Spring 5-2016

Using Intrinsic Properties of Polyaniline to Sense Expression of the microRNA Let-7

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Using Intrinsic Properties of Polyaniline to Sense Expression of the microRNA Let-7

by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2016

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Abstract

MicroRNAs are approximately 22-nucleotide long RNA molecules that function through decay and translational repression of messenger RNA. The microRNA let-7 is found to play a role in maintaining the fate of differentiated cells in humans. Thus, expression level of this microRNA is a reliable biomarker of tumor cell phenotype. However, there are significant limitations in the current profiling techniques of microRNA. The current methods like northern blotting, microarrays, RT-PCR, or using locked nucleic acid (LNA) for in-situ hybridization are either laborious, semi-quantitative, or expensive. In this research we try to address this issue by developing a fast, specific, and inexpensive method. This method utilizes the intrinsic properties of the conducting polymer polyaniline (PANI) as a sensing platform to detect specific target miRNAs.

Key Terms: MicroRNA, miRNA, Polyaniline, PANI

Acknowledgements

I would like to acknowledge and thank Dr. Alex Flynt, Dr. Partha Sengupta, and the other members of the Flynt Laboratory. Without Dr. Flynt allowing me to begin work in his lab, things would be immensely different for me now. He has helped guide me through this project and helped me achieve many things. Partha Sengupta has not only been a mentor, but also a great friend. Without his direct involvement and guidance on this work, I would be nowhere. Partha has taught me many things in science as well as in life. To the rest of the Flynt Laboratory I owe thanks for helping me adjust to this new realm and making the dull times fun.

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List of Abbreviations

PANI	Polyaniline
miRNA	micro – Ribonucleic Acid
UV	Ultraviolet
FTIR	Fourier-Transform Infrared Spectroscopy
RT-PCR	Real time-Polymerase Chain Reaction
DBSA	Dodecylbenzenesulphonic Acid
APS	Ammonium persulfate
ssDNAc	Single strand DNA complement
mDNA	Mimic DNA

Chapter I: Introduction

MicroRNAs (miRNAs) are a large group of ~22 nucleotide RNA molecules that regulate the expression of genes. Dysregulation of miRNA expression has been implicated in many diseases including, cancer (Ravo 2015); (Siegfried 2014); (Serguienko 2015); (Zhu 2015). Down-regulation of suppressor miRNAs and up-regulation of oncomiRs (miRNA associated with cancer) is linked to initiation of proliferation, invasion, angiogenesis, and metastasis of tumors (Kolenda 2014). miRNAs can be quantified and used as biomarkers for pathogenesis of disease. This detection of the changes in expression of miRNAs can be used as a test for the early diagnoses of these diseases.

The goal of this research is to create a conducting polymer based biosensor for the detection of miRNAs. Conducting polymers are organic polymers that have unique electronic properties that resemble metals. Changes in the electronic structure of conducting polymers, as when bonded to a target molecule, are shown in the altered electrical and optical properties which can be measured. This allows for the detection of the presence, or absence, of the target molecule. This biosensor uses the conducting organic polymer, polyaniline (PANI), to detect the miRNA let-7. The basis of our approach uses UV-enhanced electrostatic bonding between a probe single-strand DNA complement (ssDNAc) let-7 to the polyaniline. After hybridizing let-7 to the ssDNAc, changes in PANI fluorescence, as well as changes in the electrical properties can be used to measure expression of the complementary miRNA.

This work is significant because it provides a platform for the detection of expression of miRNAs that could lead to the early detection of many life-threatening diseases. As medicine becomes more focused on the molecular scale, new technologies will be important for real world, clinical applications. By fine tuning the biosensor so that it may detect a low threshold of miRNA expression, in the future we could have a sensor with many different probes that could test for a plethora of miRNAs and other molecule levels that could show the pathogenesis of disease and help guide physician actions to the most effective treatment regimen.

Chapter II: Literature Review

The detection and characterization of expression of microRNAs (miRNAs) in tissue samples are proving to be a new and exciting method for detecting tumor cell phenotype. miRNA dysregulation is also being implicated in the etiology of many other diseases (Li 2015). miRNAs are ~22 nucleotide long RNAs that play critically important roles in gene regulation by targeting messenger RNAs (mRNAs) for destruction and repression (Bartel 2004). miRNAs have a wide variety of expression patterns, but it all begins with the transcription of the primary miRNA (pri-miRNA). The pri-miRNA transcript is cleaved by Drosha, an RNase III endonuclease, freeing a ~60-70 nucleotide stem loop. This is the miRNA precursor (pre-miRNA). Dicer, another RNase III enzyme, then cleaves the loop off the pre-miRNA, and further prepares the miRNA complex for full maturation. After this, one strand of the duplex becomes associated with the RNA-induced silencing complex (RISC). The RISC mediates matching the miRNA's code to a complementary mRNA. The RISC then silences its target mRNA, thus fulfilling the

action of the miRNA for controlling genetic expression (Bartel 2004). By regulating the mRNA transcript, miRNAs control translation of proteins.

Let-7 miRNA family plays a critical role in regulation of development and carcinogenesis (Kolenda 2014). Let-7 is an abundant miRNA and highly conserved in animals. The miRNAs of the let-7 family act as tumor suppressors and regulate the expression of many oncogenes (Kolenda 2014). Let-7 expression is found to be dysregulated and extremely low in the more aggressive types of cancer (Serguienko 2015). Let-7 expression plays a crucial role in tissue maintenance, and dysfunction could lead to cancer.

The members of the let-7 family are connected with many features of cancer and could be applied as diagnostic, predictive, and prognostic biomarkers (Kolenda 2014). Current methods like Northern blotting, microarrays, RT-PCR, high throughput sequencing, or using locked nucleic acid for in-situ hybridization are either laborious, semi-quantitative, or expensive. While there have been several reports of conducting polymer based sensing technology of nucleic acids, most are based on covalent immobilization of biomolecules or sensing by fluorescent moieties attached to probes (Deng 2014). Our research work is based on electrostatic immobilization of probes that alter the intrinsic properties of the conducting polymer polyaniline without use of expensive labeling agents. We use mild UV irradiation to augment electrostatic bonding of probe oligonucleotides complementary to let-7. This results in measurable changes in the properties of polyaniline measured by FTIR, UV, and fluorescence. Hybridization of oligonucleotides identical to let-7 or RNA samples from animals expressing let-7 results in a reversal in the changes associated with binding probes to polyaniline. We

demonstrate that this is due to release of probes from the conducting polymer after hybridization. This phenomenon serves as the basis of our miRNA detection platform.

Chapter III: Methodology

This research will use the fluorescent and electrochemical properties of the conducting polymer, polyaniline (PANI), to detect the hybridization between miRNAs and probe ssDNAc (single-strand complementary DNA) oligonucleotides.

The first step was to synthesize PANI through emulsion polymerization as shown in Figure 1. 11 mmol of aniline was completely dissolved in 60 mL of chloroform within a 250 mL round bottom flask and the solution was stirred in a magnetic stirrer at 600 rpm. 7.44g of DBSA (dodecylbenzenesulphonic acid) was added into the aniline solution and stirred vigorously at 0-5° C. 3.072g of APS (ammonium persulfate) was dissolved in 20 mL RNase/DNase free water in a beaker and added drop by drop into the reaction mixture for 30 min. The reaction mixture was stirred at 0-5° C for 24 hours followed by stirring at room temperature for 24 hours. The reaction mixture initially turned to a milky solution and then to dark brown and finally into a dark green colored PANI/DBSA dispersion in chloroform-water mixture. The resultant PANI/DBSA solution was filtered in a Buchner funnel and then extracted with 80 mL chloroform and 120 mL of RNase/DNase free water. The dark green, higher density PANI was collected from separating funnel and unreacted DBSA and APS was left in supernatant aqueous solution.

The ssDNAc (single strand DNA complement) to miRNA let-7 was then prepared in a solution. The ssDNAc vial was centrifuged for 30 seconds at 10,500 rpm to un-compact the powder dNTPs (nucleotide). RNase water was added to create a 100 μMol solution. It was centrifuged for 30 seconds at 4,500 rpm to ensure mixture.

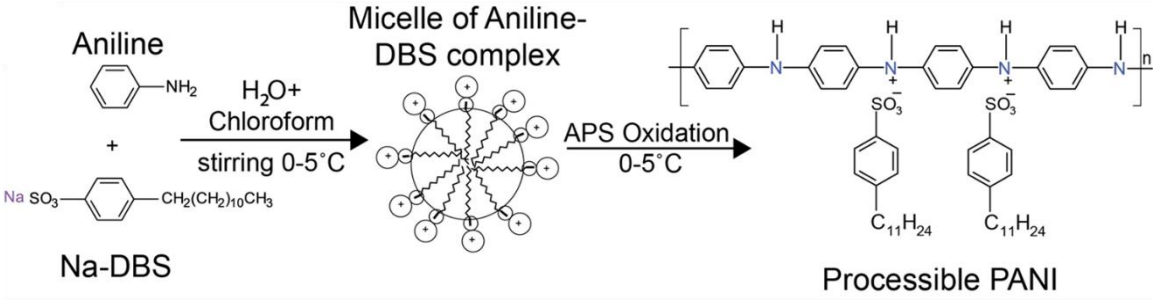

Figure 1. Molecular structural formula for the micellar-aided polymerization of Polyaniline

The synthesized PANI was then characterized. Many dilutions were created of the PANI mixture and pipetted into a microplate to have its fluorescence excitation and emission elucidated through spectroscopy using the SpectraMax M3 microplate reader. The characterization of the PANI/ssDNAc electrostatically bonded mixture was then performed. The PANI mixture was diluted by mixing 1 mL of it with 9 mL of RNase/DNase free water. 8 µL of the ssDNAc mixture was pipetted into a single vial. Into this vial 72 µl of RNase/DNase free water was also added. From this vial 8 µL of the new mixture was removed and pipetted it into another vial and 72 µL of water was added again. These dilutions were continued until there were 9 different levels of dilutions. 200 µL of the PANI solution was added into each of the vials. The new PANI/ssDNAc mixture vials were slow mixed for 15 minutes. After the time elapsed, the vials were exposed to 1 µJ/cm² UV light for 1200 ms. This optimal UV exposure time of the PANI mixtures was determined by irradiating at an intensity of 100 µJ/cm² for various time intervals as shown in Figure 2. This specific time of exposure resulted in the greatest fluorescence emission of the PANI.

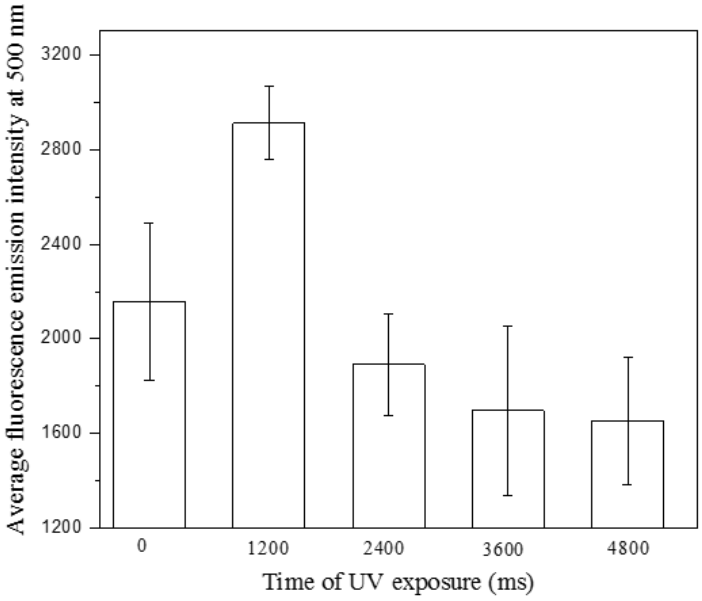

Figure 2. Optimal UV exposure for greatest fluorescent emission intensity

UV irradiation was also used to enhance the association of probe oligos to PANI. Exposure of polymer surfaces to UV can create polar species and localized charge units increasing wettability (Kadashchuk 2007). UV treatment of PANI and probe DNA solution should make the π-π* bond in PANI more labile, potentially enhancing electrostatic bonds that could form with the negative phosphate groups of probe DNA

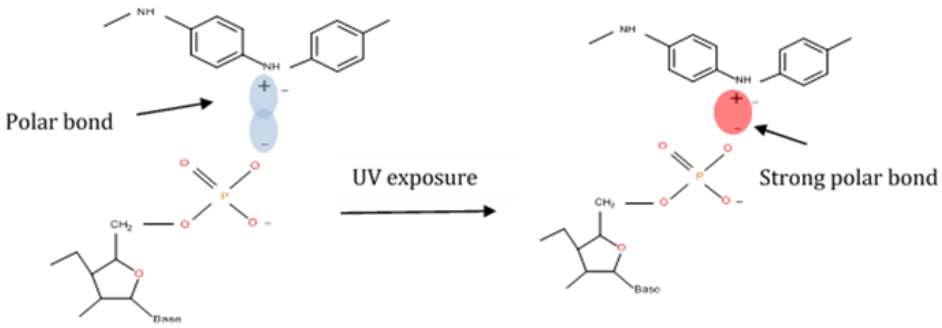

Figure 3. UV exposure greatly enhances the electrostatic bond oligos (Sengupta 2015) (Figure 3).

After the exposure to the UV light, the samples where then microplated and their fluorescent emission and excitation recorded. The PANI/ssDNAc mixture would then be hybridized to the let-7 mimicDNA (let-7 mDNA) and have its electrochemical properties observed in the same manner as the PANI/ssDNAc mixture.

The electrical properties of the pure PANI, PANI/ssDNAc, and PANI/ssDNAc/let-7 mDNA mixtures were then elucidated by coating on interdigitated electrodes and obtaining measurements through cyclic voltammetry. Interdigitated electrodes allow a platform for the electronic properties of the PANI mixtures to be measured. Copper interdigitated electrodes were soldered to two copper wires to allow for measurements. The interdigitated electrodes were then cleaned by being bathed in a RNase/DNase free water and chloroform mixture. The tip of a Fisher Sonic Dismembrator model 300 was then put into the bath and turned to 60,000 RPM. This process was done to every interdigitated electrode for 2 minutes. The interdigitated

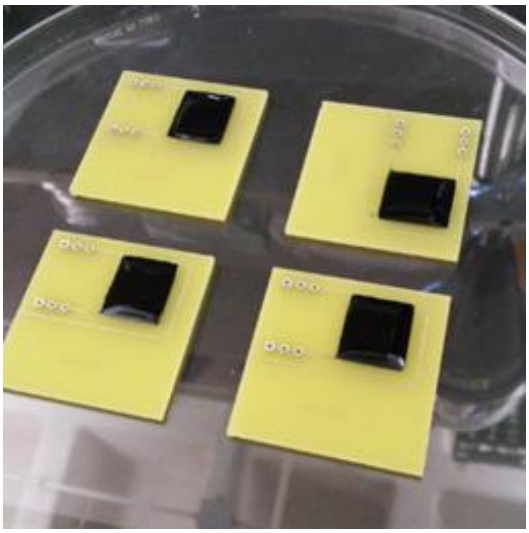

Figure 4. PANI drop-coated interdigitated electrodes

electrodes were then allowed to dry. Next, the interdigitated electrodes were drop coated, as shown in Figure 4, with 20-50 μL of PANI or PANI/ssDNAc and dried in an incubator

at 40° C for 48 hours. The interdigitated electrodes were then individually connected to a Tektronix DMM 4040 6-1/2 Digit Precision Multimeter to obtain the cyclic voltammetric measurements. Cyclic voltammetry is used to plot the current through the electrode versus the applied voltage to the electrode over time. The interdigitated electrodes coated with PANI only had their electrical properties measured. Interdigitated electrodes coated with the PANI/ssDNAc were measured, and then had 8 μL of mDNA added to their surface while the change in the electrical measurements were recorded.

Chapter IV: Results

To create a PANI- based biosensor able to detect nucleic acids, processable PANI must be generated that was dispersed in water so that association with probe oligos and targets may take place. FTIR analysis confirmed successful emulsion polymerization of PANI. Let-7 complementary DNA probe oligos were electrostatically bonded to the dispersed PANI. Let-7 was chosen as a target miRNA because of its clinical relevance. Conducting polymers spontaneously bind to nucleic acids making electrochemical changes detected indistinguishable from erroneous interactions. The problem of distinguishing target nucleic acids in complex biological mixtures was rectified through UV enhancement of the conducting polymer polyaniline. It was previously reported that hybridization caused electrostatically bonded probes to dissociate from PANI and other cationic polymers (Zhang 2011). To test if UV-enhanced bonded probes behave in the same way, hybridization was performed with complementary DNA oligos. After hybridization of complementary oligos, PANI regained its higher doped state, adding credence to the dissociation of the Probe-DNA duplex. To further elucidate that the probes dissociated from the PANI once hybridized, changes in the intensity of the

PANI's fluorescence emission was studied before and after hybridization. The hybridization of complementary oligos lowered PANI fluorescence that was caused by the association of probes with it (Figure 5).

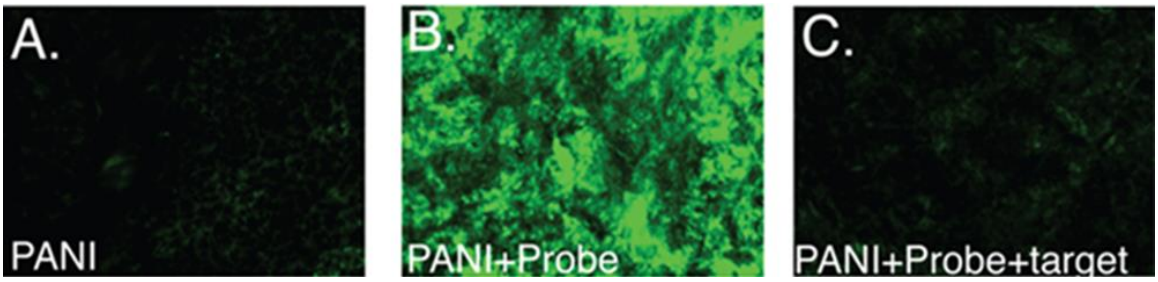

Figure 5. A. PANI Fluorescence emission is low **B**. PANI+Probe produces a significant amount of fluorescence **C**. PANI+Probe+target shows that basal fluorescence was achieved due to dissociation of hybridized duplex

This result was incredibly specific to the sequence of target oligo that was added during hybridization. Were a single mismatch introduced into the oligo, it greatly impaired the restoration of PANI's base fluorescence compared to when the hybridization was performed with a complementary oligo. A UV-enhanced PANI based biosensor shows that it is particularly specific when targeting nucleic acids (Figure 6). This sensor was able to detect a complementary oligo that is present at a concentration around 10 pM (10^{-12}M).

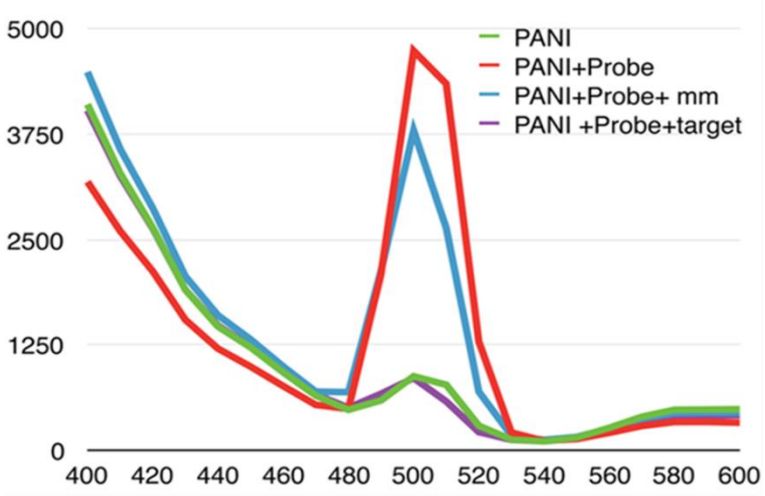

Figure 6. Fluorescence emission at optimum wavelength; PANI and PANI+Probe+target show low levels of fluorescence. The PANI+Probe+mismatch has high fluorescence still, showing the specificity of the sensor

The cyclic voltammetry shows both oxidation and reduction peaks under applied voltage. PANI shows a characteristic oxidation peak at -0.2 V with the reduction peak at 0.2 V in Figure 7. It has been found that the intensity of both oxidation and reduction peaks of PANI get diminished after immobilization with DNA. The reason for this might be because the strong electrostatic bonding between positively charged PANI and negatively charged DNA, facilitated by UV irradiation, traps the electron density at localized pockets and does not allow mobility of electrons along the PANI chain. Increased resistance of the PANI-DNA complex also observes this phenomenon. However when the hybridization takes place with mimic DNA, the driving force behind the conformity of a double helix structure causes detachment of the DNA probe from the PANI molecule causing the recovery to higher intensity of peaks of PANI-Duplex. The PANI-mimic that has one mismatch with the ssDNAc probe does not allow conformity of the helical structure formed during hybridization and detachment of the probe does not

occur, leading to lower intensity oxidation and reduction peaks. This also proves high

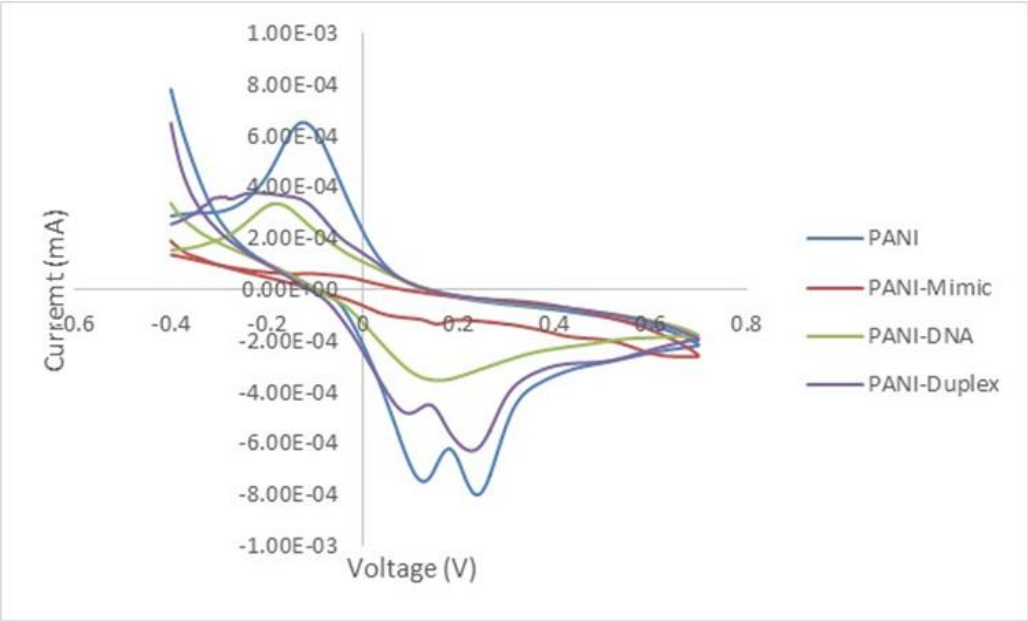

Figure 7. Cyclic Voltammetry. The PANI and PANI-Duplex show oxidation peaks at -0.2V and reduction peaks at 0.2V

specificity of the sensor measurement by the underlying principle of electrostatic DNA probe immobilization to PANI and detachment on hybridization with the help of cyclic voltammetry.

Chapter V: Conclusions

In this research, a cheap, fast, reliable, and specific miRNA biosensor was created. PANI was synthesized through micellar-aided polymerization that was able to be dispersed in water. This allowed the PANI to interact and bind with nucleic acids in solution. UV-exposure was used to enhance the electrostatic bonds between PANI and probe DNA also causing increased fluorescence which is another useful characteristic of this miRNA sensing platform. This biosensor was able to detect the hybridization of oligos to the probes with considerable specificity. The electrical properties of the PANI, PANI/ssDNAc, and PANI/ssDNAc/mDNA were investigated through measurement on interdigitated electrodes using cyclic voltammetry. Unique electrochemical

characteristics were observed that showed miRNA are able to be measured through these properties as well. Finally, this biosensor was exceedingly sensitive, and able to detect amounts of oligo lower than other comparable methods.

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