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Application Note

No. 89

Life Science

Structural Confirmation of Polyoxazoline-Conjugated Albumin (New Artificial Blood) Using a Benchtop MALDI-TOF MS System

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Life Science

■ Abstract

The demand for animal medical care, especially pet healthcare, continues to increase every year, but the infrastructure for blood transfusion therapy remains inadequate. Recently, we (Komatsu Lab team, Chuo University) have developed a new artificial blood product (artificial plasma) for pets "polyoxazoline-conjugated albumin." This formulation is created by chemically modification of readily available porcine-derived albumin with polyoxazoline and can be safely administered to dogs and cats. In this paper, we describe an example of structural confirmation of POx-PSA using a matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF MS).

1. Introduction

In the case of human healthcare, all raw materials for blood transfusion products rely entirely on donated blood. As the number of blood donors decreases due to an aging population, there is a risk of struggling to secure an adequate supply of transfusion blood for general medical use, as well as an inability to meet the demand for large-scale disasters such as earthquakes and typhoons. Indeed, the global supply of donated blood has significantly declined during the COVID-19 pandemic, leading to severe blood shortages. Consequently, there is growing interest in the practical implementation of "artificial blood" which does not depend on blood donations.

Blood comprises blood cells (such as erythrocytes and leukocytes) and plasma (a liquid component containing proteins, vitamins, etc.). Albumin, the most abundant plasma protein, plays a crucial role in maintaining colloid osmotic pressure and circulating blood volume.

Albumin isolated from donated blood is formulated and widely used in clinical practice as albumin preparation (plasma fractionation product). Patients with low albumin levels due to liver or kidney abnormalities (hypoalbuminemia) receive albumin preparations. The preparations are also used in the treatment of sepsis, refractory ascites associated with cirrhosis, refractory edema, and severe burns. While hydroxyethyl starch (HES) preparations are commercially available as an alternative to albumin for use as artificial plasma, concerns persist regarding potential side effects such as blood coagulation disorders, renal dysfunction, and increased levels of amylase.

We (Komatsu Lab team, Chuo University) developed an artificial blood product (artificial oxygen carrier), which is a hemoglobin wrapped covalently with albumin. This artificial blood, named HemoAct, has no blood type, making it suitable for administration to anyone at any time. Furthermore, HemoAct can even be freeze-dried for long-term storage as a powder. We have demonstrated its safety through animal experiments and shown its efficacy in various applications, including resuscitation from hemorrhagic shock, treatment of stroke, and cancer therapy.

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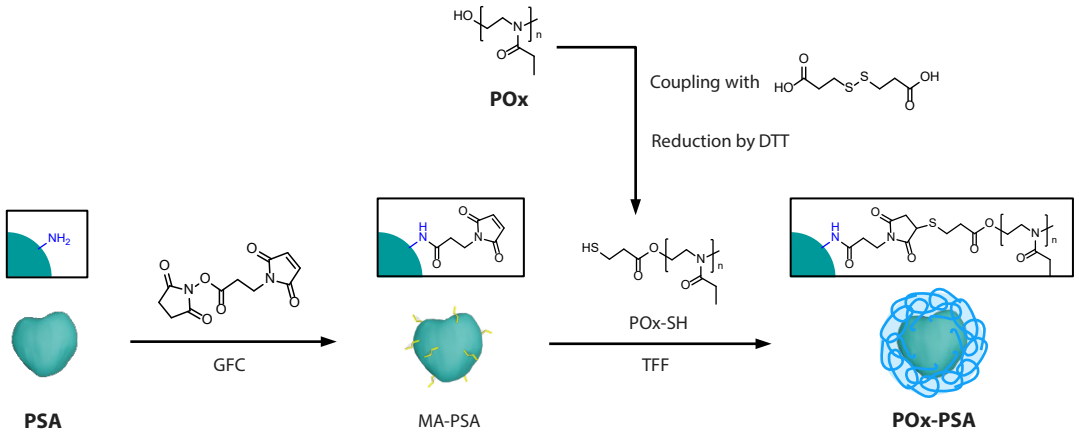

Fig. 1 Polyoxazoline-Conjugated Porcine Serum albumin (POx-PSA) Synthesis

Now, let us consider the veterinary medicine, specifically pet healthcare focusing on dogs and cats. In recent years, the aging of pets has been progressing, leading to a steadily increasing demand for veterinary care. However, when it comes to blood transfusion therapy, there is a lack of well-established systems, primarily due to the absence of a structured pet blood donation system.

For example, obtaining canine albumin preparations derived from canine blood or feline albumin preparations from feline blood is a challenging endeavor. As with humans, hypoalbuminemia in pets can only be treated by administering plasma from the same species. Nonetheless, ensuring a stable supply of canine or feline plasma presents significant difficulties.

As one potential solution, the administration of albumin from a different animal sources can be considered. For instance, readily available porcine serum albumin (PSA) is a viable option. However, porcine albumin is recognized as a xenogeneic protein for dogs and cats, which poses the risk of antibody production and potential adverse effects upon readministration. One technique for avoiding this risk is conjugating synthetic polymers such as polyethylene glycol (PEG) to the surface of proteins, preventing the production of antibodies. PEG is well-known for its biocompatibility and water-soluble properties. Nevertheless, in recent years, it has been observed that patients administered with PEG-conjugated enzymes can develop antibodies against PEG (anti-PEG antibodies). The presence of anti-PEG antibodies results in rapid elimination of PEG-conjugated drugs from the body upon administration.

Recently, we have synthesized POx-PSA (polyoxazoline-conjugated porcine serum albumin) by binding the synthetic polymer "polyoxazoline (POx)" to the surface of PSA. This formulation can be used as an artificial plasma for dogs.[1)] POx is a highly biocompatible, non-immunogenic, and non-ionic water-soluble polymer with properties that are equivalent to or superior to PEG. POx-PSA is converted into a stable powder form through freeze-drying, and the resulting white powder can be stored reliably for over a year. When reconstituted in water, the POx-PSA solution exhibits the same properties as it had before freeze-drying.

This article describes the structural confirmation of POx-PSA using the MALDI-8020 benchtop MALDI-TOF MS system.

2. Experiments

POx was covalently bound to PSA obtained from the blood of specific pathogen-free (SPF) pigs, ensuring their rearing in a hygienic pig facility and the absence of specific diseases.

First, the porcine blood was centrifuged and the resulting supernatant was frozen at −80 °C. Thawing the frozen supernatant slowly at 4 °C, followed by a second round of centrifugation, removed cryoprecipitates, resulting in porcine blood serum. Subsequently, an excess of sodium caprylate was added to the porcine blood serum, which was then heated to 70 °C to denature other unwanted proteins. After centrifugation to remove precipitates, PSA was isolated using anion exchange chromatography (AEC). The purified PSA was analyzed by SDS-PAGE and size exclusion chromatography (SEC) to verify its purity.

POx with a terminal sulfhydryl group (POx-SH) was synthesized to conjugate POx to PSA. POx-SH was obtained by coupling of 3,3′-dithiopropionic acid (DTDPA) to a commercially available POx (Mw: 5 kDa, Sigma-Aldrich 740713) and subsequent cleaving of the disulfide bonds using dithiothreitol (DTT) (Fig. 1 above).

Finally, POx-SH was bound to the amino groups on the PSA surface via a cross-linking agent (*N*-succinimidyl 3-maleimido propionic acid: SMP) (Fig. 1 below). Size exclusion chromatography showed the peak of POx-PSA in the high molecular weight region compared to unmodified PSA, suggesting the presence of multiple POx chains covalently bound to PSA. The purification of POx-PSA was accomplished using tangential flow filtration (TFF).

MS measurements of PSA, POx, and POx-PSA were conducted using the MALDI-8020 benchtop linear MALDI-TOF MS system in positive-ion mode (Fig. 2).

Fig. 2 MALDI-8020 Benchtop MALDI-TOF Mass Spectrometer

3. Results and Discussion

One of the notable features of matrix-assisted laser desorption/ionization (MALDI) is its ability to primarily generate singly-charged ions, even for high molecular weight species such as proteins. Fig. 3 shows the MALDI mass spectrum of PSA. The analytical conditions were as follows:

- ✓ Matrix: 20 mg/mL Sinapinic acid
- ✓ Pulsed Extraction: 100000
- ✓ Sample Concentration: 20 pmol/μL
- ✓ Sample Preparation: A mixture of 1 μL of the sample solution and 0.5 μL of the matrix solution was prepared on the MALDI plate, and the measurement was conducted after air-drying.

In addition to a singly-charged ion (*m/z* 66803), a doubly-charged ion (*m/z* 33444) and a dimeric ion (*m/z* 133676) were also clearly detected. This MS analysis showed the exceptionally high purity of PSA.

Fig. 4 represents a MALDI mass spectrum of the starting material, POx (commercially available, Mw: 5 kDa, Sigma-Aldrich 740713). The analytical conditions were as follows:

- ✓ Matrix: 5 mg/mL a-cyano-4-hydroxycinnamic acid
- ✓ Pulsed Extraction: 5500
- ✓ Sample Concentration: 0.04 mg/mL
- ✓ Sample Preparation: A mixture of 0.5 μL of the sample solution and 0.5 μL of the matrix solution was prepared on the MALDI plate, and the measurement was conducted after air-drying.

In the spectrum, ladder peaks with a 99 Da spacing in the *m/z* range of 3500-7000, centered around *m/z* 5000, were observed. The mass difference between the peaks is attributed to the repeating units of polyoxazoline. The ion species of polyoxazoline was found to be sodium-adducted form.

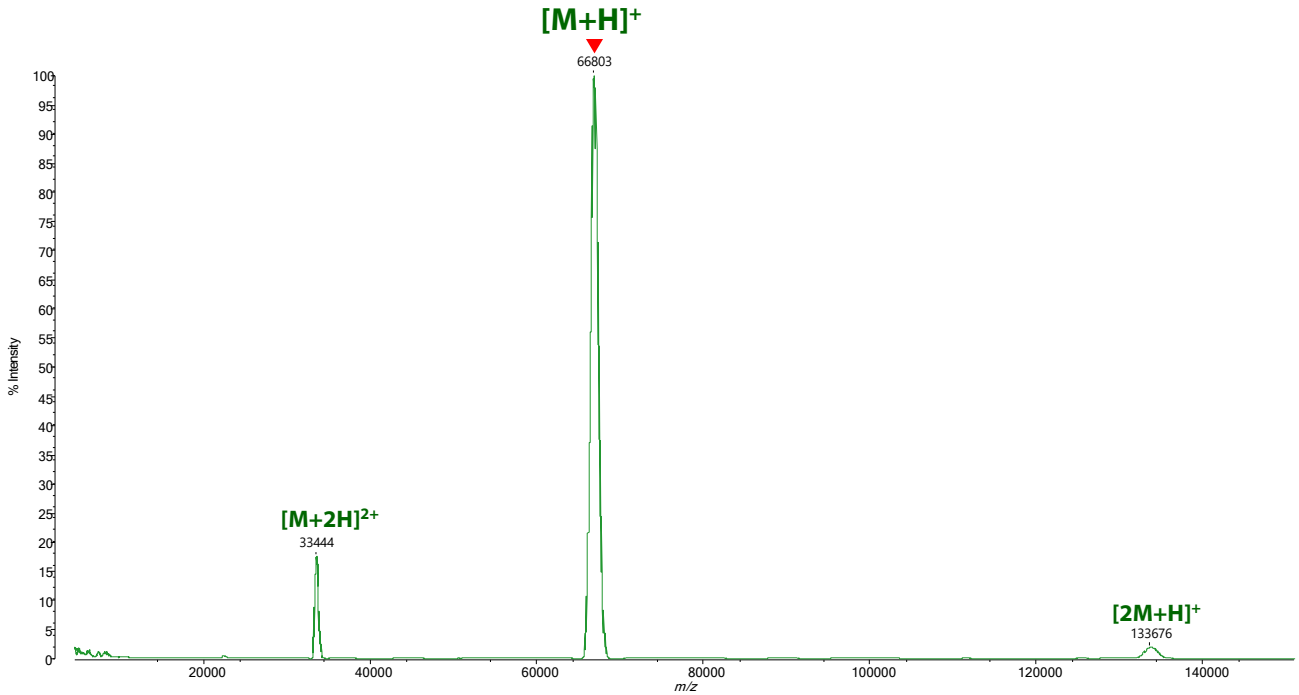

Fig. 3 MALDI Mass Spectrum of PSA Isolated from Porcine Blood

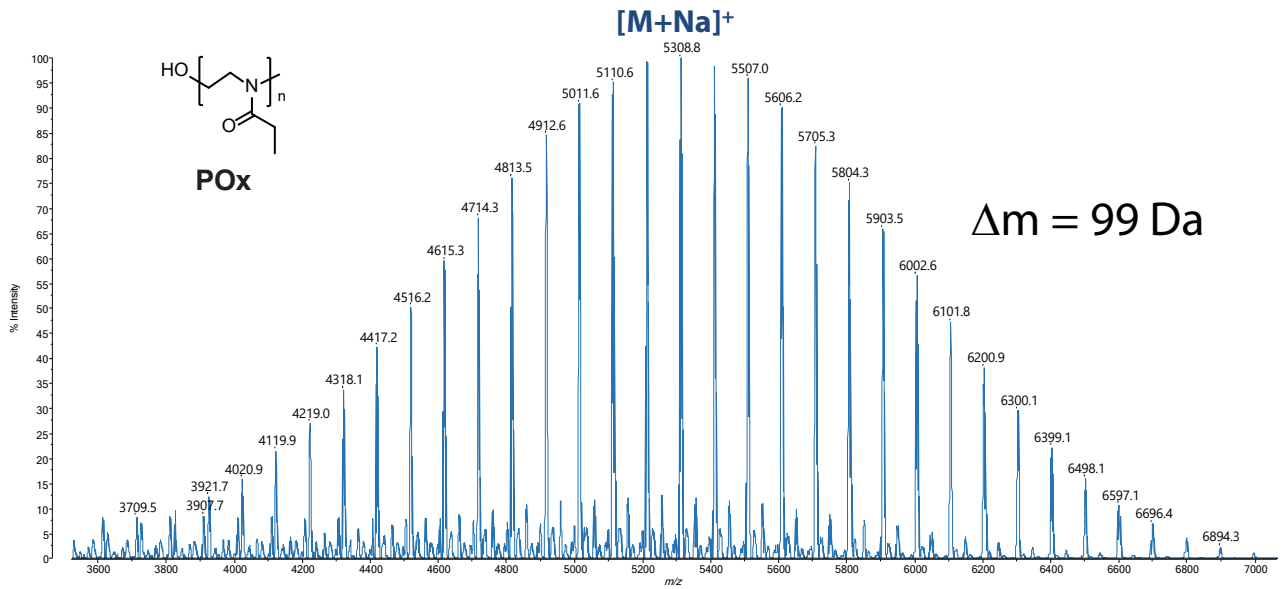

Fig. 4 MALDI Mass Spectrum of POx

Fig. 5 represents the MALDI mass spectrum of POx-PSA. The measurement conditions are as follows. For reference, the mass spectrum of PSA before POx binding (in green) is also overlaid:

- ✓ Matrix: 20 mg/mL Sinapinic acid
- ✓ Pulsed Extraction: 100000
- ✓ Sample Concentration: 20 pmol/µL
- ✓ Sample Preparation: A mixture of 1 µL of the sample solution and 0.5 µL of the matrix solution was prepared on the MALDI plate, followed by air-drying. On-plate desalting was then performed with 0.1 % trifluoroacetic acid (TFA) before analysis.

The each multiple peak in the spectrum corresponds to POx-PSA with different number of POx attachments. Broader peak widths compared to the PSA peak can be attributed to the fact that the attached POx has a distribution of molecular weights.

By examining the *m/z* values, it becomes evident that adducts with 2 to 9 POx attachments are present, with 5 to 6 attachments being the most predominant. Fitting the peak pattern by simulation revealed that the average binding number of POx was 5.7, which closely aligns with the average binding number (5.9 ± 0.4) derived from separate gravimetric analysis of the freeze-dried powder.

The LC/MS analysis tends to form multiply-charged ions. This would have produced very complex and difficult-to-interpret spectra if it had been used to analyze POx-PSA in which multiple POx molecules with a range of molecular weights bound to PSA. By contrast, MALDI typically generates singly-charged ions, making it ideal for analyzing samples of greater mass complexity because the mass values can be identified directly from the resulting mass spectrum.

Currently, animal experiments using POx-PSA are in progress. It has been observed that binding POx to PSA improves its blood retention (prolonged half-life) and no antibodies are generated against PSA or POx. Furthermore, administering POx-PSA solution to rats in hemorrhagic shock model revealed that parameters such as blood pressure, heart rate, and pH, which had decreased by hemorrhaging, were restored to pre-hemorrhage levels, with no adverse effects on organs. POx-PSA exhibits excellent efficacy in restoring circulatory blood volume due to its high colloid osmotic pressure. The safety of POx-PSA solution has been confirmed in dogs.[1)]

4. Conclusion

This Application Note has introduced an example of the detection of polyoxazoline-conjugated porcine serum albumin (POx-PSA), which is anticipated as artificial blood for pets, using a benchtop linear MALDI-TOF MS system. Given that MALDI-TOF MS system primarily detects singly-charged ions, it serves as a convenient and precise tool for the analysis of complex modified proteins with multiple attached polymers and molecular weight distribution as observed in the case of POx-PSA. This technique can be considered an effective tool for observing polymer-conjugated protein species.

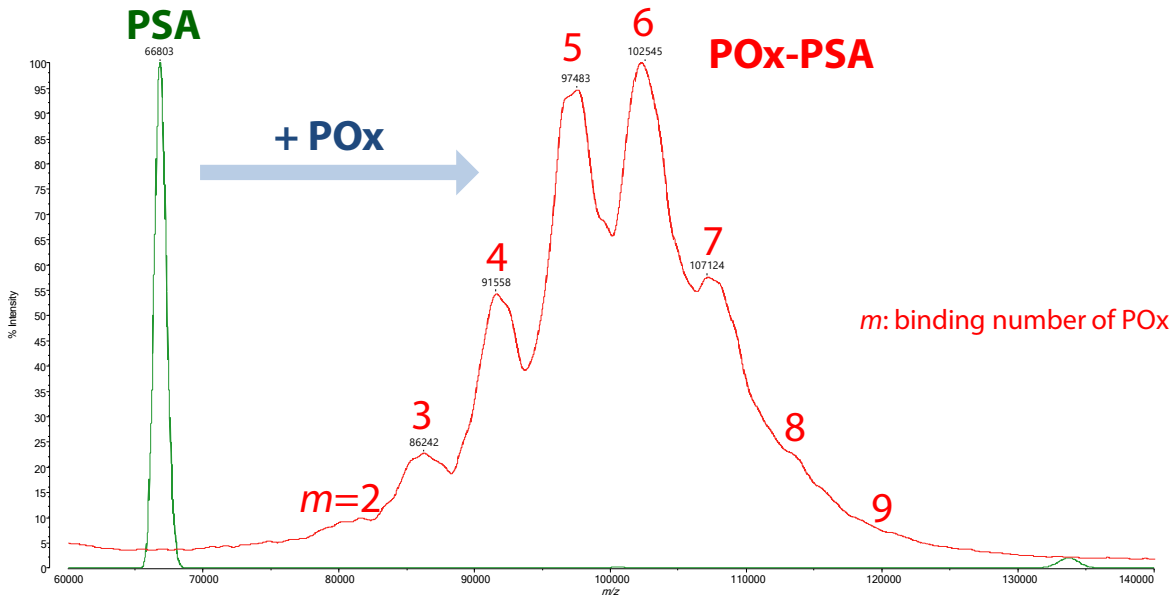

Fig. 5 MALDI Mass Spectrum of POx-PSA (Red) and PSA (Green)

Reference

1) Okamoto, W., Usui, T., Hasegawa, M., Kobayashi, T., Fujisawa, J., Taguchi, K., Matsumoto, K., Kohno, M., Iwazaki, M., Shimano, S., Nagao, I., Toyoda, H., Matsumura, N., Tomiyasu, H., Tochinai, R., & Komatsu, T. Polyoxazoline-conjugated porcine serum albumin as an artificial plasma expander for dogs. *Scientific Reports*, 13, 9512 (2023). https://doi.org/10.1038/s41598-023-35999-4

First Edition: Nov. 2023

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