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Electrodes having tip diameters in the few hundred nanometer range have been constructed by coating thin copolymer films onto suitable electrode materials that have been etched to very small dimensions. Desorption of the insulation from the exact tip of the structure is a key element in the construction of electrodes of suitable dimensions for neuronal analysis. Preliminary data obtained in collaboration with Yau Yi Lau of our laboratories shows the utility of these insulated, etched carbon fiber electrodes for analysis of neurotransmitter release from single nerve cells.

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DEVELOPMENT OF STRUCTURALLY ULTRASMALL ELECTRODES FOR ELECTROCHEMISTRY AT SINGLE NERVE CELLS

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INTRODUCTION

The molecular interactions that result in neuronal communication have been of interest to scientists for the past several decades. Recently, with the advent of microelectrodes, electrochemical study of neurotransmission, the process by which two neighboring neurons communicate, has become attractive. Furthermore, electrochemical methods can discriminate against the many components of brain fluids that are not easily oxidized. This inherent selectivity offered by electrochemical methods has lead to the employment of both electrochemical detection for separation techniques and direct in vivo electrochemistry (1,2).

HPLC with electrochemical detection has been used to determine easily oxidized components of brain homogenates from various regions of the human brain (3). Open tubular liquid chromatography with electrochemical detection has been used to determine components in homogenates of single whole nerve cells (5-7). Finally, capillary electrophoresis with electrochemical detection has been used to separate and detect easily oxidized species in picoliter volumes of cytoplasm removed from single intact neurons in the snail brain (1,4).

In vivo electrochemistry gains superior spatial resolution of neurotransmitter concentrations by use of ultrasmall electrodes that are inserted directly into neuronal microenvironments (2,8,9). Recently, electrodes small enough to place into single large nerve cells in a pond snail have been used to determine dopamine dynamics on the single cell level (10,11). However, similar analysis of smaller human brain cells has proven difficult due to limitations in the miniaturization of the voltammetric probes used. The goal of the

research presented here is to develop voltammetric probes of suitable dimension for use in single mammalian cells. In addition, an ultimate goal is to develop an electrode small enough that it can be manipulated into a single synapse between two neurons. These goals require electrodes that are sub-micron and sub-100 nm in total tip diameter, respectively. Using the technique described in this paper, electrodes having 500 to 1000 nm total tip diameter can be routinely constructed, and even smaller structures are attainable at a lower success rate.

Various electrode materials (platinum, platinum-iridium, gold, carbon) can be etched down to extremely small sizes (<100nm), but providing insulation around the electroactive tip in order to provide the necessary spatial resolution (and noise characteristics), while still maintaining small dimensions, has proven difficult. In this paper, I have examined the use of a thin poly(oxyphenylene) film for this insulation to provide total structural diameters in the sub-micron range. Historically, these types of polymer films have been electrochemically polymerized onto metal surfaces for protection from corrosion (12). More recently, poly(oxyphenylene) films have been deposited onto electrodes to provide capacitive and even passivating films on the electrode surface (13,14). Mengoli, et al. (12,15-17) have shown that insulating films can be applied by polymerization and curing of 2-allylphenol. Potje-Kalmoth, et al. (14) have used this method to coat carbon fibers with insulating films that are several microns thick surrounding the fiber. McCarley et al. (13) have developed a similar technique by polymerizing phenol to

modify electrode surfaces with an extremely thin coating of polymer film, which provides a molecular sieving effect.

By copolymerizing 2-allylphenol and phenol, I have attained thin polymer films, which are electrically insulating. By coating thin copolymer films onto suitable electrode materials that have been etched to very small dimensions, electrodes having tip diameters in the few hundred nanometer range have been constructed. Desorption of the insulation from the exact tip of the structure is a key element in the construction of electrodes of suitable dimensions for neuronal analysis. Preliminary data obtained in collaboration with Yau Yi Lau of our laboratories shows the utility of these insulated, etched carbon fiber electrodes for analysis of neurotransmitter release from single nerve cells.

EXPERIMENTAL

Electrode Construction. A schematic of our electrode is shown in Figure 1. Electrodes were constructed by aspirating 11 μm diameter carbon fibers (Thornel P-55S, 4K pitch based carbon fiber, Amoco Performance Products, Inc.) into standard glass capillaries (A-M Systems, Inc.), pulling the capillary to a tip with a glass capillary puller (Harvard Bioscience), and cutting the exposed fiber to obtain pulled glass structures with protruding carbon fibers. The capillaries were then filled with gallium (Aldrich Chemical Co.) and a nichrome wire was inserted for electrical contact. For each electrode, the protruding fiber was etched to the desired dimensions

(100nm to 5 μm diam.) by placing it in a hot methane-oxygen torch. The electrodes were coated with a copolymer of phenol and 2-allylphenol by potentiostating at +4.0 volts versus a platinum counter/reference electrode with a Bioanalytical Systems CV-1A potentiostat. The coating solution consisted of 0.075 M phenol (liquefied 90% in carbonic acid, Aldrich chemical Co), 0.105 M 2-allylphenol (98%, Aldrich Chemical Co.) and $1x10^{-5}$ M ammonium hydroxide, all in a solvent system of 0.190 M Butylcellusolve (2-butoxyethanol 99+%, Aldrich Chemical Co.) in 1:1 methanol/water. As many as six electrodes were coated simultaneously by connecting their leads together in parallel. Polymer curing was accomplished by heating the structures to 150°C for 30 min. All chemicals were used as received without further purification.

In vitro Voltammetry. In vitro voltammetry was performed with a locally constructed low-noise, 3-electrode potentiostat and a Hewlett Packard x-y recorder. All in vitro voltammetry presented in this paper was performed using 1.0×10^{-4} M solutions of dopamine or 4-methyl catechol (Sigma Chemical Co.), a neutral analog of dopamine, in a citrate/phosphate buffer solution (pH 7.4).

Laser Activation of Electrodes. The apparatus used for laser treatment of the microelectrodes employed a VSL-337ND pulsed nitrogen laser obtained from Laser Science Incorporated. All activation procedures were carried out at a laser frequency of 20 Hz and each pulse had a duration of 3 ns. Electrodes were held at -0.200 volts vs SSCE during laser irradiation, which consisted of 150 to 200 pulses. The laser beam was reflected through a prism and focused with a 150 mm focal length lens before passing though the bottom of the

electrochemical cell to strike the electrode. All optics were constructed of fused silica and were obtained from the Newport Corporation. The laser spot size was approximately 100 μm in diameter and had a peak pulse power of 280 MW/cm^2.

In vivo Electrochemistry. Planorbis corneus were obtained from NASCO (Fort Atkinson, WI) and were maintained in aquaria at room temperature until used. The snails were pinned in a wax-filled petri dish and dissected under snail Ringer's solution to reveal the left and right pedal ganglia. A micromanipulator (de Fonbrune, Curtin Matheson) was used to place the working electrode on the cell wall of the identified dopamine neuron (18). Electrochemical experiments performed *in vivo* employed an Ensman Instruments EI 400 potentiostat controlled by an IBM personal computer, which also served for data acquisition. Local bathing of the outside of the neuron with 3.0 M KCl was achieved by pressure injection through a glass micropipet. The injector consisted of standard capillary glass (A-M Systems, Inc.) pulled down to small diameters with a microelectrode puller (Harvard Bioscience) and cleaved with a scalpel to approximately 100 μm tip diameter. The tip of the injector was then manipulated (Prior manipulator, Medical Systems) to a distance of approximately 50 μm from the outside of the cell body.

RESULTS AND DISCUSSION

The amperometric response during anodic polymer deposition onto a group of six etched carbon fibers is shown in Figure 2. This response is typical for this type of polymerization (12,14-17) with the magnitude of current passed being proportional to the exposed surface area of the electrode(s). The procedure used to insulate the electrode with polymer is sensitive to monomer concentration, pH of the monomer solution, and the time of deposition. First, relative monomer concentrations greatly influence the characteristics of the polymer film that is formed. At least a 50% ratio of the 2-allylphenol to phenol is required to insure insulating property of the polymer film. Higher phenol content results in increased charging currents and peak shaped voltammetry, indicating that the polymer film is not insulating, and the diffusion of the electroactive species is considerably slower inside the polymer film. Conversely, higher 2-allylphenol content results in strong insulating films of considerable thickness (>2 μm). Copolymerization of these monomers in the proper proportions results in a thin (100-500 nm), insulating polymer film. The use of high concentrations of both of the monomers results in thicker films formed on the electrode surface in order to insure insulation. Although the films are not always uniformly deposited, there is an obvious correlation with film thickness and deposition time. Concentrations of 0.105 M 2-allylphenol and 0.075 M phenol provide insulating films while maintaining reasonable deposition times. Secondly, this procedure is extremely sensitive to pH. A target pH of about 9.0 is ideal for the thinnest films. Higher

pH results in rampant polymerization and thick films, which are insulating, but are too thick for our end use. Finally, the time of deposition is a key element in obtaining a thin, as well as insulating polymer layer. Electropolymerization at 4.0 V for at least 8 minutes is typically required to insure the deposition of an insulating film. Longer times of deposition insure insulating films, but produce somewhat thicker films. A scanning electron micrograph of an 11 μm carbon fiber with an insulating copolymer film is shown in Figure 3. In this case the electrode was held at +4.0 V for 16 minutes, which resulted in a polymer thickness of about 300 nm. The electrode tip has been cleaved with a scalpel to expose the tip of the carbon fiber.

Smaller structures have been obtained by applying this type of film to etched carbon fibers. There are several ways to etch carbon fibers to small dimensions, but the simplest way (and most effective way in our laboratory) is exposure to a methane/oxygen flame. A scanning electron micrograph of a carbon fiber that has been "flame etched" in this manner is shown in Figure 4. The large bar represents 1 μm, so the extreme tip of the fiber is about 100 nm in diameter. Application of a thin, insulating polymer film onto these small carbon fibers should allow electrodes having extremely small total tip diameter to be constructed. A scanning electron micrograph of a fire etched carbon fiber, which has been anodically polymer coated for 8 min., is shown in Figure 5. The large scale bar represents 1 μm, so the total structural diameter of this electrode is just under 2 μm, and the polymer thickness appears to be about 100 nm. Although considerably smaller electrodes have been constructed, they have

proven too fragile for the mounting and handling procedures prior to SEM imaging.

After coating the etched carbon fiber with insulating polymer, desorption of this material from the exact tip of the electrode is necessary to obtain a small electroactive area. Initially, polymer removal was accomplished by simply cleaving the electrode tip with a scalpel; however, that requires extreme care on the part of the experimentalist to remove as little of the tip as possible while preserving the small tip size. Additional methods of polymer desorption have included lowering the tip onto a glass plate to physically displace the polymer off of the tip, and the use of a field emission arc originating at the electrode tip. The latter method provides the most consistent results as well as the smallest final structures. Field emission removal of the insulating polymer is accomplished by micromanipulating the electrode tip very close to a platinum plate and applying a potential between the electrode and the plate. An arc of current passes between the electrode tip and the platinum anode, resulting in desorption of the polymer from the extreme tip of the electrode structure. A schematic of this process is shown in Figure 6. The entire apparatus is placed beneath a stereomicroscope to aid in proper electrode placement. The voltage applied is varied with polymer composition and thickness, but is typically between 1 and 10 V for the smaller electrodes. Voltammetric responses of several relatively large electrodes (4-8 μm) with the polymer removed by each of the three methods discussed above are shown in Figure 7. In each of these cases, the voltammetric response to $1x10^{-4}$ M dopamine is reasonably well-behaved. It should be noted that

many electrodes constructed by these procedures do not display well-behaved voltammetry and, hence, cannot be directly used for analytical experiments. These electrodes can, however, be activated by high energy laser pulses focused onto the carbon surface (19,20). The voltammetric response of one electrode before and after laser irradiation is shown in Figure 8. Laser pulses from a 337 nm nitrogen laser focused down to a small spot size (ca 100μm) provide adequate power density for the activation process at the carbon electrode.

Placement of a well-behaved voltammetric electrode into biological media can be accomplished by use of micromanipulators and a microscope. Information can be obtained regarding the dopamine concentration and dynamics with the use of these ultrasmall electrodes.

The amperometric response of an electrode that was placed on the exterior of the cell membrane of the dopamine neuron of Planorbis corneus (pond snail) is shown in Figure 9. The electrode was held at +0.8 V vs a SSCE reference electrode. The dopamine neuron was stimulated by bathing with 100 μL of 3 M KCl solution at 90 sec., and the corresponding apparent release of dopamine was monitored. Since the cell in question is known to contain large amounts of dopamine (18), the electrochemical response observed in Figure 8 is attributed to dopamine. However, we have not yet obtained conclusive evidence for this. The peak amperometric response is 264 pA, which corresponds to approximately 10^{-5} M dopamine based on a precalibration experiment. In addition, dopamine dynamics can be examined using this type of experiment. The initial decline in Figure 8 seems to indicate that the KCl is diffusing away from the cell and less dopamine release is

observed. The later, more steep decline in the amperometric signal could be attributed to the depletion of dopamine containing vesicles at the inner walls of the cell, resulting in the sudden halt in dopamine release. Further experiments on this system promise to provide details of dopamine release and reuptake dynamics in this system. In addition to studies of the dopamine system of Planorbis corneus, experiments of this nature using still smaller electrodes are in progress for the analysis of cultured human neurons. Furthermore, ongoing experiments involve the placement of an extremely small carbon electrode into a synaptic cleft between two neurons to study the dynamics of neurotransmission.

By constructing and characterizing electrodes in the sub-micron regime, analysis of neurotransmitter concentrations in microenvironments previously unobtainable is now possible. Analysis of these microenvironments promises to add new and exiting data to the frontier of neuroanalytical chemistry.

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FIGURE LEGENDS

Figure 1. Schematic of an etched carbon fiber, poly(oxyphenylene) coated electrode. W, nichrome wire; GA,Gallium; C,Carbon Fiber; P,Poly(oxyphenylene) coating.

Figure 2. Typical amperometric response for anodic polymer deposition onto the carbon fibers obtained during the deposition of poly(oxyphenylene) onto six etched carbon fibers simultaneously.

Figure 3. Scanning electron micrographs of the tip of an electrode constructed with an unetched 11 μm carbon fiber which was anodically polymer coated with poly(oxyphenylene)for 16 min. Image B is a close-up of image A. The large scale bar in each case represents 1 μm.

Figure 4. Scanning electron micrograph of a carbon fiber which has been etched in a hot methane/oxygen flame. The large scale bar represents 1 μm.

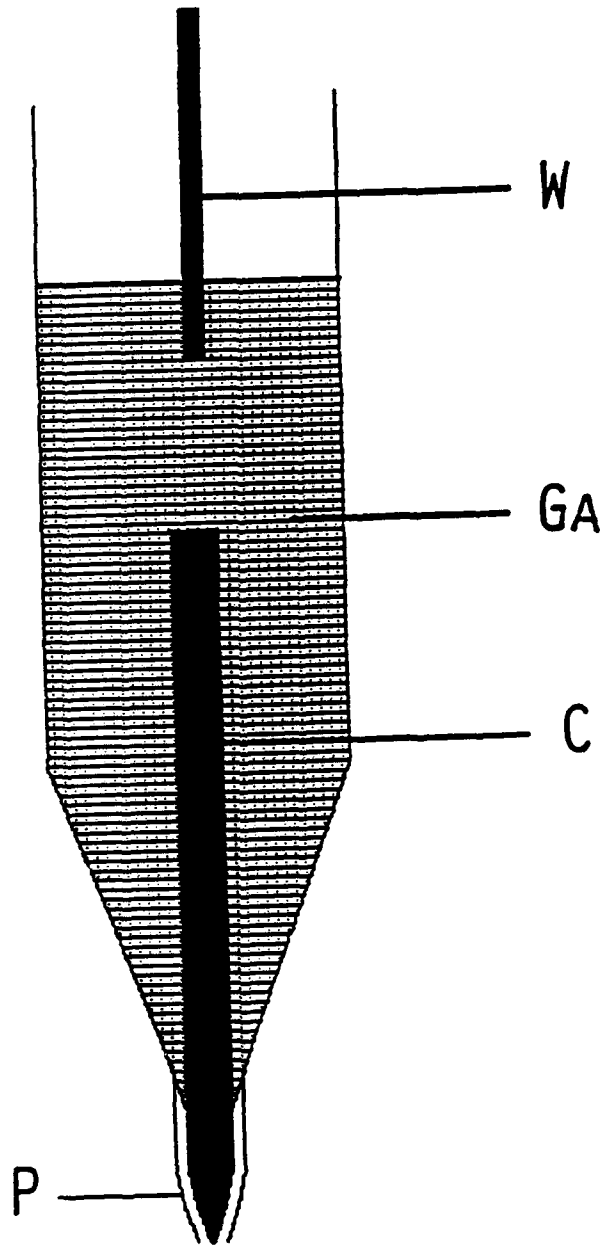
Figure 5. Scanning electron micrograph of a fire etched carbon fiber which has been anodically polymer coated with poly(oxyphenylene) for 8 min. The large scale bar represents 1 μm.

Figure 6. Schematic of the field emission arc method of polymer desorption from to electrode tip.

Figure 7. Oxidation of 1.0 x 10^{-4} M dopamine at flame etched carbon fiber electrodes with polymer desorbed from the electrode tip by A) scalpel cleavage, B) hitting electrode on a glass plate, and C) field emission arcing.

Figure 8. Example of electrode activation by 337-nm laser pulses focused to high power density (280 MW/cm^2). Voltammetry of 1.0 x 10^{-4} M 4-methyl catechol at A) electrode with polymer desorbed by field arcing B) same electrode after 7 s. of pulsed laser treatment.

Figure 9. Amperometric response of a 5 μm diameter electrode placed on the exterior wall of the giant dopamine neuron of Planorbis corneus. The cell was bathed with 100 μL of 3 M KCl at 90 s. The working electrode was held at +0.8 V vs SSCE.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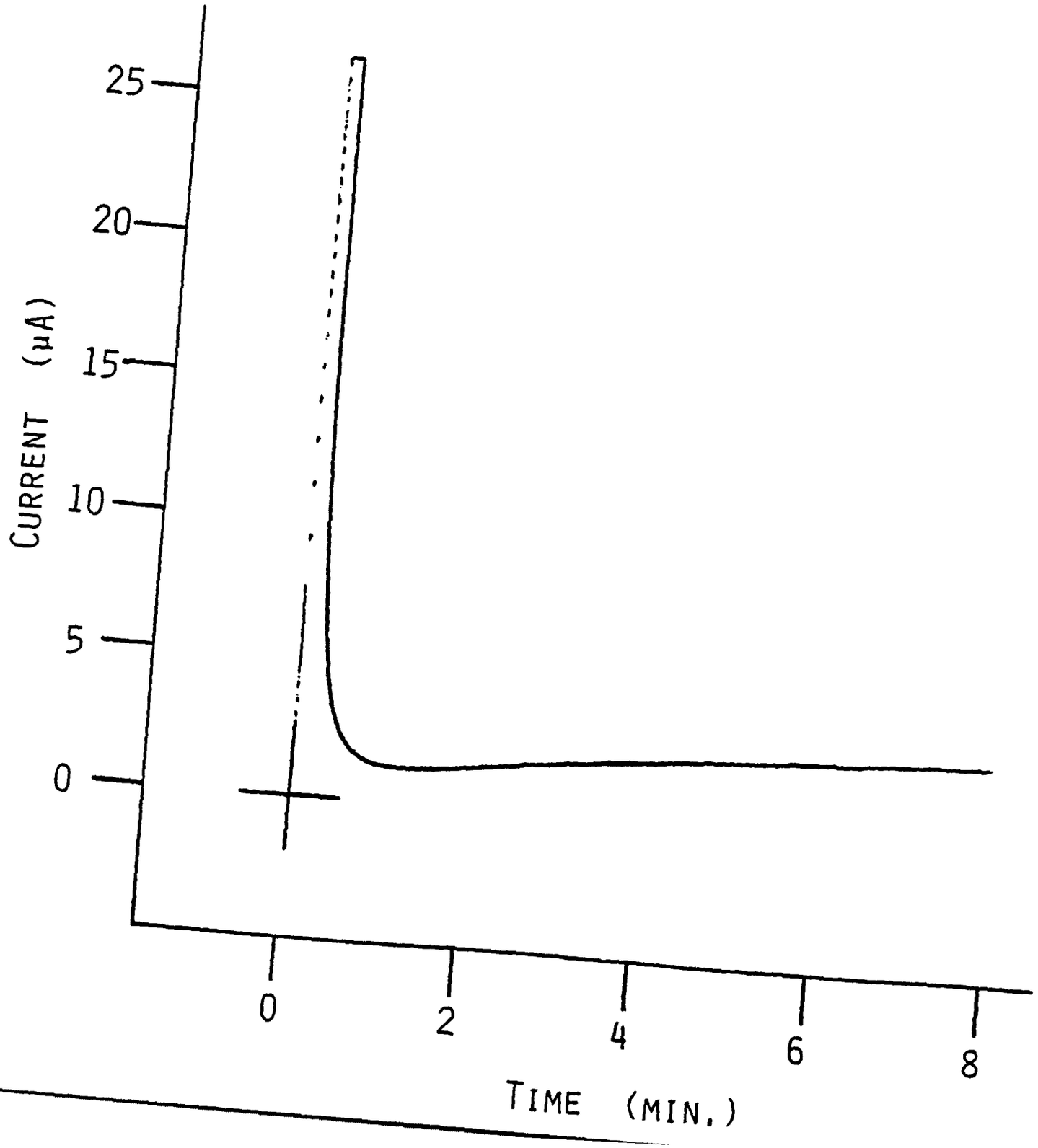
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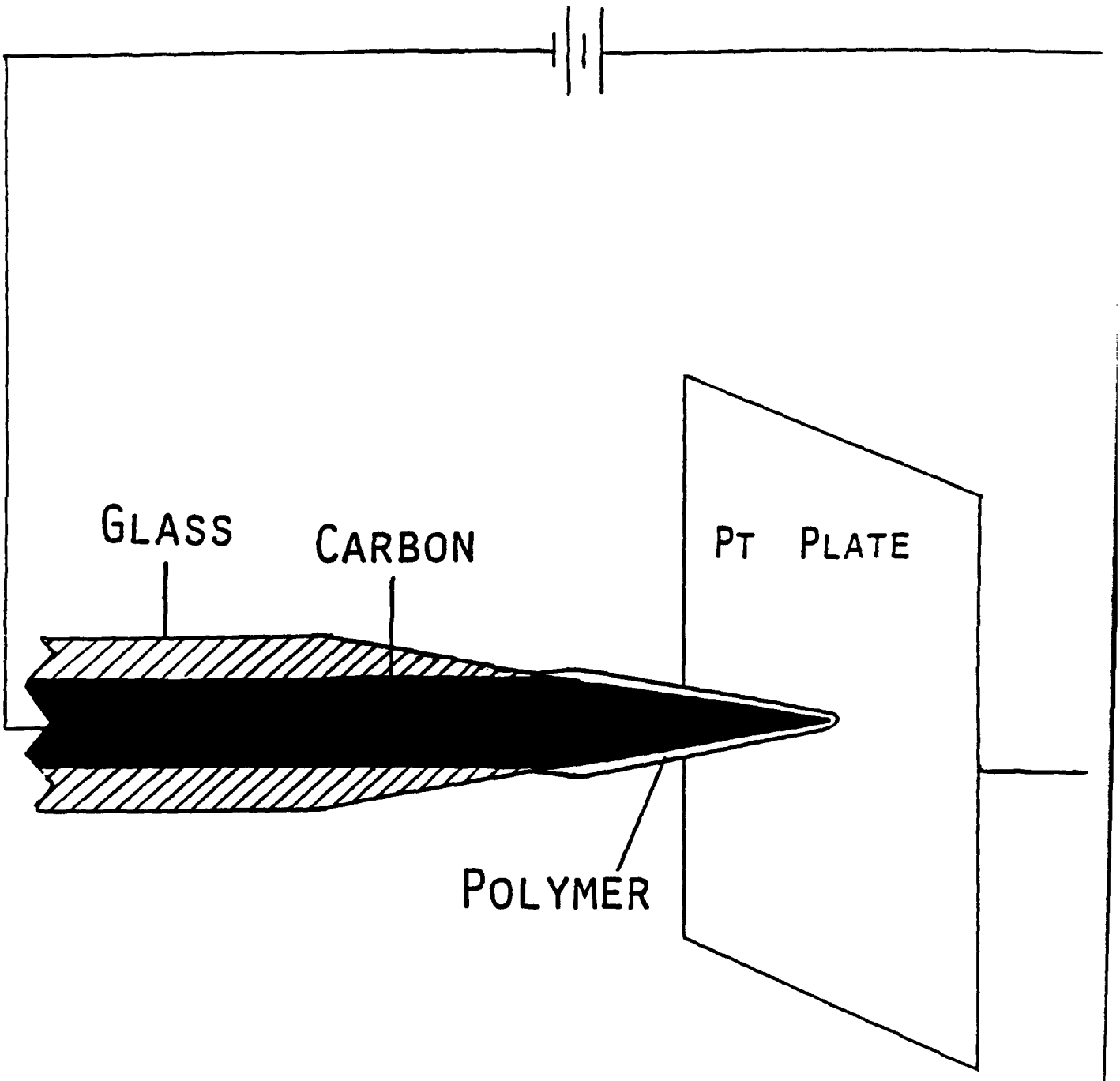
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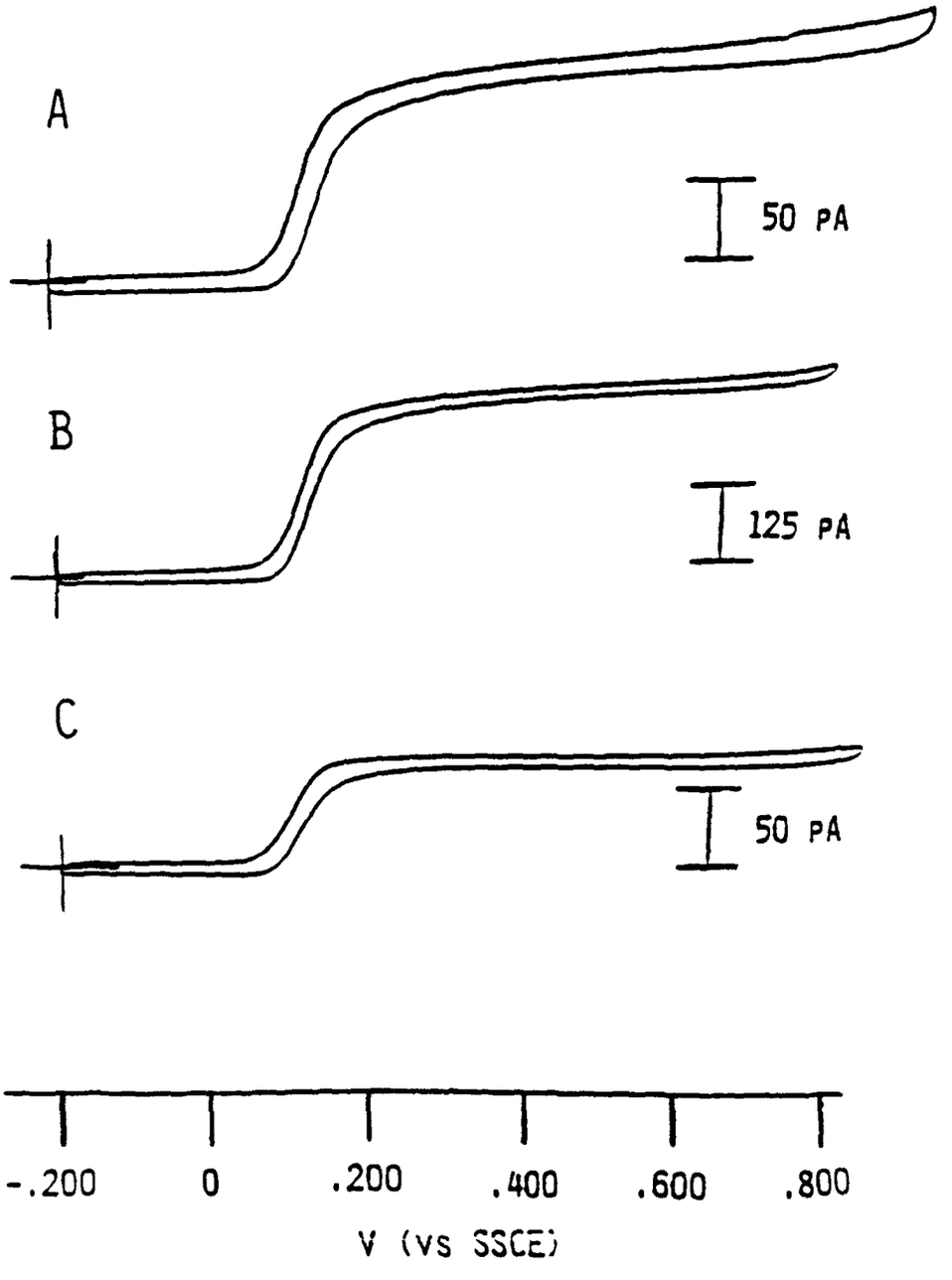
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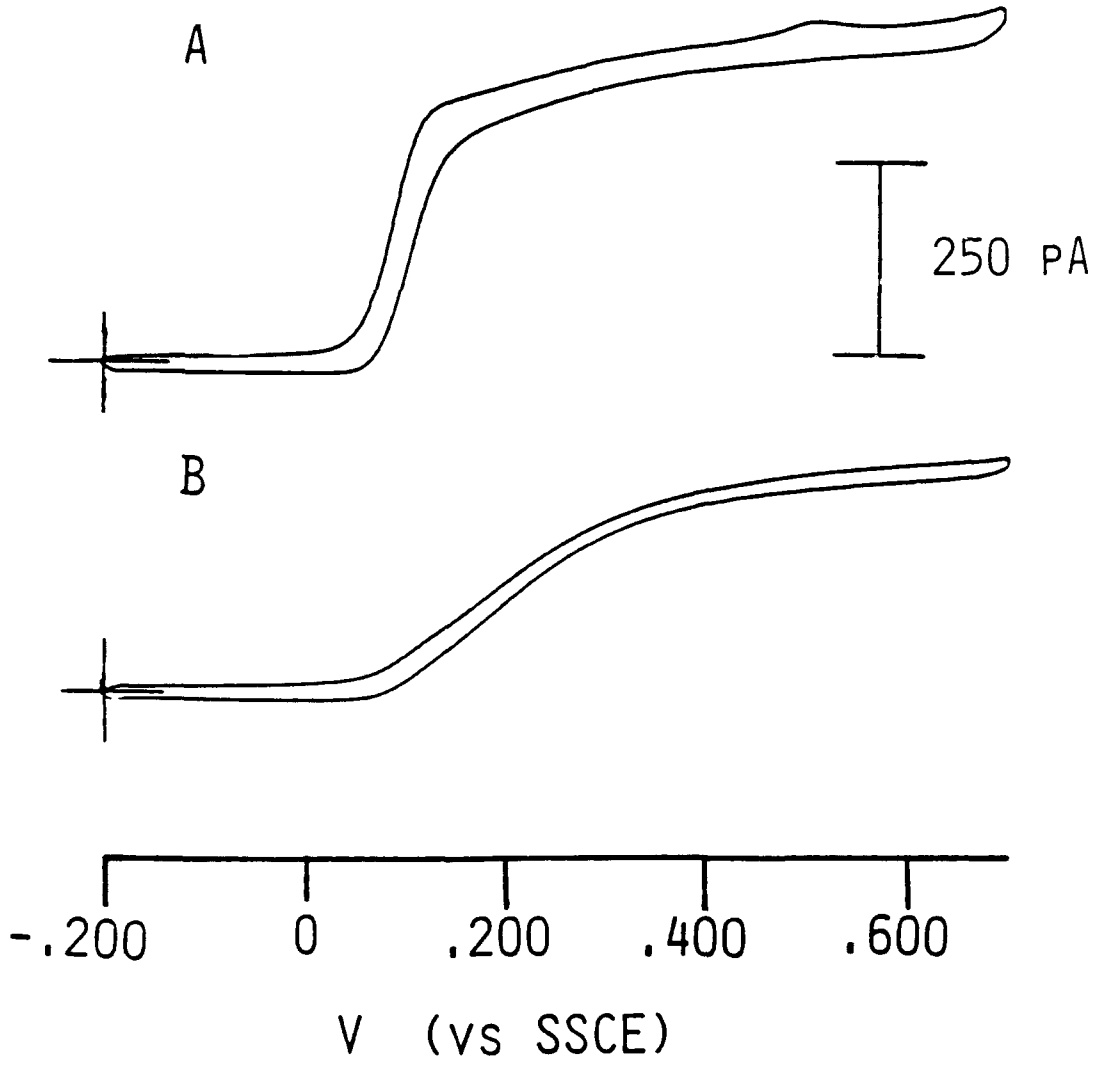
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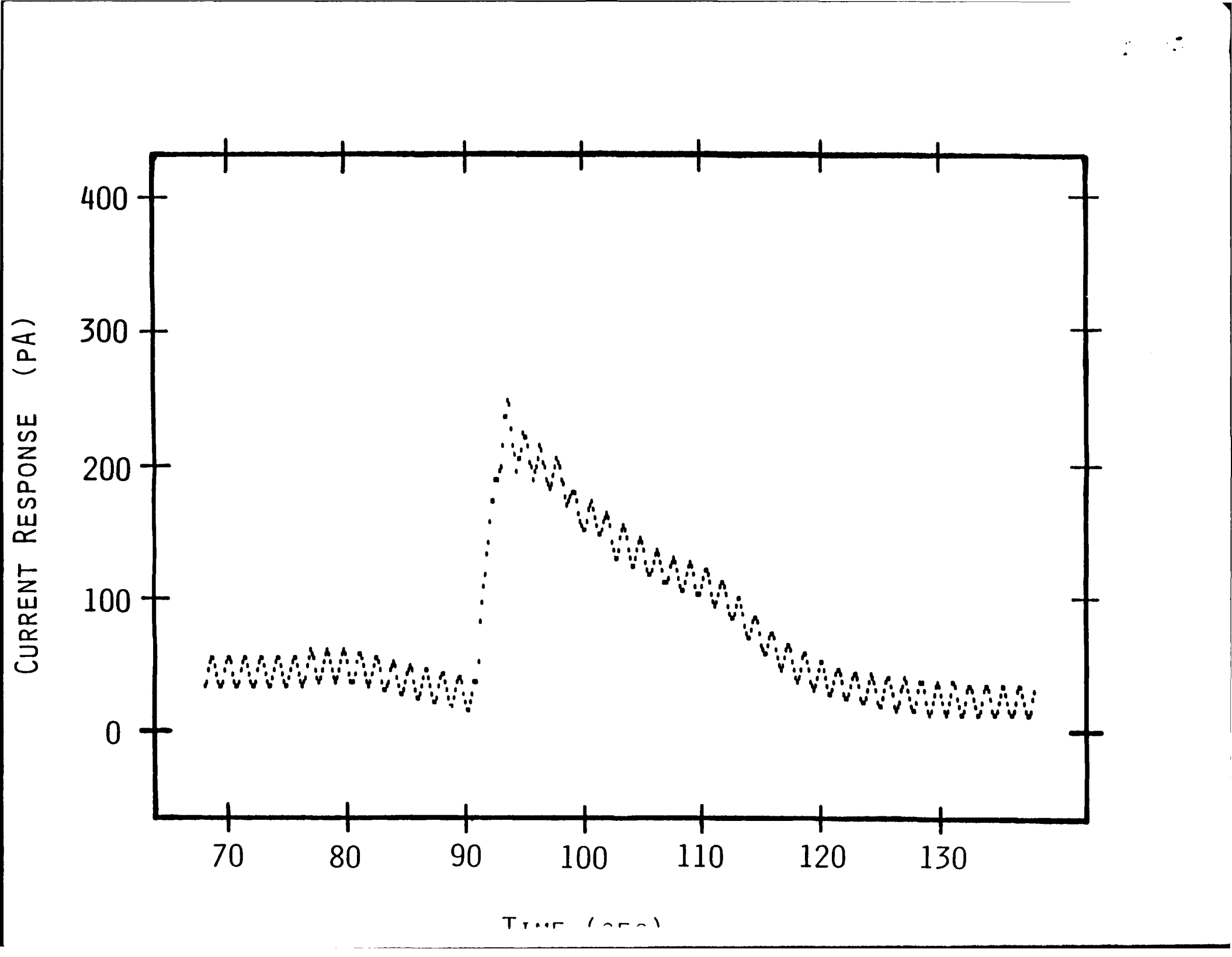
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