Studies on plasma induced bio-chemical reaction inside liquid for biological application

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Abstract: Applications of low-temperature atmospheric pressure plasma to biological fields are attractive. Considering the effects on living organisms, it is important to understand the chemical reactions under wet conditions. Some of the chemical species delivered into the liquid by the plasma react chemically with biomolecules, resulting in macroscopic effects. Through the research of plasma sterilization with bacterial suspension and plasma-treated water, we have finally developed a new chemical disinfectant with peroxynitric acid.

Keywords: plasma medicine, disinfection, atmospheric plasma, plasma-treated water.

1. Biological applications of atmospheric plasma

Applications of low-temperature atmospheric pressure plasma to biological fields have attracted attention. While various kinds of applications have been reported in many fields, including plasma medicine and plasma agriculture, not enough research has been conducted on their mechanism of elementary reaction processes. It would not be scientifically valid to assume that plasma itself has a direct effect on living organisms, such as healing, disinfecting, treating cancer, or promoting plant growth. Through the study of the application of plasma-treated water (PTW) for sterilization, we have conducted research on the elementary processes of reactions at various phases. Considering the effects on living organisms, it is important to understand the chemical reactions under wet conditions.

In most cases, the plasma itself does not directly affect the living organism, but the chemical species supplied by the plasma cause chemical reactions with some biomacromolecules (amino acid, protein, lipid, polysaccharide nucleic acid, etc.) in the organism, which eventually lead to some macroscopic effects. Various reactions occur during plasma irradiation, but it is necessary to exclude side reactions and find the key reactive processes for the macroscopic effects.

2. Plasma induced bio-chemical reaction inside liquid

When atmospheric pressure plasma is generated in the atmosphere, various chemical species are produced by reactions inside the plasma and around gases surrounding the plasma. The half-life of each chemical species are different, and if it is sufficiently long relative to the duration of diffusion, the chemical species could be effective. Some chemical species, including ions, have sufficiently long lifetimes and can exist in space far enough away from the light-emitting part of the plasma. Ions in that area are sometimes referred to as atmospheric ions.

Next, chemical reactions in the liquid phase must be considered. In some cases, chemical species are supplied from the gas-liquid interface, but in other cases, the plasma contacts the liquid and induces plasma chemical reactions in the liquid phase. Reacting objects, such as biomolecules, are present in liquids, so it is basically sufficient to consider reactions with chemical species supplied in the liquid. They are essentially the same whether or not plasma is in contact with them. In considering these reactive chemical species, it is important that spatial concentration gradients of the chemical species due to its own half-life, chemical reactions with others and diffusion occur in both the gas and liquid phases. In particular, very steep concentration gradients occur at the gas-liquid interface. Moreover, when a large amount of biomolecules are present in the liquid, many chemical species supplied by the plasma are quickly trapped, so the reaction is mostly limited to the surface.

There are two ways of utilizing the chemical species produced by plasma: the direct irradiation of the target solution and the mixture of PTW to target. Chemical species produced by plasma can be roughly divided into short-lived and long-lived species, and the criterion for their separation is just the time required for the target chemical species to act, which requires a sufficient half-life and diffusion distance. It is clear to think of PTW as a case where chemical species with sufficiently long half-time is in effect compared to the direct irradiation of plasma to water, and there is essentially no point in distinguishing between them. Furthermore, in order to examine the effects of plasma on cells, it is important to consider the cell structure in which the outer and inner water layers are physically separated by the cell membrane. The permeability of the cell membrane, which often affected by electrical neutralization due to acid dissociation equilibrium, is also important in determining whether chemical species can enter the cell to bring intracellular oxidative stress.

Considering the effect on cells present in a wet environment, it is useful to consider the indirect reaction field where chemical species supplied into the liquid by the plasma act with some biomolecule of cells (plasma itself does not directly act with cells). In such solution, chemical reactions occur simultaneously with biomolecules of cells and other substances. For chemical reactions between various chemical species and biomolecules, it is possible to find reaction rate constants in much of the references, but it is difficult to determine which pathway is key and important. Key reactions are difficult to identify because various side reactions occur simultaneously, and a series of chain reactions may be important in some cases.

Among chemical modifications to biomolecules, we believe that the effect on proteins is particularly important to evaluate the potential for interesting effects on the cells. In proteins, polymers of peptide-bound amino acids, chemical reactions occur likely with the amino acid residues. Reactions between amino acid residues and chemical species are often predictable from reaction rates, and in many cases, specific amino acid residues are preferentially chemically modified. Chemical modification of some of the amino acid residues disrupts the conformation near the enzyme's active center and reduces enzyme reaction. Thus, on the micro level, biomolecules are chemically modified, and on the macro level, some effect on the cell occurs. On a more macroscopic level, an effect is promoted on the individual.

3. Completely new sterilization technique discovered through plasma research using peroxynitric acid

With the above concepts in mind, we have continued our research on plasma sterilization in liquids for more than 15 years, considering the elementary processes of plasma-induced reactions especially inside liquid, and have finally developed a completely new chemical disinfectant.

At first, we have developed the reduced-pH method with direct plasma exposure to bacteria suspension [1] and cryo-preserved PTW [2], which brings about 100 times higher bactericidal activity under the specific acidic condition (pH < 4.8). Our PTW, characterized by the reduced-pH method and the cryo-preservation, has the strongest bactericidal activity among the other reported PTWs. This is thought to be brought by HOO• generated from O_2^{-}• in the acidic condition (the acid dissociation equilibrium of HOO• and O_2^{-}•, pKa is 4.8), because electrically neutral HOO• can easily permiate into cell membrane. As half-life period of O_2^{-}• is much shorter than experimentally obtained one of PTW activity, we assumed that PTW contains some precursor of HOO•. Although PTW contains many chemical components, respective components in PTW were evaluated according to whether it had bactericidal activitis by ion chromatography [3]. In addition to peaks of H_2O_2, NO_2^{-} and NO_3^{-}, $HOONO_2$ (PNA: peroxynitric acid) was detected and only this fraction had bactericidal activity. HOO• would be released from PNA by radical cleavage. We conclude that PNA is a key chemical species of cryo-preserved PTW with the reduced-pH method.

Although the existence of PNA was reported about a century ago [4], the usage of PNA to disinfection we propose is the first in the world. PNA is known to be chemically synthesized by mixing HNO_2 and H_2O_2 under extremely low pH condition [4], and simplified chemical reactions are shown as follows.

$$HNO_2 + H_2O_2 \rightarrow HOONO + H_2O \qquad (1)$$

$$HOONO + H_2O_2 \rightarrow HOONO_2 + H_2O \qquad (2)$$

These raw chemical materials can be supplied to PTW by plasma exposure. Our chemical synthesis experiments showed that pH should be less than 2, preferably around 0. It is not concordant with the fact that pH of the whole PTW is about 2-4 in the plasma experiments, indicating that PNA synthesis would not proceed in the whole PTW. Considering the dynamic supply of plasma-generated chemical species to water surface, these concentration around the water surface layer (the interface between plasma and liquid) would be very high due to localization during plasma exposure. It is thought that PNA is generated only in the thinner surface of the plasma-irradiated solution, where pH is extremely low, and PNA is stored in PTW if the temperature is sufficiently low. Peroxynitric acid chemistry should be considered in the field of PTW research. P. Lukes proposed peroxynitrite acid (HOONO) chemistry [5]. This is quite related to our peroxynitric acid chemistry. Peroxynitrite acid chemistry belongs to post discharge PTW according to only reaction (1). HOONO is generated slowly (for several hours) to kill bacteria. We think PNA was synthesized in usual PTW experiment including excellent experiments by P. Lukes. However, usual PTW experiments were done at room temperature and no PNA remains at such condition after a few minutes.

Our PTW has the highest bactericidal power compared to similar researches, equivalent to 100% hydrogen peroxide, and it takes only a few seconds to kill *Bacillus subtilis* spores by 6 LogR. Through the research of plasma sterilization, we have developed a new chemical sterilization method of peroxynitric acid. To obtain peroxynitric acid practically, chemical synthesis method is better than plasma treatment in consideration of cost and quantity. 2 M PNA obtained by chemical synthesis has a bactericidal power equivalent to 20,000% hydrogen peroxide, and animal experiments and other studies have confirmed that at least 0.1 M PNA is safe for living organisms. Fortunately, the bactericidal properties of PNA are much better than those of conventional disinfectants with respect to the ratio of bactericidal power to safety, and we have already started application research in many fields, including medical equipment, human body, agriculture, and food. Plasma was used as a tool to produce PNA in PTW, but it can be said that it was a tool to develop this completely new chemical sterilization technique.

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