Capillary Electrophoresis Analysis of Alkaline Phosphatase

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Alkaline phosphatase is a vital enzyme used in separation studies and as a biomarker for liver, bone, and certain cancer conditions. Its stability and specific properties enable insights into enzyme behavior, aiding in the development of detection methods with broader applications in various scientific fields. Alkaline phosphatase has four main isoenzymes: germ cell alkaline phosphatase (GCAP), intestinal alkaline phosphatase (IAP), placental alkaline phosphatase (PLAP), and tissue-nonspecific alkaline phosphatase (TNAP), each with distinct roles. TNAP is found in the liver, kidney, and bones, playing a role in bone mineralization. Separation techniques like electrophoresis and chromatography are valuable for studying enzymes and proteins, revealing insights into their structure and function in pharmaceutical research and post-translational modification (PTM) studies.

alkaline phosphatase capillary electrophoresis post-translational modifications whole cell analysis

1. Introduction

Alkaline phosphatase is an important enzyme that plays a crucial role in various biological processes. It is commonly used as a model enzyme in separation studies and has several advantages for enzyme detection. Alkaline phosphatase is found in many living organisms, including bacteria, plants, and animals, making its molecular weight (MW) differ. However, human ALP has an MW of 86,000 Da. Its widespread occurrence makes it a convenient enzyme for research and detection purposes. In the medical field, alkaline phosphatase is used as a biomarker for various health conditions, especially those related to the liver and bones. Detecting its presence and activity can help to diagnose and monitor diseases like liver disorders, bone diseases, and certain cancers. Alkaline phosphatase is relatively stable under certain conditions, allowing researchers to study its behavior without significant degradation or loss of activity. This enzyme has been extensively studied and characterized, making it easier for researchers to compare their experimental results with existing knowledge and data. Detection methods for alkaline phosphatase can be highly sensitive, allowing for the detection of small amounts of the enzyme even in complex biological samples. Alkaline phosphatase detection methods can be designed to be highly specific, ensuring that the measured activity or presence of the enzyme is not influenced by other interfering substances. Alkaline phosphatase exhibits specific enzymatic properties, such as substrate specificity and reaction kinetics, which can be studied during separation experiments to gain insights into enzyme behavior [1][2][3]. Overall, using alkaline phosphatase as a model enzyme in separation studies helps researchers to develop and optimize

detection methods that can be applicable to other enzymes as well. The insights gained from these studies can have broader implications in various scientific fields, including biotechnology, medicine, and biochemistry.

In addition, alkaline phosphatase is responsible for the dephosphorylating process in cells. ALP comprises a group of enzymes that facilitate the breakdown of numerous organic phosphate esters under alkaline pH conditions, with zinc serving as a crucial cofactor. It therefore plays a significant role in various human pathological and normal conditions. The serum alkaline phosphatase activity primarily emanates from three primary sources: the liver (accounting for more than 80 percent), bone tissue, and, in some cases, the intestinal tract. This turns it into four main isoenzymes: germ cell alkaline phosphatase (GCAP); intestinal alkaline phosphatase (IAP); placental alkaline phosphatase (PLAP); and tissue-nonspecific alkaline phosphatase (TNAP), whereby all of which have their own physiological role. For example, TNAP is expressed mostly in the liver, the kidney, and bones. The functional role of bone isoenzyme is seen in bone mineralization, while the function of liver and kidney isoenzymes remains unknown [4][5]. The functional roles of the other isoenzymes—GCAP, IAP, and PLAP—are still not known [6].

2. Capillary Electrophoresis (CE)

The history of CE is relatively short compared to other separation techniques, but it has seen rapid advancements and improvements. In 1983, Lukacs and his professor, Professor Jorgenson, published a seminal paper that demonstrated the potential of using capillaries to overcome the limitations of traditional gel electrophoresis methods. They built upon the earlier work of Hjerten and Catsimpoolas, who had developed capillary zone electrophoresis, and identified ways to reduce interferences between bands, thus enhancing the resolution and efficiency of separations [7][8][9][10]. This breakthrough opened the door for further research and development in the field of capillary electrophoresis. Over the years, CE has become a complementary method to other modern separation instruments and has found applications in various scientific fields, including biochemistry, pharmaceuticals, environmental analysis, and more.

Capillary electrophoresis (CE) is an advanced form of electrophoresis that uses narrow capillaries filled with a conductive buffer to separate molecules under an electric field. CE offers higher separation efficiency and speed due to the small capillary size and the ability to generate higher electric fields. It requires smaller sample volumes, leading to enhanced sensitivity and lower detection limits. CE systems can be automated and integrated with other techniques, like mass spectrometry [11], for comprehensive enzyme analysis. CE has broad applications in high-throughput analysis, protein characterization, and monitoring enzyme activity in various fields, including pharmaceutical research, clinical diagnostics, and biotechnology. CE can utilize different modes of separation, such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), or capillary gel electrophoresis (CGE), depending on the specific application. In CE, the movement of charged molecules through a narrow capillary tube occurs under the influence of an electric field. The electrical double layer forms at the capillary wall, consisting of an inner layer of counterions and an outer layer of co-ions. As analytes migrate through the capillary, they experience electroosmotic flow driven by this double layer, allowing for the separation of molecules based on their charge and size. **Figure 1** shows a diagram illustrating the components of CE.

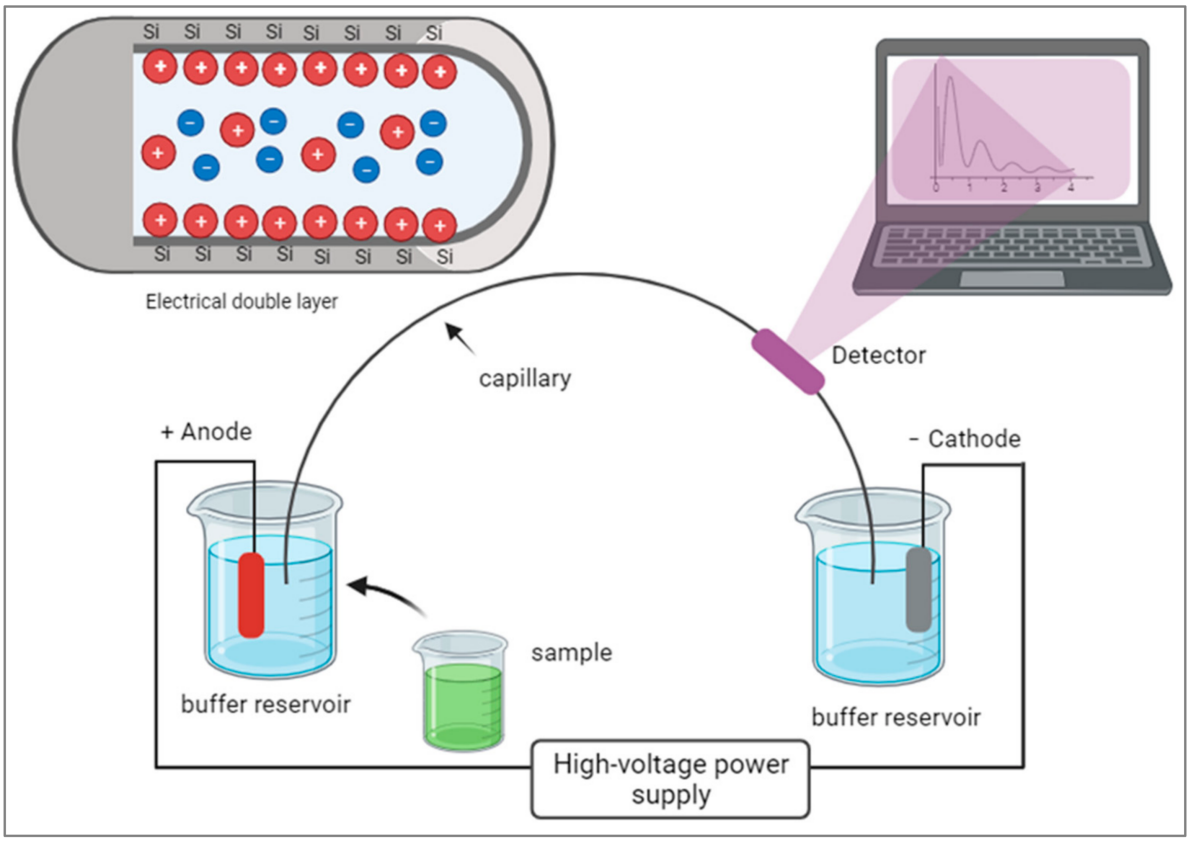

Figure 1. Diagram of capillary electrophoresis showing the electrical double layer formed when voltage is applied.

However, it is acknowledged that CE may be less efficient than some modern separation methods in terms of limit of detection and reproducibility. Researchers continue to investigate and refine capillary electrophoresis to improve its analytical separation capabilities. Efforts are being made to enhance sensitivity, increase throughput, and optimize the reproducibility of results. Additionally, advancements in microfluidics and miniaturization have led to the development of microdevices for CE, which further expand its applications and potential for future research [12][13][14]. Overall, capillary electrophoresis has come a long way since its inception, and ongoing research aims to make it an even more powerful and efficient technique for analytical separations in various scientific disciplines.

The capillary is usually made with glass. The glass is limited by high temperature and relatively low flexibility and robustness, but all of which are improved by using fused silica. The silica's outer surface is sometimes coated with a polymer. The thinner fused silica capillary allows for small volumes of samples. This enhances the ratio of surface-to-volume. The small size of the capillary tube also reduces the heat and voltage, which reduces the denaturing of the protein during separation [15].

Alkaline phosphatase is an enzyme that catalyzes the hydrolysis of phosphate esters with an alkaline pH. Fused silica is negatively charged [16] and the alkaline buffer in the two reservoirs has a negative charge. This means that, combined, they are electrostatic repulsive forces [17]. In capillary electrophoresis, the electrostatic repulsive forces between the buffer and the capillary wall help to prevent the adsorption of proteins onto the capillary wall, which could lead to loss of the analyte and distorted results. This is particularly important for proteins like alkaline

phosphatase, as adsorption could lead to a decrease in the detected enzyme concentration and affect the accuracy of the analysis.

Its detection in capillary electrophoresis can be achieved using various methods, including indirect detection and on-column detection [18]. In indirect detection, a substrate is used to generate a detectable product when it reacts with the enzyme (alkaline phosphatase). The product formed from the enzymatic reaction is then detected in the capillary. One common approach is to use a fluorescent or UV-absorbing substrate that, upon hydrolysis by alkaline phosphatase, produces a fluorescent or UV-absorbing product. The formation of the product is proportional to the enzyme concentration, allowing for quantification. In on-column detection, the analyte itself is directly detected as it migrates through the capillary. For proteins like alkaline phosphatase, the detection is based on their intrinsic properties, such as absorbance, fluorescence, or refractive index. With regard to capillary electrophoresis separation, the prepared protein sample is injected into a narrow capillary filled with an electrolyte solution. An electric field applied across the capillary charges protein molecules to ensure that they can migrate through the capillary; then, based on their charge-to-mass ratio, they separate proteins. Smaller and more highly charged proteins will move faster than larger or less charged ones, leading to the separation of the protein mixture. As the protein molecules pass through the detection point in the capillary, they are exposed to a UV or visible light source, for instance. Proteins, like many other biomolecules, absorb light in the UV-Vis range due to the presence of aromatic amino acids (such as tryptophan, tyrosine, and phenylalanine) and chromophores. The extent of light absorption depends on the concentration of proteins in the capillary. The detected absorbance signals are recorded and analyzed to generate an electropherogram, which is a graph showing the peaks corresponding to different proteins in the sample. The height of each peak represents the relative concentration of the corresponding protein in the mixture. Through the use of appropriate detection methods, the protein's migration through the capillary can be monitored and quantified without the need for derivatization or reactions with substrates.

3. Indirect Detection of Alkaline Phosphatase

In the case of a UV-Vis detector, p-nitrophenol (pNP) is the product of the enzyme reaction of alkaline phosphatase and the substrate p-nitrophenol phosphate (pNPP). pNP has chromophores that make it sensitive for spectrometric detection and it has two functional groups: one is the OH and the other is NO_2. These occupy opposite ends of the benzene ring. The absorption spectra occur in 405 nm for this compound. By following the steps in **Figure 2**, capillary electrophoresis with a UV-Vis detector can be used to detect and quantify ALP. Indirect detection is typically performed using discontinuous assays with pre- or post-reaction sampling, as it involves stopping the enzymatic reaction at specific time points to quench the reaction and prevent further product formation. The reaction mixture is then analyzed at each time point to measure the accumulated product or remaining substrate concentration. So, here, the enzyme reaction is initiated by mixing the enzyme with its substrate and other necessary components. After a specific incubation period, the reaction is stopped (e.g., by changing the pH or adding a quenching reagent). A small volume of the reaction mixture is sampled and analyzed via capillary electrophoresis using indirect detection methods. The concentrations of the product(s) or remaining substrate are determined, and the enzyme activity or concentration is calculated based on these measurements. Discontinuous

assays provide information about the extent of the enzymatic reaction at specific time points, allowing for the determination of initial reaction rates and steady-state kinetics.

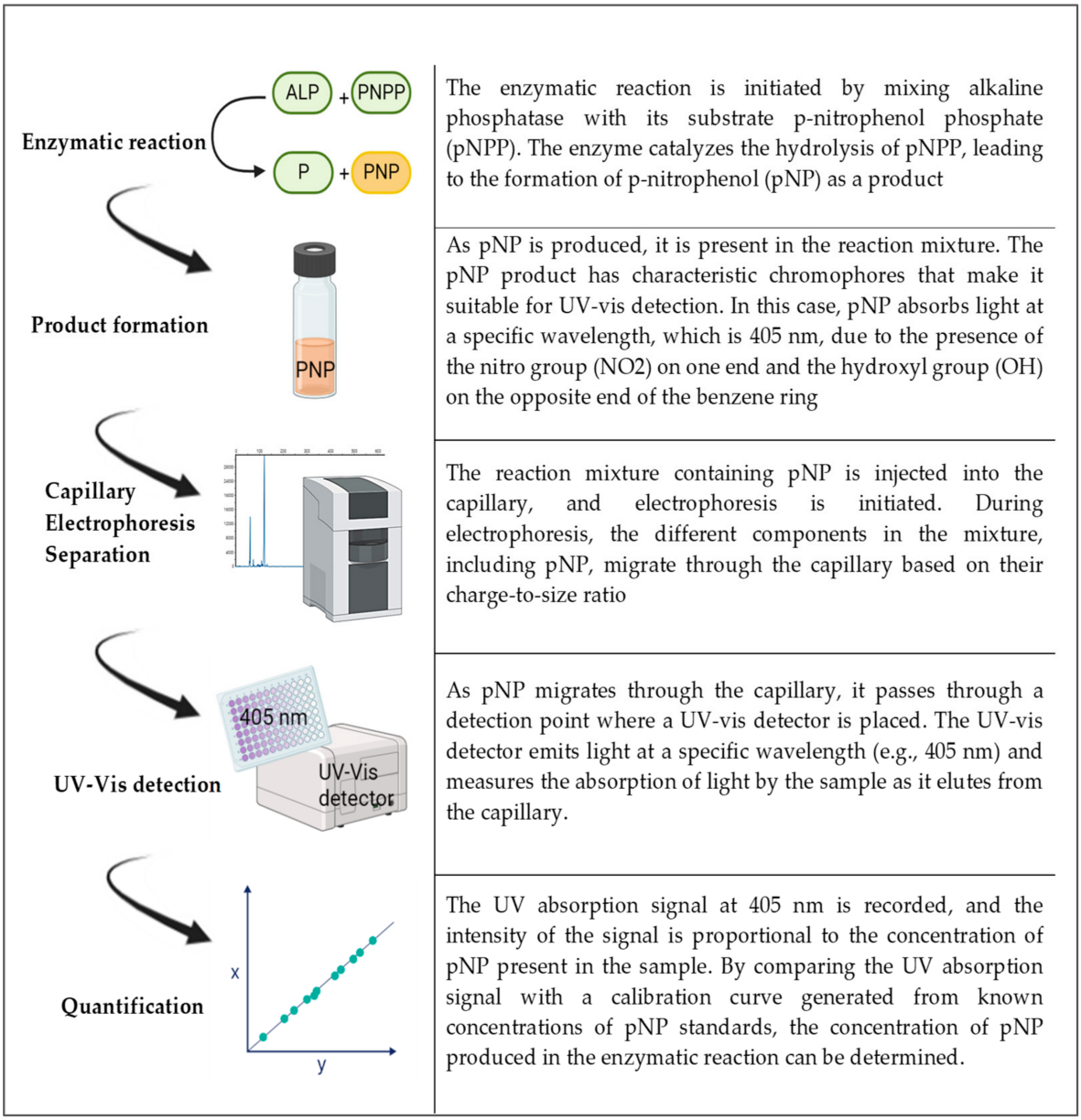

Figure 2. The steps of capillary electrophoresis with a UV-Vis detector can be used to detect and quantify pNP, the product of the enzymatic reaction involving alkaline phosphatase and pNPP.

4. On-Column Detection of Alkaline Phosphatase

In this detection method, the substance of interest is detected directly as it moves through the capillary and during its migration it can be real-time-monitored during the enzymatic reaction; therefore, it is called a continuous assay. **Figure 3** shows that continuous assays provide kinetic information about the enzymatic reaction and are useful for studying reaction rates, enzyme inhibition, and enzyme kinetics. It involves continuously monitoring the enzymatic reaction as it progresses over time. This approach allows for real-time observation of the reaction kinetics. The

enzyme reaction is initiated by mixing the enzyme with its substrate and other necessary components. The reaction mixture is injected into the capillary, and electrophoresis is started. As the reaction progresses, the products of the enzymatic reaction, or any other species involved in the reaction, move through the capillary with different electrophoretic mobilities. These products are detected as they elute from the capillary, and their concentrations are monitored over time, usually through absorbance or fluorescence detection. The rate of product formation or substrate consumption is then used to determine the enzyme's activity or concentration.

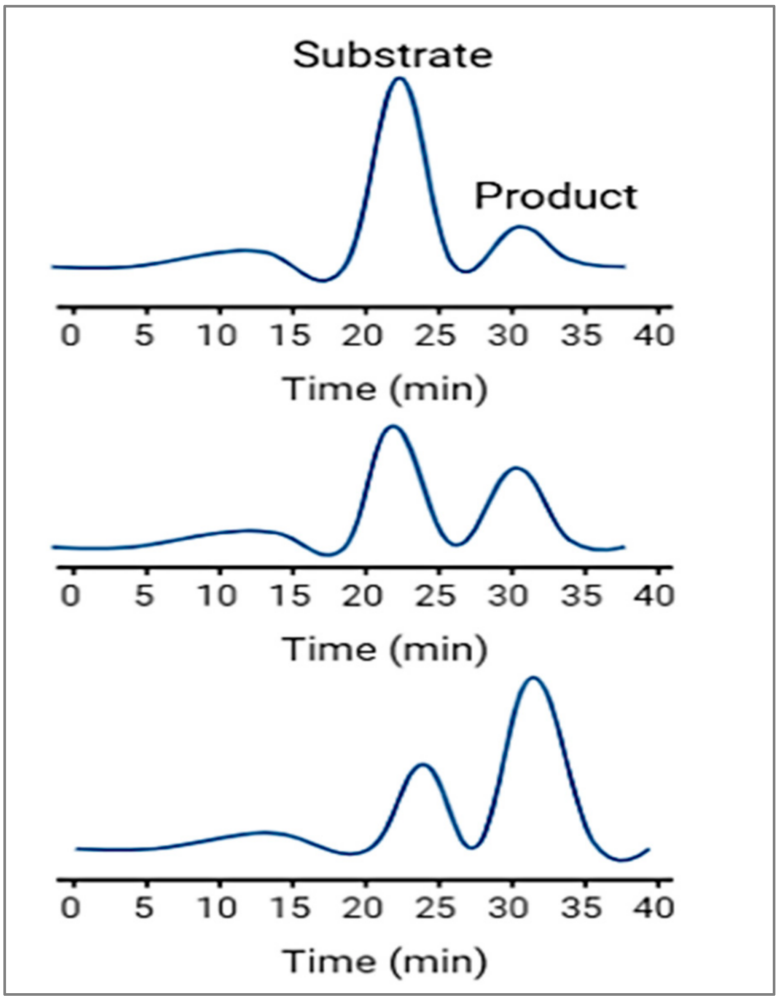

Figure 3. Capillary electrophoresis diagram of continuous assays which offer kinetic details about enzymatic reactions and are valuable for investigating reaction rates, enzyme inhibition, and enzyme kinetics.

Using a laser-induced fluorescence detector for ALP detection is another powerful approach in capillary electrophoresis. This method is particularly popular in the literature, especially when researchers focus on affinity binding for ALP analysis [19] and ALP concentration [20]. In laser-induced fluorescence detection, ALP enzyme reactions using fluorogenic substrates are monitored based on their fluorescence signals. Various aspects need to be considered and troubleshooted when using this technique. Some of the challenges include the adhesion of ALP in the capillary, the activation of ALP, inhibition using different metal chelators, and the selection of suitable labels (e.g., glycosphingolipids) in different buffers [21][22][23][24][25]. The integration of laser-induced fluorescence detection with capillary electrophoresis allows for the highly sensitive analysis of enzymes and proteins. However, a major

limitation of this method is that most fluorogenic dyes are limited in their compatibility with specific functional groups within compounds. For instance, Fluorescein isothiocyanate may be less reactive at low concentrations of amine groups, whereas rhodamine dyes might be more efficient with succinimidyl ester groups. This selectivity of fluorogenic dyes can limit their universal application. Moreover, the lack of standardization for ALP assays with fluorescence detection can pose challenges. To overcome these limitations, researchers have turned to nanomaterials, such as nanoparticles, to enhance the performance and sensitivity of the technique. Quantum dots [26] and magnetic nanoparticles [27] have been reported in ALP enzyme assays with capillary electrophoresis. These nanoparticles possess unique physical properties that can enhance the immunoaffinity within the capillary and improve the accuracy and efficiency of the analysis. By leveraging nanomaterials and addressing the limitations of fluorescence detection, researchers can further enhance the capabilities of capillary electrophoresis in enzymatic and protein analysis, including the detection and characterization of ALP. These advancements contribute to the continuous improvement and expansion of capillary electrophoresis as a powerful tool in various scientific fields.

5. Alkaline Phosphatase Detection from Whole Cells

It is important to note that the successful detection of ALP from whole cells using capillary electrophoresis depends on optimizing the sample preparation, separation conditions, and detection methods. Moreover, the sensitivity of the technique should be considered, as the concentration of ALP in whole cells may be relatively low, requiring careful optimization to achieve reliable results. Additionally, the use of complementary techniques like mass spectrometry [28] can provide more comprehensive information about PTMs in complex biological samples. Studying PTMs of ALP using capillary electrophoresis can provide mechanistic insights into how specific modifications regulate the enzyme's activity, substrate specificity, subcellular localization, and protein–protein interactions.

Capillary electrophoresis enables the identification of protein isoforms with distinct post-translational modifications (PTMs) due to their slightly different migration times, allowing for differentiation. Moreover, the intensity of electropherogram peaks in the capillary electrophoresis reflects the protein's concentration in the sample, enabling researchers to quantify the abundance of specific PTMs and understand the extent of modification. Coupling capillary electrophoresis with mass spectrometry enables comprehensive PTM profiling, facilitating the identification and characterization of specific PTMs on proteins and shedding light on their regulatory roles in various cellular processes. By correlating PTM patterns with specific cellular conditions or disease states, scientists can gain insights into the functional implications of PTMs on protein activity and cellular signaling pathways. **Figure 4** shows detecting post-translational modifications (PTMs) in capillary electrophoresis from whole cells, which involves several steps. After releasing cells' contents in the appropriate buffer, proteins are extracted from the lysate using appropriate solvents. Proteins are then likely to denature and reduce to break down their tertiary and quaternary parts. For targeting PTM-specific enrichment, proteins are modified using different methods, including immunoprecipitation [29] or affinity chromatography [30]. For ALP, PTM-specific detection phosphorylation [29][30][31]

[32][33] or glycosylation [34][35][36] can be used. After injecting the protein mixture, data can be analyzed and compared to the untreated cell and other controls in the test.

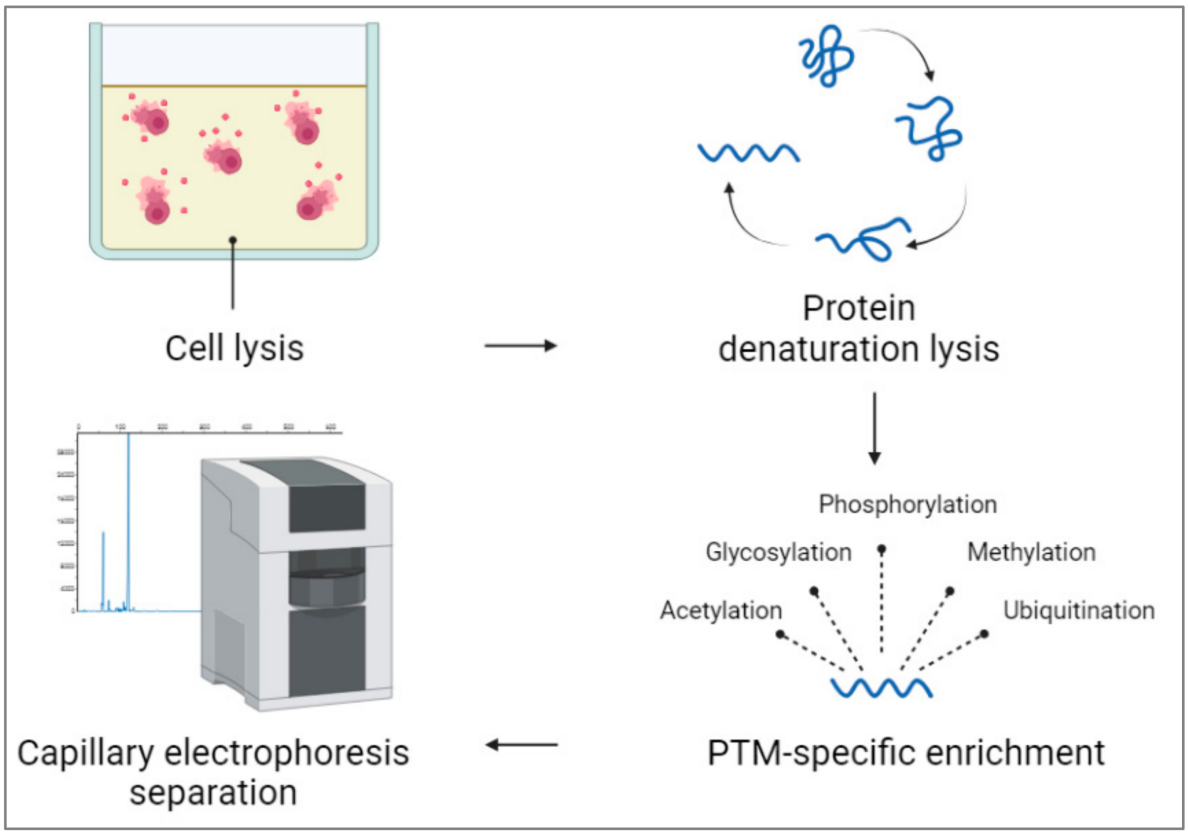

Figure 4. Modified ALP protein for post-translational modifications (PTMs) in capillary electrophoresis from whole cells.

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