mg protein for fetuin, lectin, and BSA, respectively, within 20-hr (cytochrome c was not measured because of an interference from background color).

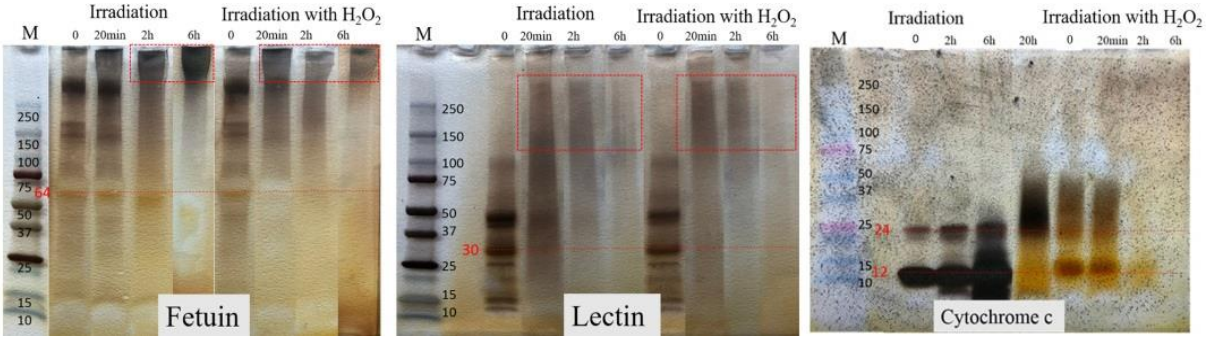

Fig. 5 The by-products from irradiated fetuin and lectin were resolved on SDS-PAGE. Model proteins were dissolved in artificial seawater (ASW), and were irradiated in quartz vials (125 mL) under 400W xenon lamp (Suntest system). H2O2 (with the mass ratio of 1:1 to protein) was added into half of samples to create a robust oxidative condition. Subsamples were taken at 20 min, 2h, and 6h. M: standard marker with 10-250kDa bands [39].

In addition, the H_2O_2 accelerated the formation of carbonyl content, with the carbonyl content reaching a maximum within 2-hr.

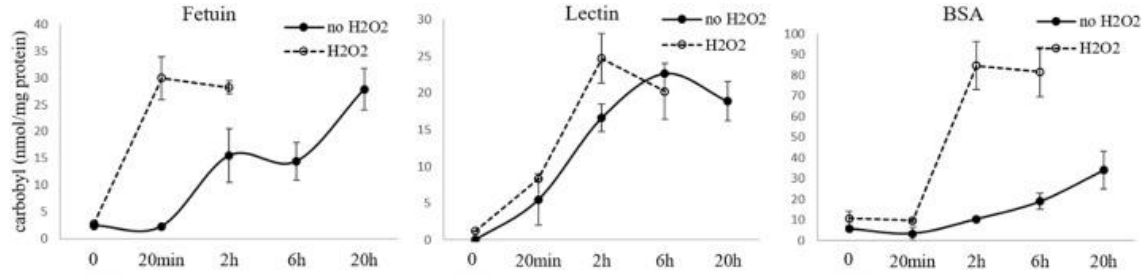

Fig. 6 The carbonyl content (nmol/mg protein) of by-products from irradiated fetuin, lectin, and BSA [39].

Our field-collected surface seawater samples, from San Francisco Bay and Gulf of Mexico, corroborated that proteinaceous material is the key component accounting for DOM microgel size increase by 25% and 18%, respectively, during the irradiation (Fig. 7), whereas this photo-oxidation induced microgel formation had been inhibited if ROS quencher was added (Fig. 3) [28].

The progression of protein unfolding, hydrophobic interactions, oxidation, and chemical crosslinking is depicted in Fig. 8 [42]. Other biomolecules such as lipids and sugars may also take part in the cross-linking of proteins. It is well documented that the end-products of lipid peroxidation, such as malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal, cause protein damage by means of reactions with lysine amino groups, cysteine sulfhydryl groups, and histidine imidazole groups [26], [43]. The heavily oxidized product, containing both lipids and proteins, is known as “lipofuscin”, which is highly resistant to proteolysis [44]. Furthermore, the formation of cross-linked products can be strongly dependent on the degree of fatty acid unsaturation [42]. Besides lipids, cross-linking could occur between proteins and reduced sugars in vitro, through a pathway known as Maillard reaction [45]. This pathway has been suggested for the partial preservation of proteins in the marine environment [9], although it is unknown if photo-oxidation takes part in this process. Thus, the cross-linking may incorporate other biomolecules into proteinaceous materials.

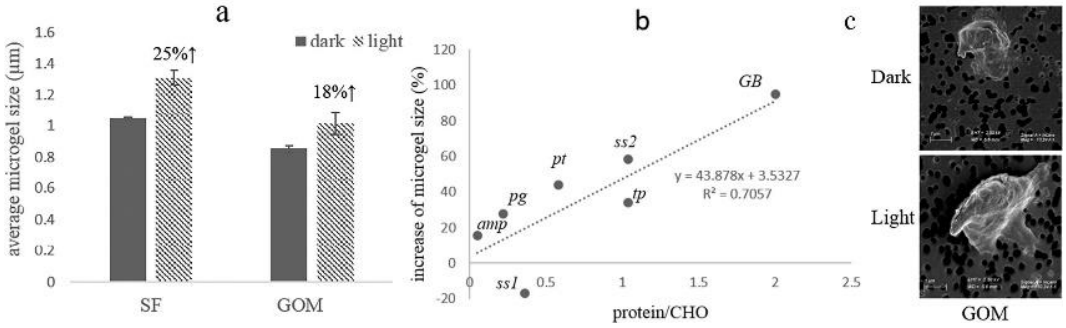

Fig. 7 a) an increase of a subgroup of particle size around 1 μm observed in San Francisco (SF) and Gulf of Mexico (GoM) seawater after 1 h irradiation (by flow cytometer); b) particle size increase (%) was positively correlated to protein-to-carbohydrate ratio of particles; c) GoM particles taken by scanning electron microscopy (SEM) in dark and after irradiation [39].

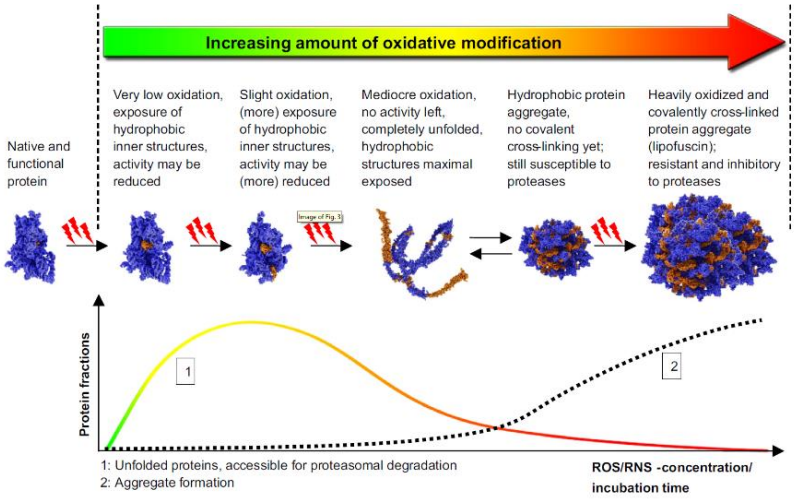

Fig. 8 The transformation reactions of proteins from labile to refractory. With increasing extent of oxidative modification, the proteins become more labile initially but then more refractory [42].

3. Production of more Refractory Proteins during Photo-oxidation

In the marine environment, oxidation/photo-oxidation of proteins have complicating and contrasting effects on their lability. Obernosterer, Reitner [46] observed a decreased bioavailability in most irradiated samples with addition of a protein (i.e. bovine serum albumin), while no distinct pattern was observed in surface waters from the Mediterranean Sea compared to the corresponding dark controls. In their later study [47], they observed increased bioavailability in the protein-amended chlorophyll maximum layer seawater, but decreased bioavailability in deep water from the Southern Ocean. Keil and Kirchman [48] suggested that photo-oxidation can transform a labile protein (i.e. Rubisco) to a refractory one in seawater. Nevertheless, the effects of photo-oxidation on the refractory nature of proteins in marine environments are still not well understood and are still being debated.

In biochemical and health studies, however, more light has been shed on the lability or refractory nature of proteins [18]. The first step in the biological degradation of proteins is by peptide hydrolysis using proteases [17]. The proteases cleave the proteins into small primary amides (<600Da), which are available for microbial uptake and utilization [5], [49]. The accessibility of proteins to proteolysis depends on the oxidative extent of proteins. On one hand, with increasing oxidative modification (Fig. 8), the proteins become unfolded, with more hydrophobic structures being exposed, thus becoming more accessible to proteolysis. On the other hand, with stronger oxidative modification, ROS mediated cross-linking occurs and the proteins

eventually become resistant and inhibitory to proteases. In natural seawater, the ROS are generated from different sources, and the oxidative condition may vary greatly at different depths or at different micro-environments, thus it may explain the observations of differences in the lability of proteins during photo-oxidation.

Our photo-oxidation experiments employed proteolysis rates as an index of the refractory nature of proteins (i.e., resistance of protein to proteases), after they had experienced irradiation of different duration lengths [39]. The protease assay has previously been used in crosslinking studies and marine sediments studies [9], [50]. At environmental relevant protein concentration, the proteolysis rates varied among different native proteins, with the highest rate for fetuin, indicating differences in the refractory nature of proteins depending on their sources. Generally, the proteolysis rates increased or remained unchanged at the early stages of irradiation (<6 h) but decreased after long-term irradiation (6~20 hr) (Fig. 9). The addition of ROS (i.e. H_2O_2), at an environmental relevant concentration (at a mass ratio of protein to H_2O_2 at 1:1) accelerated the occurrence of the decline of proteolysis rate.

The proteolysis rates started to decrease after 20 min past addition of ROS, compared to 6 hr for fetuin. The time point (2 hr) for proteolysis rate to decline differed for lectin, after addition of ROS, compared to 6h without the addition of ROS. The overall results indicate that with increasing extent of oxidative modification, proteins ultimately become refractory, even though some became more labile at the early stages (Fig. 5) [39], [44]

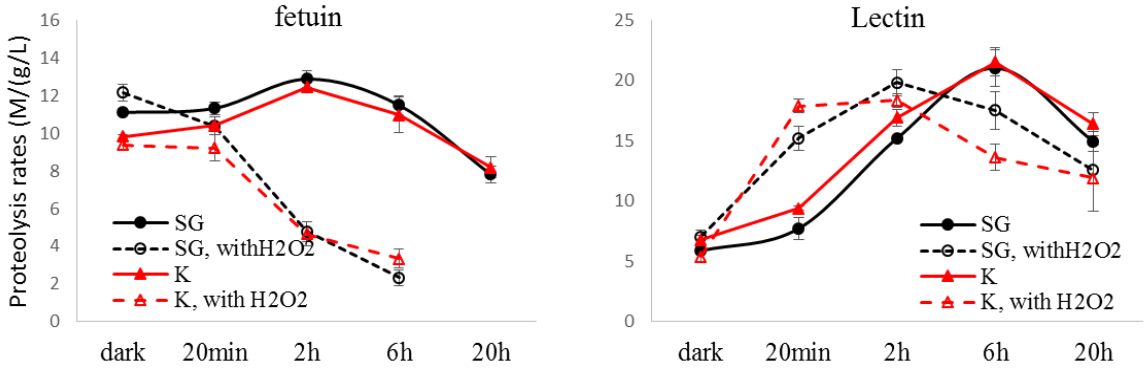

Fig. 9 Proteolysis rates of irradiated fetuin and lectin with or without H2O2 addition. The proteases, from Streptomyces griseus (SG) and protease K were added into the colloidal fraction to cleavage the modified/unmodified proteins into small primary amines. Proteolysis rates were calculated as the difference between sample values (primary amines concentrations (M) with proteases addition) and blank values (without protease addition), normalized to the protein content (g/L). As can be seen, similar features are noticeable with and without H_2O_2 [39].

4. Photo-Induced Aggregation of Don

Aggregation of proteins can also occur during oxidation [23], [44] (Fig. 10). Under weak oxidation conditions, the proteins become unfolded. With more hydrophobic structures being exposed, the proteins become more prone to aggregate. At stronger oxidative modification, cross-linking occurs to form HMW products. Since the stability of tangled networks of microgels drastically increases with longer polymer length [42], the polymers with HMW assemble faster and form more stable gels. Both increased hydrophobicity and molecular weight lead to aggregation, and the unfolding and cross-linking processes can occur simultaneously [50]. A few recent studies have shown that photo-oxidation can induce aggregation of protein-containing extracellular polymeric substances (EPS) [51]-[55]. It has been found that DON, specifically amides, accumulate within irradiation-derived aggregates [56]. Our previous studies [28], [32] have shown that proteins play important roles in light-induced aggregation, and the aggregation process is dependent on ROS. Moreover, N was relatively more enriched compared to C in the photo-produced aggregates. In our experiments on model proteins fetuin and lectin under axenic

condition, aggregate formation was observed in irradiated samples, but was not evident in the dark controls (Fig. 10). Both turbidity and aggregates mass of irradiated samples were higher than dark control, especially after 2h-irradiation. The irradiated samples had 200% higher turbidity values for fetuin and 150% for lectin than the dark control after 6h-irradiation.

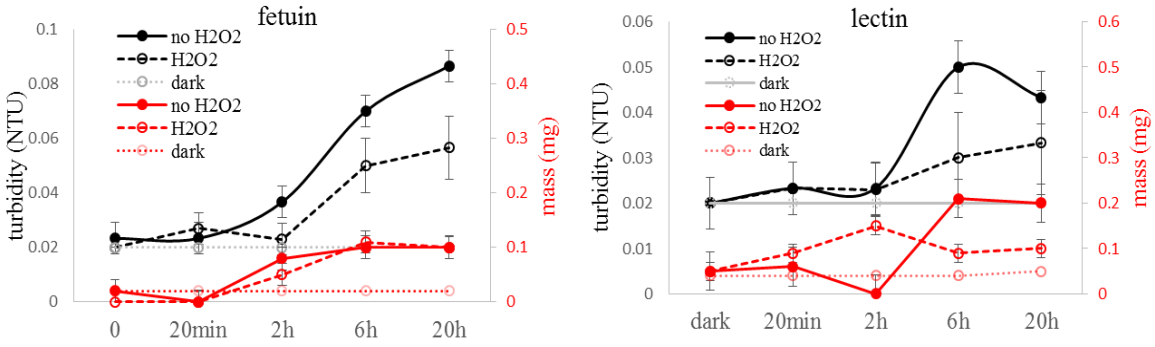

Fig. 10 Turbidity (black line) and aggregates mass (red line) of fetuin and lectin collected at different irradiation time points. Turbidity was used as an indicator of particle concentration [38].

In the ocean, the aggregation of proteins leads to faster formation of microgels [28]. The divalent bonds between the anionic biopolymer moieties and metals (e.g. Ca^{2+}) can stabilize microgels through bridging [57]. The concentrated Ca^{2+} inside the gels can lead to crystalline mineralization of the gel's matrix, and thus facilitate aggregation. Accordingly, the aggregation is a physicochemical pathway for preservation of proteins, while some proteins are protected by mineral sorption and escape from further oxidation and biodegradation. The particulate proteins can thus maintain their native lability. Previous studies have shown that proteins extracted from marine sediments are accessible to proteolytic cleavage [9]. It is likely that ROS-facilitated photo-oxidation induced cross-linking of the DOM, with part of the proteinaceous material becoming refractory. Some of this cross-linked proteinaceous material form aggregates as a result of irradiation (MW change, unfolding, hydrophobicity change, etc., Fig 8) and thus, can facilitate the preservation and export of partially refractory DON to the deep ocean.

5. Summary and Conclusions

Our very recent evidence of cross-linking (shown as increased molecular weight by SDS-PAGE and FTICRMS) under oxidative conditions of several model proteins, combined with the increased carbonyl content and concomitant increase of their refractory nature by using their proteolysis rates is novel evidence and lays the solid groundwork to provide the details of this crosslinking mechanisms. The model proteins were selected for their extensive applications in studies of the bioavailability of NOM in ocean waters. Aggregates form through reactions of proteins with ROS. This evidence, and that for limited degradability of the crosslinked material explains part of the chemical transformation and preservation pathways of the proteinaceous materials as HMW DON in the ocean. Furthermore, our previous studies [28], [32], [39], [56] have shown that sunlight can induce aggregation of DOM into microgels (0.5-1.5 μm) and further into aggregates (> 1 μm) in the marine environments (both from Gulf of Mexico and San Francisco Bay seawater) [28]. The fact that proteinaceous components play a key role is direct evidence that such processes can occur within hours under natural conditions (e.g., sunlight, DOM concentrations, ambient seawater). It is thus likely that the proteinaceous material that was involved in the formation of microgels and aggregates in the sun-lit surface layer is preserved at depth.

6. Acknowledgements

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