Effects of non-thermal atmospheric plasma on protein

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Currently, the advancement in non-thermal atmospheric plasma technology enables plasma treatments on some heat-sensitive targets, including biological substances, without unspecific damage caused by thermal effect. The significant effects of non-thermal atmospheric plasma modulating biological events have been demonstrated by considerable studies. Protein, one of the most important biomolecules, participates in the majority of the life-sustaining activities in all organisms, whose functions are derived from the diverse biochemical properties of amino acid compositions and four-tiered protein structure hierarchy. Therefore, the knowledge of how non-thermal atmospheric plasma affects protein greatly benefits the understanding and application of the non-thermal atmospheric plasma effect in biological area. In this review, we summarize recent research progress on the effects of non-thermal atmospheric plasma, particularly its reactive species, on biochemical and biophysical characteristics of proteins at different structural levels that lead to their functional changes. Moreover, the physiological effects of non-thermal atmospheric plasma at cellular or organism level driven by the manipulations on protein and their relative application prospects are reviewed. Despite the exceptional application potential, the exploration of the non-thermal atmospheric plasma’s effect on protein still confronts with difficulties due to the limited knowledge of the underlying mechanisms and the complexity of non-thermal atmospheric plasma operation systems, which requires further studies and standardization of non-thermal atmospheric plasma treatments.

Key Words: non-thermal atmospheric plasma, protein, amino acids, protein structure

Plasma, the fourth state of matter, is an ionized gas that comprises a variety of active charged particles, such as electrons, ions, free radicals, metastable excited state of matter, and vacuum ultraviolet (UV) radiation. These reactive plasma components are able to initiate and control a series of chemical reactions. According to the average generation temperature, plasma can be divided into two types, thermal plasma and non-thermal plasma. In contrast to the high temperature, up to several thousand Celsius degrees, for the generation of thermal plasma, non-thermal plasma is obtained around ambient temperature, which makes it suitable to treat heat-sensitive materials. Non-thermal Atmospheric Plasma (NTAP) is produced by applying the electric energy to the one or more working gases [oxygen (O₂), nitrogen (N₂), argon (Ar), air, etc.] filled between two electrodes under low pressure or atmospheric pressure. There are many reports on the interactions between NTAP and biological systems, such as microorganism inactivation, tumor inhibition, cell proliferation, and so on.

Protein is one of the most important macromolecules constructing biological systems, which is composed of multiple amino acids linked together by peptide bonds. All proteins in all species are constructed by the same set of twenty amino acids. The twenty amino acids have a common structure consisting of a central α-carbon atom (Cα) with linkages to an amino group (α-NH2), a carboxyl group (α-COOH), a hydrogen atom and a distinctive R group. A dehydrated condensation reaction occurring between an α-COOH and its neighboring α-NH2 forms a peptide bond and further leads to a polypeptide chain. An R group, being referred to as a side chain, varies in size, shape, polarity, charge and hydrophobicity among the twenty amino acids and confers unique structures and chemical properties to each amino acid. A functional protein is formed through folding and/or assembly of single or multiple polypeptide chains to a unique structure which is driven by a series of interactions between sidechains and polypeptide backbone(s), such as van der Waals force, hydrogen bonds, electrostatic interactions, and hydrophobic effect. Protein structure is divided into four levels, primary structure, secondary structure, tertiary structure, and quaternary structure. The primary structure refers to the arrangement of amino acid residues in a polypeptide chain. Some regular hydrogen-bonding patterns form according to the arrangement of amino acids in a polypeptide chain, resulting in the folding of protein to the secondary structure including α-helix, β-sheet, and random coil. Multiple secondary structures are packed together via interactions between side chains to form compact independent three-dimensional structures called domains. The tertiary structure of a protein is composed of one or several relatively independent domains. Usually, a properly folded tertiary structure constructs the basic functional unit of a protein. Furthermore, some larger proteins consist of more than one tertiary folded protein chain (subunit), where subunits interact with each other to form complexes called quaternary structure. Biochemical properties of proteins and their derived functions are largely determined by protein structure at these four levels. The diversity of protein structures delivers a wild variety of roles played by proteins in cellular and physiological activities, such as structural proteins, enzymes, carrier proteins (membrane proteins), hormones, antibodies, and so on.

This review gives an overview on the biochemical and biophysical effects of NTAP on proteins and the resultant functional changes as well as the derived applications, which aims to assist in directing further researches and exploring potential applications.

NTAP

The principle of the generation of NTAP. The application of electric field on working gases results in the elastic and inelastic collisions of electrons, gas particles and atoms. The transferring of kinetic energy among particles occurs through...
their elastic collisions, which keeps NTAP at a lower temperature. Meanwhile, the inelastic collisions lead to the excitation, dissociation, and ionization of gas atoms and molecules, which generates NTAP with the accompanying formation of active components, such as UV light,\textsuperscript{40,41} charged particles,\textsuperscript{42,43} reactive oxygen species (ROS), and reactive nitrogen species (RNS).\textsuperscript{17,18} RONS (ROS and RNS) comprise some short-lived reactive species. With the typical half-lifetime from 1 nanosecond to a few seconds, such as hydroxyl radicals (OH), superoxide (O$_2^-$), singlet oxygen (O$_2^*$), nitrogen dioxide (NO$_2$), atomic oxygen (O), and nitric oxide (NO), and some long-lived reactive species, such as hydrogen peroxide (H$_2$O$_2$) and ozone (O$_3$), the half-lifetime of which are respectively several hours and 20–30 min.\textsuperscript{18–22}

**NTAP devices.** According to the different discharge modes, NTAP devices are mainly divided into six types as introduced in the following section.\textsuperscript{4,25} Dielectric barrier discharge (DBD). DBD generates NTAP between two electrodes separated by an insulting material (e.g., ceramic, quartz, etc.) as a barrier layer.\textsuperscript{4,25} Because of limiting current, DBD is regarded as one of the safest devices without spark and arc discharges.\textsuperscript{41} The ignition voltages of DBD are relatively high, and the flow rate of the applied gas is relatively low.\textsuperscript{23} There are two typical DBD devices, namely planar and cylindrical DBD.\textsuperscript{23} The DBD device generates wide NTAP, which makes it suitable for treating samples with large area.\textsuperscript{24} Corona discharge. This device generates NTAP at the tip of a sharp electrode in the presence of a heterogeneous electric field.\textsuperscript{4} Corona discharge is the partial self-sustaining discharge of the working gas.\textsuperscript{25,25} According to the connection mode of ground electrode, Corona discharge can be divided into two types, which are spark discharge and glow discharge.\textsuperscript{26,27} Gliding arc discharge. The distinctive characteristics of gliding arc discharge are utilization of arc electrode and its higher discharge temperature than that of corona discharge and DBD.\textsuperscript{4,4} The gliding arc device is composed of two separate principal electrodes, two auxiliary electrodes and a generator.\textsuperscript{4,28} The working gas inlet is at the top of the device, and the gas is broken down in the narrowest area of arc electrode gap which is applied with high voltage power (up to 9 kV or higher).\textsuperscript{4,4} Finally, the generated NTAP is transported to the sample surface by airflow.\textsuperscript{28} Atmospheric-pressure plasma jet (APPJ). APPJ is a self-sustaining discharge phenomenon in the working gases, including helium (He) or Ar, under an extremely low electric field.\textsuperscript{4} In APPJ devices, an dielectric hollow tube is used as the layer, which is surrounded by a metal cathode.\textsuperscript{4,28} The generated NTAP is sustainedly released to samples by the continuous flow of the working gas.\textsuperscript{4,23,24,29,30} Compared with the DBD device, the APPJ device is more suitable for treating a small area on a sample.\textsuperscript{24} Radio frequency (RF) discharge. The RF discharge device is comprised of three main components, including a RF generator, a ceramic nozzle with a needle electrode and a grounding ring electrode, and an air supply system.\textsuperscript{4,24} The NTAP is generated at the tip of the needle electrode and extended to the grounding ring electrode outside the ceramic nozzle at a typical frequency of 13.56 MHz.\textsuperscript{23,24} The RF plasma is not limited by the space of the electrode.\textsuperscript{4,23,31,32} Microwave discharge. Microwave discharge is a kind of non-polar discharge, which is triggered by the electromagnetic waves being derived from a magnetron with a cooling system.\textsuperscript{4,24} The generated wave is sent to a processing chamber filled with working gas to produce NTAP.\textsuperscript{4,24} When the power level is 50–1,000 W, the generated microwave intensity and density are 2.45 GHz and 0.25 W/m² respectively.\textsuperscript{4,23,33,34} **Key technical parameters of devices affecting the generation of NTAP.** The energy of NTAP exposed to the samples is represented by NTAP dose, which is determined by excitation voltage, power, frequency, and treatment duration. Different NTAP generation devices vary markedly in excitation voltage, power, and frequency, showing a voltage range from 0.4 kV to 90 kV;\textsuperscript{35,36} a power range from 0.4 W to 3 kW;\textsuperscript{37,38} and a frequency range from 50 Hz to 2.45 GHz.\textsuperscript{39,40} As other conditions remain unchanged, the increase in voltage, power or frequency of the NTAP device output higher energy, which usually enhances the treatment potency of plasma.\textsuperscript{41,42} For example, polyphenol oxidase (PPO) activity decreased by 70.2%, 85.7%, and 94.2% after air-DBD plasma treatment for 3 min at 19.4, 26.4, and 32.6 W, respectively.\textsuperscript{43} On the other hand, higher voltage, power, or frequency promotes the load of the equipment and generates more heat. Therefore, the applied voltage, power, and frequency need to be tuned according to the characteristics of different samples. Generally, the effect of NTAP increases along with the treatment duration until it reaches its maximum effect.\textsuperscript{44,45} Under APPJ treatment of Ar-O$_2$, a two-stage reduction of PPO activity was observed: after a rapid decline to about 20% in the first 120 s, the residual activity gradually approached about 10% in 120–360 s.\textsuperscript{46} When PPO was treated with air-DBD, its activity decreased to about 40% in 3 min at the power of 19.4 W, and it showed a similar two-stage decline to about 15% at the power of 32.6 W.\textsuperscript{46} Currently, gas is the main working medium of NTAP devices, and the type of working gas determines the constitution of NTAP.\textsuperscript{47,48} Rare gases, such as He and Ar, are often used as the working gases to generate the NTAP that contains metastable states of He, Ar, and so on.\textsuperscript{4,44,48,49} When using O$_2$ and N$_2$ for discharge, RONS are generated by the excitation of O and N atoms. Mixed gas is also used as the working gas for NTAP generation. For example, He and O$_2$ mixture is used for the production of DBD-discharged plasma which significantly reduces the activity of the lactate dehydrogenase (LDH).\textsuperscript{50} The activity of peroxyde (P DO) in fresh-cut apples can be reduced by 62% under the exposure of the discharged mixture of nitric oxide (NO) and nitrogen dioxide (NO$_2$) by microwave NTAP.\textsuperscript{56,40} Air is the most common and cheapest mixed gas, so it is of great application value to use air as the working gas to study the effect and mechanism of NTAP on proteins. It has been demonstrated that air-NTAP can increase the activity of α-amylase in brown rice and significantly reduce the activity of POD in fresh-cut melon.\textsuperscript{51,52}

Surowsky et al.\textsuperscript{46} treated PPO by APPJ using pure Ar as well as mixtures of Ar and 0.01 to 0.1% O$_2$ as working gas, and the results showed the combination of Ar and O$_2$ had better inactivation effect on PPO than pure Ar.

**Plasma activated solution (PAS).** Although direct NTAP irradiation of biological systems is promising, there is a growing need for more flexibility in utilizing NTAP-inherent RONS as putative injections.\textsuperscript{53} One way to achieve this need is activating the solutions such as water, phosphate-buffered saline (PBS), normal saline, cell medium, and other aqueous media, using NTAP devices to obtain PAS.\textsuperscript{21,24,55–57} PAS is mainly composed of RONS which are formed within solution or at the gas-liquid interface through a number of processes, for instance, interactions between gaseous reactive species and liquid molecules, as well as the gas-liquid transportation and solvation of reactive species (Fig. 1).\textsuperscript{58–63} Additionally, the chemical reactions occurring among the NTAP-derived RONS in the solution generate some secondary reactive species with long lifetime, such as nitrates (NO$_3^-$) and nitrates (NO$_2^-$) with the corresponding half-lifetime of years and several days.\textsuperscript{64} Compared with NTAP treatment, the utilization of PAS is able to evenly treat the entire surface of samples, and PAS is easier to carry and preserve.\textsuperscript{65,66}

**NTAP Affects Biochemical and Biophysical Protein Characterizations**

NTAP may affect amino acid residues, amino acid sequence...
and spatial structure of proteins through various mechanisms. First of all, the samples and working parameters of NTAP treatments in this section are exhibited in Table 1.

**Effects on amino acids.** So far, it has been reported that fourteen essential amino acids can be oxidized at their side-chain groups by RONS in NTAP treatment and converted into a variety of derivatives. Among them, aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and sulfur-containing amino acids (cysteine and methionine) show the highest reactivity with NTAP reactive species that include hydroxyl radicals, superoxide, hydroperoxyl radicals, singlet oxygen, ozone, and nitric oxide. As results of these oxidation reactions, aromatic groups are hydroxylated or nitrated, and methionine and cysteine are respectively converted into methionine sulfone or sulfonated methionine, and sulfonated cysteine. The oxidative environment provided by NTAP also facilitates the formation of disulfide bond between cysteines. Because of these outstanding reactivities displayed by aromatic and sulfur-containing amino acids, to a certain extent, the composition of the two amino acid groups specifies the sensitivity of protein to the oxidative effect exerted by NTAP. Other amino acids whose side-chain groups have been observed as the targets of NTAP comprise the ones with either hydrophilic (lysine, arginine, histidine, glutamate, and glutamine) or hydrophobic side-chains (valine, leucine, and isoleucine), as well as proline where the special cyclic structure is broken and the α-carboxylic group is amided by NTAP. It is worth noting that, due to the direct bonding of side-chain and amino group, the rotation of proline related to its neighbouring amino acid residues is considerably constrained, which in turn results in the stop of the extension of α-helix and β-sheet. Therefore, proline residues play a special role in regulating the arrangement of secondary structures.

In addition to oxidative modifications, direct exposure to NTAP results in degradation of the -COOH and -NH₂ groups of alanine and valine, which leads to a variety of decomposition products, such as acetone, formic acid, acetic acid, threo-methyl aspartic acid, red methyl aspartic acid, and pyruvate.

**Effects on primary structure.** Breakages of peptide chains are observed in several studies on the effect of NTAP on protein. As shown in Fig. 2, the hydroxyl radicals and hydroperoxyl radicals produced by NTAP probably play an important role through diamide pathway in the cleavage of protein peptide bonds, which degrade proteins into polypeptides of different segments. In the presence of D-mannitol, which is the scavenger of hydroxyl radicals, the concentration of the amino terminus formed in initial 10 min of NTAP treatment is reduced.

![Diagram of NTAP-reactive species](image)

**Table 1. Working parameters of NTAP treatments on various samples**

| Sample                           | NTAP device | Working parameters          | References |
|----------------------------------|-------------|-----------------------------|------------|
| 20 amino acids                   | APPJ        | He; 13.9 kHz; 0–10 min      | (67)       |
| Lysozyme                         | APPJ        | He and O₂; 13.9 kHz; 5–30 min | (68)       |
| Capsid protein                   | APPJ        | 99% Ar + 1% O₂; 2.5 W, 20 kHz; 15 s–2 min | (69)       |
| Proteins from T4 bacteriophage   | DDB         | 79% N₂ and 21% O₂; 230 V, 13.56 MHz; 20–120 s | (70)       |
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | APPJ | He + 0.6% O₂; 230 V, 13.56 MHz; 1–100 s | (71)       |
| L-Valine                         | DDB         | Air; N₂ and O₂; 11.2 kV, 690 fHz; 0, 10, 20, and 40 s, and 1–20 min | (72)       |
| L-Alanine                        | RF          | Ar; 13.56 MHz; 2–52 s       | (73)       |
| Bovine serum albumin (BSA), lysozyme, RNase A, and superoxide dismutase (SOD) A and B | DDB | Air; 13.5 kV and 300 Hz; 10 min | (74)       |
| Soybean protein isolate (SPI)    | DDB         | Air; 40 kV, 80, 100, and 120 Hz; 1–10 min. | (75)       |
| Membrane-bound proteins and intracellular proteins in K. Pneumoniae | DDB | He; 3 kV and 8 kHz; 1–5 min. | (76)       |
| PPO and POD                      | APPJ        | Ar and 0.01 to 0.1% O₂; 65 V; 60–360 s. | (77)       |
| Proteins of wheat flour          | DDB         | Air; 60 and 70 kV; 5–10 min | (78)       |
| Bovine milk casein and whey proteins | Corona discharge | Air; 8 kV and 25 kHz; 10–30 min | (27)       |
| Hemoglobin (Hb) and Myoglobin (Mb) | DDB         | Ar, Ar-O₂ (at different ratios), and Ar-N₂ (at different ratios); 1 kV; 1–4 min | (79)       |
| Hb and Mb                         | APPJ        | Air, N₂, and Ar; 8 kHz; 3 min | (79)       |
| Hb                               | DDB         | O₂, N₂, Ar, He, NO (10%) + N₂, and air; 9.2 kV and 10 kHz; 10 min | (80)       |
| YgaP and swc7                     | APPJ        | Air; 10 kV and 5 mA; 10 min | (81)       |
| RNase                            | DDB         | Air; 13.5 kV, 300 Hz; 1–600 s | (82)       |
| Arginine vasotocin                | Microwave   | Ar; 2.45 GHz and 600 W; 10–30 min | (83)       |
by 96%. It is found that the sensitivity of peptide bond in proteins of different molecular weights to NTAP is different. Proteins of large molecular weight (>44 kDa) are easily degraded, while proteins of small molecular weight (<20 kDa) are not significantly degraded.

**Effects on secondary structure.** The effects of NTAP on protein secondary structure have been confirmed by considerable studies, which is mainly exhibited as various alterations or destructions of protein folding. Following NTAP treatment, the percentage of β-sheet is increased in a number of proteins, such as PPO and POD; while for some other proteins (e.g., alkaline phosphatase, gluten), β-sheet folding is diminished. Similar to what are observed for β-sheet, under NTAP effect, the composition of α-helix can also be constructed or deconstructed.

As an emerging research area, the variety of the effects of NTAP on the secondary structure has not been fully explained by experimental studies. Nonetheless, according to the available data, it is found that, changes in protein folding resulted by NTAP appear to be specific to different protein types. In addition to protein type, the condition of NTAP (e.g., gas type, discharge type) seems to be another major determinant of alteration in secondary structural folding. The research on the effects of NTAP on hemoglobin (Hb) and myoglobin (Mb) distinctly shows that the α-helix in Hb and Mb increases in air NTAP but decreases in N₂ and Ar NTAP. As well, in certain cases, different discharge types exhibit different effects, for example, DBD-NTAP decreases α-helix and promotes the formation of the β-sheet, while the effects of APPJ-NTAP exerts the opposite effect. This difference may be attributed to the variation in the plume impact strengths and affecting areas of different NTAP types. More specifically, ROS, as the main product of NTAP, predominantly result in the carbonylation on amino acid residues and in turn affect the secondary structure, and the types and levels of ROS produced by NTAP are varied under different working conditions. Compared with air-NTAP, N₂-NTAP and Ar-NTAP yield more reactive species, and correspondingly more secondary structural changes are observed in the NTAP treatment with the presence of N₂ or Ar. The concentrations of hydroxyl radicals, hydrogen peroxide, nitrates, and nitrites generated by O₂ admixture NTAP are less than the ones generated by N₂ admixture NTAP, while O₂ admixture NTAP causes greater structural changes, which indicates that the role of reactive oxygen atoms may be important for structural changes of proteins. Additionally, RNS play a minor role in the structural changes, which may affect secondary structure via mediating the pH of plasma system to accelerate the reaction rate of other free radicals.

**Effects on protein tertiary and quaternary structures.** The structural hierarchy of protein determines that alterations and modifications of amino acid residues and secondary structures can be very likely magnified as changes in the structural features at higher levels, namely tertiary and quaternary structures. With this concept in mind, the investigations on the effects of NTAP on protein structure have been expanded to look into the responses of protein conformation and assembly to NTAP treatment.

Aboubakr et al. studied the impact of NTAP exposure on...
feline calcivirus (FCV) viral capsid protein and revealed that NTAP-induced oxidation occurred at specific amino acid residues located at the areas, which are key to the attachment of FCV with its host cell receptors. With oxidative modifications, these areas, including the dimeric interface between two capsid monomers, a hinge region connecting shell, and protrusion domains of viral capsid protein, which determine if the virus can recognize and bind to host cells normally, lost their native conformation and flexibility.\textsuperscript{(35)} Similarly, the alteration of local structural organization resulted by modifications of specific residues is observed in the study on the effect of NTAP on lysozyme.\textsuperscript{(35)} This study verifies that residues Trp62 and Trp108 and their surroundings which are sited in the enzyme active sites are modified following NTAP treatment.\textsuperscript{(35)} As a result, two loop regions, loop3 and loop6, contributing to the formation of substrate-binding pocket respectively, exhibit a detectable shift and distortion from their original conformation (Fig. 3), which is accompanied by significant structural rigidifications of these regions.\textsuperscript{(35)} In contrast to the local spatial rearrangements, some proteins respond to the impact of NTAP by overall conformational changes. Zhang et al.\textsuperscript{(81)} utilized dynamic light scattering to examine the NTAP-treated model proteins, YgAP and swc7, exogenously expressed respectively within the cell membrane and cytoplasm of E. coli, and demonstrated that the molecules of both proteins underwent notable compactness in the overall size under the environment induced by NTAP.

At the quaternary structural level, the effects of NTAP have been demonstrated as induction of aggregations or disruption of assemblies of certain proteins to impair their functions.\textsuperscript{[50,67,75,82,83]} The mechanisms underlying the NTAP-mediated quaternary structural changes involve a complex of modifications of amino acids, electrostatic interactions, and hydrophobic interactions where the manipulation of sulfhydryl group of cysteine residues plays an outstanding role. It is well known that the sulfhydryl group of cysteine is reactive and readily oxidized to form disulfide bond between two different cysteine residues either in the same or different peptide chains. The cross-link formed by disulfide bond exerts a crucial function in the maintenance of spatial organization of secondary structural elements and assembly of protein oligomer or complex. In the moderate oxidative environment created by short period of NTAP treatments, free sulfhydryl groups within target protein incline to be oxidized to disulfide and form intra and/or inter-chain cross-links, which leads to the formation of protein aggregates and a reduction in the protein solubility.\textsuperscript{[67,75,83]} However, if protein is subjected to a long exposure of NTAP, disulfide would be very likely over-oxidized and transformed to sulfonic and sulfonic acid, resulting in the breakage of disulfide bonds and the disassembly of some protein complexes like antibodies.\textsuperscript{(75,82,83)} As well as sulfhydryl group-mediated quaternary structural variations, the carboxylation amino acid side-chain groups introduced by NTAP can generate cross-links between protein molecules and cause aggregation.\textsuperscript{(75)} In addition, modifications of residue side-chains like the oxidation of sulfhydryl group and carboxylation alter the hydrophilicity of protein surface, which may in turn result in some hydrophilic areas being buried in the interior of proteins. In some cases, extensive NTAP treatment even leads to exposure of some hydrophobic amino acid residues via partial protein denaturation.\textsuperscript{(41,75)} These alterations in protein surface significantly increase hydrophobic interaction between protein molecules and promote the formation of aggregates.\textsuperscript{(50,75)}

**Effects of NTAP on properties and function of proteins.**

The structural changes of proteins by NTAP result in the alterations of physicochemical properties and functions. Due to air-DBD treatment, the foaming and emulsifying capacity of whey protein dramatically decreases, while the foam stability increases.\textsuperscript{(84)} The emulsifying activity index of actomyosin extracted from king prawn increases by 5 min of APPJ generated in argon gas, while the emulsifying stability index increases with the prolongation of exposure time, and begins to decrease until 3 min after exposure.\textsuperscript{(85)} These outcomes suggest that unfolding of actomyosin, which is accompanied by exposure of hydrophobic residues, enhances its emulsifying capacity.\textsuperscript{(85)} Lackmann et al.\textsuperscript{(71)} reported the inactivation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using APPJ with He and 0.6% O\textsubscript{2} for 10 min. The inactivation of lysozyme using DBD and APPJ has been studied by Choi et al.,\textsuperscript{(55)} and the results also suggested that N\textsubscript{2}-APPJ treatments had strongest action on the lysozyme activity among DBD and APPJ generated in N\textsubscript{2} and air. However, the POD activity of artichoke seed was increased 1.5 times by RF nitrogen plasma.\textsuperscript{(86)} Continued exposure to He-O\textsubscript{2} DBD results in the loss and recovery of LDH activity, which is related to the formation and decomposition of supramolecular.\textsuperscript{(50)}

NTAP treatments also have effects on the antigenicity of protein. For example, the antigenicity of tropomyosin in king prawn and Ara H1 protein in peanut is reduced by 76% and 43% under APPJ and DBD treatment, respectively.\textsuperscript{[87,88]} However, the retention antigenicity of α-casein and whey solution is not affected by RF argon plasma.\textsuperscript{(89)}

Due to the complexity of NTAP devices and working
Table 2. Effects of NTAP on properties and functions of proteins

| Protein                | Source/substratum | NTAP device | Working parameters          | Effects                                   | References |
|------------------------|-------------------|-------------|-----------------------------|-------------------------------------------|------------|
| Glycerol dehydrogenase | Klebsiella pneumoniae | DBP         | He; 13 kV and 8 kHz; 5 min | Increase in activity                      | (76)       |
| Viral capsid protein (VP1) | Feline calicivirus (FCV) | APPJ       | 99% Ar + 1% O₂; 2.5 W, 20 kHz; 15 s-2 min | Loss of infection ability                | (69)       |
| GAPDH                  | Escherichia coli  | APPJ        | He and 0.6% O₂; 230 V, 13.56 MHz; 10 min | Decrease in activity                      | (71)       |
| α-amylase              | Wheat             | RF          | Air; 13.56 MHz; 60–240 s    | Increase in activity                      | (52)       |
| Pea protein isolate    | Grain pea flour   | DBD         | Air; 8.8 kV; 3.0 kHz; 10 min | Increase in water and fat-binding capacities; Increase in solubility | (56)       |
| SPI                    | Soybean           | DBD         | Air; 40 to 60 kV, 80, 100, and 120 Hz; 1–10 min | Increase in emulsifying and foaming properties; decrease in IgE-binding level | (70)       |
| PPO and POD            | Agaricus bisporus and horseradish | APPJ       | Ar; 65 V; 60–360 s        | Decrease in activity                      | (46)       |
| LDH                    | rabbit            | DBD         | He or O₂; 14 kV; 24 kHz; 60–300 s | Increase in activity                      | (50)       |
| Phytase                | Pichia pastoris   | APPJ        | He; 15 kV; 10 kHz; 30–240 s | Increase in activity                      | (91)       |
| Whey protein isolate   | Solution          | DBP         | Air; 70 kV; 1–60 min       | Decrease in foaming and emulsifying capacity; increase in foam stability | (94)       |
| RNases                 | Bovine            | DBD         | Air; 13.5 kV, 300 Hz; 1–600 s | Terminally inactivation                   | (62)       |
| Type I collagen        | Human             | APPJ        | Air; 20 kHz; 10–90 min     | Increase in stability                     | (52)       |
| Lysozyme               | Egg white         | APPJ        | Air; He; Os; He-O₂; 5–30 min | Increase in molecular weight; decrease in activity | (68)       |
| Arginine vasotocin     | Lower vertebrates | Microwave   | Ar; 2.45 GHz and 600 W; 10–30 min | Increase in activity                      | (80)       |

parameters, as well as the diversity of objective proteins, physicochemical properties, and functions of proteins present different or even opposite changes after NTAP treatments. Table 2 presents a summary of literatures related to the effects of NTAP on properties and functions of proteins.

NTAP Regulates Protein Abundance in Cells

The synthetic quantity of proteins was observed being affected by NTAP in many kinds of cells. ROS, which are important signal molecules in cells, result in biological responses, such as altered metabolism and programmed cell death. NTAP-generated ROS are considered to be the main cause of its regulating effects on protein level. In addition, long-lived species, such as NO₃⁻ and NO₂⁻, generated in PAS were also observed to enhance the expressions of the genes involved in the secretory protein pathway.

Gene regulation. NTAP treatment can affect gene expression at the transcriptional level. mRNA levels of adipogenic-associated genes including ACC, FAS, and FAT SC1 in 3T3-L1 pre-adipocytes were significantly down-regulated by plasma activated cell medium (PAM). Treatments of PAM on keratinocyte cell (HaCaT) significantly increased the mRNA level of NADPH oxidase (NOX)1, NOX5, and dual oxidase (DUOX) 2.

Changes of protein abundance. Most of the protein abundance changes by NTAP are the results of the changes of mRNA level in cells. Kang et al. used He-02 APPJ to treat cell medium, and the obtained PAM reduced the protein abundance of CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) in 3T3-L1 pre-adipocytes through the down-regulation of their mRNA levels. It was observed that the mRNA levels of HIF-1α and VEGFA in MDA-MB-231 cells (a typical TNBC cell) and their corresponding protein abundance were both attenuated by PAM.

Besides the mRNA level, some other post-transcriptional regulatory mechanisms, such as RNA silencing, translation and protein degradation control protein abundance. As a result, in some cases, the tendency of the changes of protein expression levels is not corresponding to the alterations of mRNA transcription levels. For example, the modifications of AMPKα mRNA level and mTOR mRNA level in immature chicken Sertoli cell by air-DBD treatment were not reflected on corresponding protein abundance.

Physiological Effects

One of the main ways that NTAP plays its role in affecting the metabolism of cells and organisms is changing the properties, function and expression of proteins.

Physiological effects on microorganisms. NTAP can kill bacteria, fungi and their spores through interruption of functions of crucial proteins including membrane proteins and intracellular proteins or activation of apoptosis microbial cells. Intensive or prolonged NATP irradiation could degrade the membrane-bound proteins of microorganisms and further lead to complete bacterium disruption. Klebsiella pneumoniae was devasted by 5 min treatment of He-DBD through decomposition of the bacterial membrane proteins. Compared to NTAP irradiation, PAS results in less damage on membrane proteins. After 10 min treatment of the air-APPJ activated PBS, the membrane protein YgA of E. coli was changed in its secondary structures and condensed, which leads to the damage on the microstructure of the cell membrane. In addition to membrane protein, some intracellular proteins which are vital to the survival of microorganisms are more sensitive to the inactivation effect of mild NTAP or PAS treatments. For example, the GAPDH from E. coli was fully inactivated by PAW (He-02 APPJ) within 10 min through due to the overoxidation of the key cysteine residue at the enzyme’s active site. Because bacterial GAPDH is a highly conserved enzyme family, NTAP is probably able to work on GAPDH from other bacteria through the similar mechanism. Lunov et al. found that the short term exposure (15 s) of He-APPJ leads to the programmed cell death of bacteria, such as E. coli, Pseudomonas aeruginosa, Staphylococcus aureus and

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Bacillus subtilis, and the underlying mechanism involves the activation of proteins as caspase-like substrate.

On the other hand, in some cases, the promoted cell proliferation, growth and movement for some microorganisms under the effect of NTAP are also observed. The activities of glycerol dehydrogenase and glycerol dehydratase in K. pneumoniae were increased by 12% and 62% respectively under 1 min of He-DDB, which leads to increased survival rate of K. pneumoniae. The spore germination of Aspergillus oryzae spores which are submerged in potato dextrose broth (PDB) was enhanced by 5 min N2-DDB treatment, through the enhancement of activity of monophenols and/or the host cell. Mitogen-activated spore Bacillus subtilis leghaemoglobin content in nodules. min N leads to the activation of rhizobia activities of lemon balm, and SODs proteins, including lipid and phenol, in the fruits of chicory, which is a key enzyme in the plant antioxidative system and is involved in the process of cell stress response, such as salt, cold, and drought.

Physiological effects on plants. Phenylalanine ammonia lyase (PAL) is a key enzyme in the phenylpropanoid pathway that regulates the biosynthesis of phenolic plant antioxidants such as flavonoids, anthocyanins and tannins, and is involved in plant responses to a variety of environmental stimuli, including pathogen infection, injury, UV and other stress conditions. PPOs, a group of oxidase enzymes, catalyze the hydroxylation of monophenols and/or o-diphenols to the o-quinones, and finally leads to the yield of melanin. PODs catalyzes the oxidation of various substrates, including lipid and phenol, in the presence of H2O2. Superoxide dismutases (SODs) are a family of enzymes that catalyze the superoxide anion radical disproportionation into O2 and H2O2. PPOs, PODs, and SODs are the enzymes in plants defending against the abiotic stresses, such as salt, cold, and drought.

Air-DDB treatments for 120 s stimulated PAL and POD activities of lemon balm, and improved the tolerance of lemon balm to selenium (Se) nanoparticle. A similar effect of mitigating the Se nanoparticle phytotoxicity, as well as, were monitored in chicory after air-DDB treatments for 120 s, as well as the increment of POD activity. Ling et al. observed an increase in SOD activity in oilseed rape for air RF plasma against the damage caused by drought stress.

Enzymatic browning of fresh-cut fruits and vegetables is a common retractive phenomenon during storage. PPO and POD are two critical enzymes that regulates browning and promotes the destruction of organic integrity. PPO and POD in freshly cut fruits and vegetables were directly exposed to NTAP irradiation and their activity was reduced. The activity of PPO was reduced to about 10% after the treatment of APPJ, while POD was reduced by about 85% after 240 s. In addition, it was found that the NTAP using Ar and O2 mixture at different concentrations ratio has better deactivation effect than the one using Ar solely. Bußer et al. reported that both PPO activity and POD activities in fresh cut apple and potato tissue were reduced by NTAP. POD activity in fresh cut melon was slightly inhibited by the air-DDB treatment, be decreased by up to about 17%.

NTAP treatments are also reported as they are able to enhance starch and protein content of plants. Nitrogenase activity of rhizobia in nodules of soybean was increased by 1.4 fold after 3 min N2-DDB treatment and 1.6 fold after 2 min O2-DDB treatment, which leads to a nearly 2 fold increase in leghaemoglobin content in nodules. The starch content and grain protein of wheat were enhanced by 3 min RF plasma being fed with air, which slightly activate α-amylase and protease.

Physiological effects on animals and human. Compared with microbe and plant, cells of animals and human lacks the protection of cell wall and are more sensitive to NTAP and fragile. Thus, intensive and prolonged NTAP irradiation always leads to indiscriminate collapse of cells, which has poor scientific value and applying potential. To solve this problem, many researchers used transitory NTAP or PAM to treat cells of animal and human. Anti-tumor effect and mechanism of PAM is one of the hot spots in this research field. PAM treatment had a significant inhibitory effect on cancer cells, including pancreatic cancer, lung cancer, and brain cancer, while it had little devastating effect on healthy cells, indicating a good targeting effect on cancer cells. PAM can exert its effect on proteins that play key roles in the proliferation and metabolism of cancer cells to exclusively disrupt the cell growth. Hypoxia is one of the main features of cancer microenvironment. In order to resist hypoxia, more than half of ATP in cancer cells is synthesized though glycolysis pathway, which does not require oxygen. Therefore, cancer cells have a higher requirement for NAD, an important coenzyme in glycolysis pathway, than normal cells. Poly(ADP-ribose) polymerases (PARPs) family is consisted of structurally and functionally diverse enzymes catalyzing the cleavage of NAD into nicotinamide and contributing to the synthesis of poly(ADP-ribose), which are catalytically activated in the presence of damaged DNA and act as damage sensors. Activation of PARPs decreases intracellular NAD levels and results in impairment of glycolysis of cancer cells. Cancer (Ar-APPJ) effectively inhibited cell proliferation of human lung adenocarcinoma epithelial A549 cells through activating poly(ADP-ribose) polymerase-1. Akter et al. demonstrated PAM (Air-APPJ) induced activation of PARP by activating p38/MAPK signaling, which led to apoptosis of U87 MG brain cancer cell.

Immunogenic cell death (ICD) of cancer cells can initiate an adaptive immune response that is specific for that cancer, which was activated by numerous damage-associated molecular patterns (DAMPs), including Surface exposed calreticulin (ecto-CRT), annexin A1 (ANXA1), type I interferon, and high-mobility group box 1 (HMGB1). Ecto-CRT is a resident chaperone of endoplasmic reticulum that mediates protein folding and promotes storage and homeostasis of cellular Ca++. After DDB treatment for 10 s on A549 lung carcinoma cell (O2), CT26 colorectal cancer cell (air) and A375 melanoma cell (air), the ecto-CRT expression of these cells was up-regulated resulting in adaptive immune responses against these types of tumors. In addition to affecting energy metabolism and triggering ICD of cancer cells, PAM can inhibit cancer cells through alterations on other signal transduction network in cells. For example, PAM downregulates the expression of AKT kinase and subsequently weakens the inhibitory effects of the phosphatidylidylinositol 3-kinase/phosphatase and tensin homologue (PI3K/Pten)-AKT pathway on programmed cell death to induce the apoptosis in glioblastoma cells. The effect of NTAP on other types of mammalian cells also becomes an emerging research area. He-APPJ was irradiated directly to a culture medium containing pre-osteoblastic MC3T3-E1 cells for 5 s and 10 s, and the results showed that He-APPJ stimulated the osteoblastic differentiation of the cells through activating alkaline phosphatase. Kang et al. revealed that PAM (He-O2, APPJ) inhibited differentiation and lipogenesis in 3T3-L1 pre-adipocytes via down-regulation of C/EBP-homologous protein. A microsecond pulsed DBD device was designed and used to treat mouse aortic rings for 10 s, and the treatment induced angiogenesis of the aortic rings through the up-regulation of the expression of vascular endothelial growth factor, matrix metalloproteinase-9, and CXCL 1 in cells.
Application Prospect

Based on the literatures summarized above, NTAP has significant physiological effects on cells of a large range of organisms even viruses through affecting the properties, functions, and expressions of proteins. Meanwhile, compared to other physical and chemical methods, including UV, ozone, alcohol, and chemotherapy, it is generally accepted that NTAP is environmentally friendly, energy-efficient, and low toxic.⁶,113,123,124 Compared with the ROS derived from chemicals, the NTAP-inherent ROS do not introduce chemical contamination after the treatment.⁷⁰ Since exhibiting lethal effect on diverse pathogenic microorganisms, NTAP is a promising technology for disinfection of environment, wound, food and plant.¹⁰¹,¹０２,¹２３,¹２５ NTAP can also be applied to improve the quality of plants by taking advantage of its effect on certain symbiotic bacteria associated with plants.¹³⁰ As reported in several previous studies, the NTAP-induced adaptive response towards specific growth stresses conferred excellent tolerant ability to plants to survive in the corresponding stress environments.¹⁰⁷,¹２９ During fruit storage, some enzymes, such as PPO and POD, cause nutrient loss through enzymatic browning reactions. The inhibition of the activities of these enzymes by NTAP effectively protects the nutrients in fresh-cut fruits and prolongs their storage period.⁵４,¹３０ Besides food preservation, the improvement of food quality by NTAP is another application in food industry. NTAP has demonstrated its capacity of significantly reducing the antigenicity of some proteins triggering allergy in foods, including shrimp tropomyosin, albumin, and Ara h1 of peanut, which protects people from the incidence of food allergy.⁶,⁷,¹０２,¹０３ NTAP shows significant anti-tumor effect on many cell lines from different cancer types including triple-negative breast cancer, glioblastoma, breast adenocarcinoma, leukemia, head and neck cancer, colorectal cancer, and lung carcinoma, indicating it a useful new anticancer therapy targeting various cancers.⁶,⁷,¹０２,¹０３ NTAP shows significant anti-tumor effect on many cell lines from different cancer types including triple-negative breast cancer, glioblastoma, breast adenocarcinoma, leukemia, head and neck cancer, colorectal cancer, and lung carcinoma, indicating it a useful new anticancer therapy targeting various cancers.⁶,⁷,¹０２,¹０３ Furthermore, NTAP inhibits the formation of scar tissue through reducing the expression levels of α-smooth muscle actin (SMA) and type I collagen.⁶,¹０２ All in all, NTAP not only has the effects of wound disinfection, but also inhibits scar formation.¹３２ In addition to these main areas, the application of NTAP effect has been being explored for serving various medical purposes, such as the treatment on obesity and obesity-related diseases⁹⁷ and promoting bone regeneration.¹３２

Conclusion and Discussion

The considerable ROS and RNS derived from NTAP are able to react with and modify certain groups of amino acids, which is the basic construction unit of protein. This dramatic effect of NTAP is subsequently propagated to higher structural orders of proteins, namely secondary, tertiary and quaternary structure, which adjusts or changes biochemical properties and functions of protein. Due to the crucial role of protein in various life-sustaining physiological activities, the NTAP’s effect can be amplified to cellular or even individual organism level to intervene in a range of vital movements, which have been demonstrated by a lot of studies so far. As an energy-efficient and environment-friendly technology with the capability to exert manipulations at different biological levels, NTAP has been being extensively explored for applications on a wide range of areas covering decontamination, plantation, animal husbandry, food industry, and medicine. Although NTAP shows outstanding application potential, currently there have been some limitations on putting this technology into practice. The main concerns include high cost of rare gas used for the generation of NATP, requirement for additional safety precautions against high voltage produced by NTAP devices, and difficulty of carrying devices and storing reactive species.²５,¹２３,¹２４,¹２５ Another notable issue arising from the application of the effect on protein is the limited understanding of the mechanism underlying the effect and the poorly predictable response of protein to NTAP, which hinders the relative research and application. In some cases, given that the same type of NTAP is applied, different or even opposite corresponding alterations in protein properties or physiological phenomena could be observed for varied targets, such as, increase vs decrease in the percentage of α-helix, promotion vs reduction of protein expression or function, and induction of cell apoptosis vs proliferation. Apart from the diverse nature of the treatment targets, the variation of the type of NTAP devices and the applied operating parameters result in high uncertainty and poor reproducibility of the treatment effect. Therefore, to obtain an absolutely controllable and predictable effect on a given target, it is necessary to establish a uniformed standard for NTAP treatment. However, the working parameters of NTAP show a fair complexity, involving combinations among different working gases, voltages, powers, frequencies, and treatment times, which greatly increase the difficulty of standardization of NTAP working system. To attenuate this issue, a possible solution could be integrating voltage, power, frequency, and treatment time as one parameter indicated by NTAP dose, considering that NTAP energy is the ultimate output of the combination of these parameters. In addition, the study on the mechanism of NTAP’s effect on protein is still in its infancy and is not in-depth enough. It has been revealed that the sensitivity of protein to NTAP is closely associated with molecular weight of protein, amino acid sequence and conformation, but the specific target amino-acid sequences of NTAP and the underpinned sequence-recognition mechanism are still unknown. The majority of the available reports about the structural effects of NTAP on proteins only describe the observed phenomena without in-depth investigations on the underlying mechanisms. On the basis of the standardization of NTAP treatment, the exploration of the interaction mechanism between NTAP and protein will not only greatly facilitate NTAP applications in the aforementioned potential fields, but also be probably applied to assist in protein science research and protein engineering.

Author Contributions

Conceptualization, YX and QX; writing—original draft preparation, YX; writing—review & editing, YB, CD, HL, XZ, and QX; funding acquisition, QX and XZ. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

APPJ atmospheric-pressure plasma jet
BSA bovine serum albumin
C/EBPα CCAAT/enhancer binding protein α
DBD dielectric barrier discharge
FCV feline calicivirus
GAPDH glyceraldehyde 3-phosphate dehydrogenase
Hb hemoglobin
ICD immunogenic cell death
LDH lactate dehydrogenase
MAPK mitogen-activated protein kinase
Mb myoglobin
NTAP non-thermal atmospheric plasma
PAL phenylalanine ammonia lyase

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Conflict of Interest

No potential conflicts of interest were disclosed.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.
by atmospheric pressure cold plasma based on dielectric barrier discharge. 

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