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Capillary electrophoresis-based immunoassay for insulin antibodies with near-infrared laser induced fluorescence detection

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Abstract

A noncompetitive capillary electrophoresis (CE)-based immunoassay with near-infrared laser induced fluorescence detection (NIR-LIF) for insulin antibodies has been developed. In the assay, insulin was derivatized with a NIR fluorescent dye (NN382; LI-COR). Insulin antibodies were detected via the formation of an immunocomplex. Parameters affecting the separation such as pH, voltage and ionic strength were investigated. Furthermore, it was found that increasing the ramp time of the applied voltage improved the detection limit of the assay by an order of magnitude. The detection limit of the assay was 1.1 n*M*.

Keywords: Insulin antibodies

1. Introduction

Immunoassays are highly selective analytical techniques due to the specific reaction between an antigen and antibody. Immunoassays are seeing more applications in pharmaceuticals, clinical, and environmental chemistry. Attractive attributes of immunoassays include high selectivity and low detection limits [1]. The high selectivity of immunoassays allows one to measure trace amounts of the compound of interest in the presence of structurally or chemically related compounds. Consequently, immunoassays are useful for the analysis of samples in complex matrices. Fluorescence, chemiluminescence, enzyme amplification and radioactivity are common labeling methods used with immunoassays [2]. Immunoassays do have disadvantages; they are often tedious and slow [3,4]. Furthermore, immunoassays suffer from poor reproducibility due to difficulties in attaching the reagent to the support surface [2].

Capillary electrophoresis (CE)-based immunoassays have recently been used as a viable alternative to conventional immunoassays [5–13]. CEIA (capillary electrophoresis immunoassay) has the advantages of minimal reagent consumption, rapid separations, automation and the ability to simultaneously determine the presence of multiple analytes [2]. The use of laser induced fluorescence (LIF) detection allows for high sensitivity. CEIA has been applied to a wide range of compounds including hGH [14], thyroxine (T4) [15], digoxine [16], phencyclidine (PCP) [9], morphine [9], methamphetamine [17], and cortisol [8]. CEIA with LIF combines the high

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efficiency of CE, the high selectivity of immunoassays and the high sensitivity of LIF detection.

All CEIA LIF applications thus far have used visible fluorescent dyes as derivatization reagents. The advantages of using NIR dyes as labels in CEIA are numerous. NIR dyes have high molar adsorptivities (ϵ~150 000–200 000) and reasonable quantum yields (ϕ~0.05–0.5). The quantum yields of NIR dyes increase upon complexation due to shielding effects [18,19]. Detection in the NIR region (~670–1000 nm) is characterized by low levels of background interference, since few molecules posses intrinsic fluorescence in the NIR region of the spectrum [20–27]. This makes the NIR region of the spectrum ideal for bioanalytical applications, where autofluorescence in the visible region is a possible source of interference [20]. Scatter noise (Raman and Rayleigh) is reduced in the NIR region due to its dependence on the detection wavelength by $1/\lambda^4$ [20]. NIR-LIF detection allows for the use of solid state excitation and detection components. GaA1As laser diodes may be used for excitation. These laser diodes are inexpensive, compact, have long operating lifetimes, and have low maintenance costs [20]. Detection is accomplished via silicon avalanche photodiode detection (APD). Quantum efficiencies of 80% are typical of APD when used in the NIR. APD has low noise characteristics as well as long operating lifetimes [20].

Kennedy and co-workers have developed CEIA methods using insulin as the analyte; however, all of their work has been done using LIF in the visible region [5,6,28,29]. In this paper we demonstrate the feasibility of using NIR dyes as tracers in the separation of an antigen from its immunocomplex. The use of the NIR dye NN382 (Fig. 1) as a peptide-labeling reagent for angiotensin has previously been reported [20]. In this work, NN382 was used to label insulin. In the assay, monoclonal insulin antibodies were detected as the complex with the NIR dye labeled insulin. Excess labeled insulin is used such that the complex peak is directly proportional to insulin antibody concentration.

2. Experimental

2.1. Chemicals

Sodium phosphate, tris[hydroxymethyl]aminomethane (Tris), potassium sulfate, sodium chloride, potassium chloride, insulin and monoclonal anti-insulin antibodies were purchased from Sigma (St. Louis, MO, USA). The near-infrared dye NN382 was a gift from LI-COR (Lincoln, NE, USA). NN382 is the dye that LI-COR uses in its commercial DNA sequencers. All water used was nanopure grade (Barnstead Model D4751 ultrapure water system). Derivatization buffer was prepared by dissolving the appropriate amount of phosphate buffer into water and adjusting the pH with sodium hydroxide. Run buffer was prepared by dissolving the appropriate

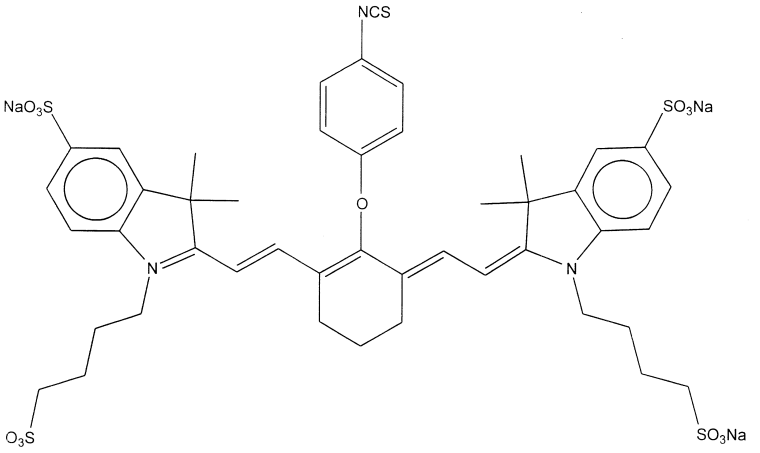

Fig. 1. Structure of NN382.

amounts of sodium phosphate and potassium sulfate into water. The proper pH was achieved by adding the appropriate amount of sodium hydroxide. The final run buffers were filtered through 0.45-μm nylon membrane filters and sonicated for 10 min prior to use.

2.2. Preparation of NN382-labeled insulin

Insulin was labeled with the near-infrared dye NN382 to allow for visualization and quantitation of the immunocomplex. The derivatization procedure consisted of adding 1 mg of insulin and 1 mg of NN382 to 1 ml of 100 m*M* phosphate buffer (pH 11.5). The reaction was mixed for 1 h and then quenched by the addition of 25 ml of Tris buffered saline (pH 8.3): 100 m*M* Tris, 138 m*M* sodium chloride, and 2.7 m*M* potassium chloride. The labeled insulin was stored at 4°C prior to use. The purity of the labeled insulin was checked by high-performance liquid chromatography (HPLC). Labeled insulin and unlabeled insulin were injected at a concentration of 0.2 m*M*. The chromatograms were compared and no peak overlap was observed. Consequently, there is little, if any, unlabeled insulin still present after the derivatization procedure.

2.3. Preparation of monoclonal insulin antibody

Stock antibody solutions were prepared by addition of 25 μl of 6.9 mg/ml of monoclonal insulin antibody to 25 ml of Tris buffered saline (pH 8.3): 100 m*M* Tris, 138 m*M* sodium chloride, and 2.7 m*M* potassium chloride. The antibody mixture was stored at 4°C prior to use.

2.4. Apparatus

A modified P/ACE 5000 CE instrument (Beckman Instruments, Fullerton, CA, USA) was used for all experiments. The instrument was interfaced with a proprietary microscope and laser assembly manufactured by LI-COR. The laser assembly consists of a GaAlAs laser diode and a focusing lens (f=46 mm). The laser emits at 787 nm and has a 20-mW output power, modulated with a 50% duty cycle. The output of the laser was focused directly onto a fiber optic connector. The arrangement gives a 4 mW output power at the capillary interface. The detector used in the experiments was a Peltier cooled, three-stage APD system. Three identical bandpass filters (820±10 nm) are used in the detector in order to reduce background noise. The APD signal was demodulated by a lock in amplifier. The analog signal was then filtered before it traveled to a Beckman model 406 A/D converter. The digitized signal was collected by chromatographic software (Beckman System Gold, version 8.01) run on a 486-33 personal computer. A more in depth description of the instrument is available [30].

2.5. Capillary electrophoresis

All experiments were performed at 23°C. The electrophoresis run buffer consisted of 50 m*M* phosphate with 25 m*M* potassium sulfate (pH 7.0). All separations used fused-silica capillaries coated with polyimide (Polymicro Technology, Phoenix, AZ, USA). All capillaries used were of 37 cm×50 μm I.D.×360 μm O.D. The inlet to detector length was 30 cm for all capillaries. At the beginning of each day the capillary was rinsed with 1 *M* sodium hydroxide for 30 min, followed by 15-min rinses of deionized water and run buffer. The samples were introduced by pressure injection (5 s at 0.5 p.s.i.; 1 p.s.i.=6894.76 Pa). The injected volume, calculated by the Hagen–Poiessuille equation, was 7 nl. The applied voltage was 25 kV and the current was 250 μA. The voltage ramp time was 4 min. After each run, the capillary was rinsed with water for 2 min and run buffer for 2 min.

2.6. Noncompetitive immunoassay protocol

A calibration plot was obtained by mixing 250 μl of 200 n*M* NN382-insulin with 250 μl of 0–250 n*M* insulin antibodies. All dilutions were made using Tris buffered saline (pH 8.3). The solutions were vortex-mixed on slow speed for 5 min at room temperature. Peak areas were calculated with chromatographic software.

3. Results and discussion

3.1. Noncompetitive CEIA with NIR-LIF detection

The electropherograms of NN382-insulin and mixtures of NN382-insulin and insulin antibodies are shown in Fig. 2A–D. A calibration curve for the immunocomplex was constructed by measuring the peak area of the immunocomplex at various insulin antibody concentrations (Fig. 3). The average relative standard deviation (RSD) for all points was 6.3%. The curve was linear over two orders of magnitude, with a correlation coefficient of 0.992 from 1 n*M* to 100 n*M*. At higher antibody concentrations, the curve deviates from linearity, presumably due to saturation of NN382-insulin. The

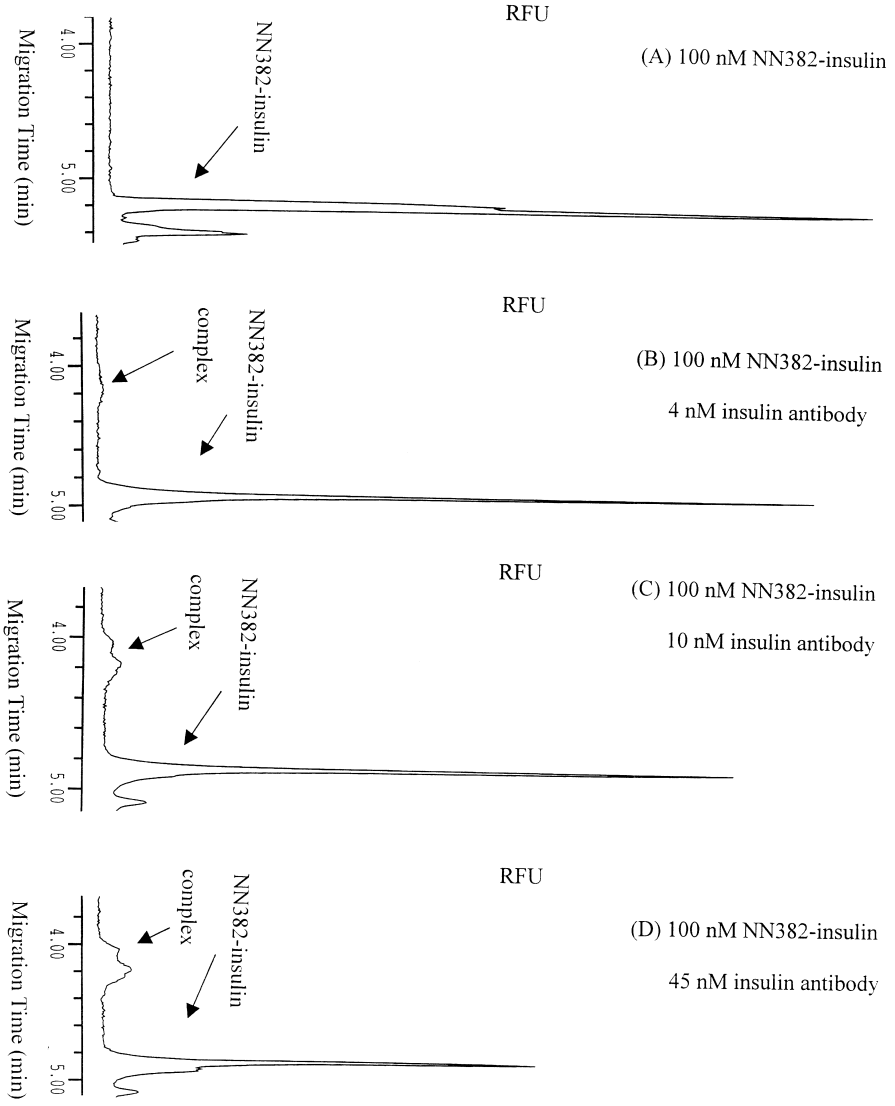

Fig. 2. Comparison of electropherograms of noncompetitive CEIA. (A) 100 n*M* NN382-insulin; (B) 100 n*M* NN382-insulin and 4 n*M* monoclonal insulin antibody; (C) 100 n*M* NN382-insulin and 10 n*M* monoclonal insulin antibody; (D) 100 n*M* NN382-insulin and 45 n*M* insulin antibody. Conditions; capillary 37 cm×50 μm I.D.; applied voltage, 25 kV; ramp time, 4 min; 50 m*M* phosphate and 25 m*M* potassium sulfate, pH 7.0; 5-s injection.

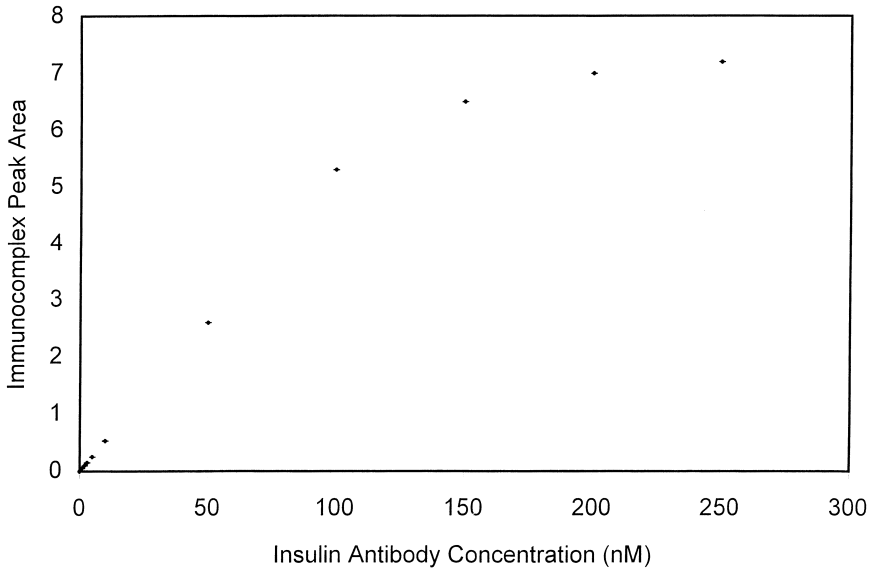

Fig. 3. Calibration curve for the determination of insulin antibodies by the noncompetitive assay. The error bars (one standard deviation) are within the points.

detection limit of the assay, defined as the concentration of insulin antibodies that gave a signal equal to three times the standard deviation of the baseline noise, was 1.1 n*M*. Multiple peaks were observed for the labeled insulin, as seen in Fig. 4. A peak resulting from excess is dye was also observed (Fig. 4). The NIR dye NN382 has an isothiocyanate functionality that is reactive towards amines. Insulin has more than one site available for labeling, explaining the multiple insulin peaks in Fig. 4.

Addition of insulin antibody resulted in the appearance of an additional peak, presumably due to

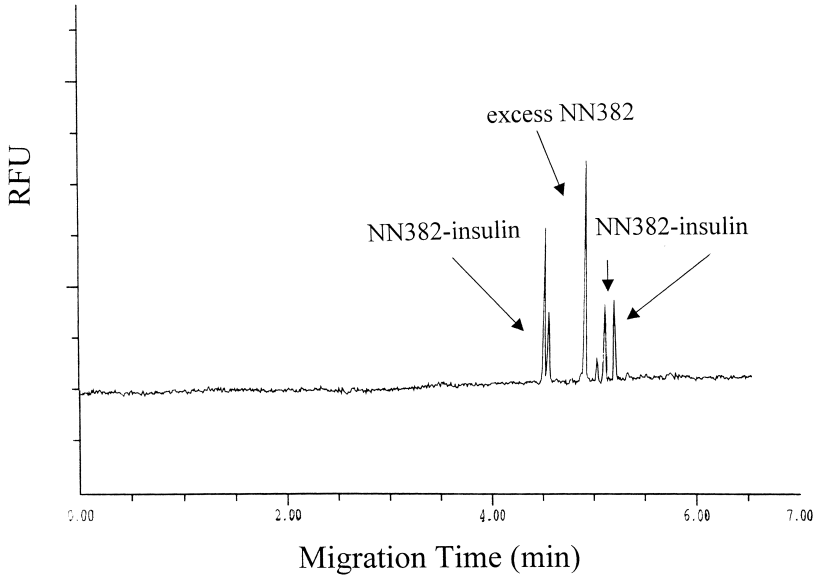

Fig. 4. Electropherogram of 10 n*M* NN382-insulin. Conditions as in Fig. 2.

the formation of the immunocomplex. Increasing the concentration of insulin antibody resulted in a decrease in the peak height of NN382-insulin. Simultaneously, increasing concentrations of insulin antibody resulted in an increase in the peak height of the complex (Fig. 2B–D). The complex peak was only observed for one of the insulin peaks, suggesting that the dye might interfere with the epitope. This scenario is not unlikely since the dye is roughly one fifth the molecular mass of insulin and carries a fair amount of charge due to the sulfonate groups (Fig. 1). The peak shape associated with the complex was generally poor. The antibody could be interacting with more than one antigen, resulting in the observed peak shape. It might be possible to improve the peak shape by using just the Fab fragment as opposed to the whole antibody [8]. This would result in a one to one stoichiometry for the antibody–antigen interaction.

3.2. Effects of capillary length and internal diameter

The effect of capillary length on the separation of the immunocomplex from the labeled insulin was investigated. When doing CE-based immunoassays, it is advantageous to use the shortest capillary length possible. The reasons for doing so are twofold. Shorter capillary lengths result in shorter analysis times. Secondly, if the immunocomplex is not stable over the time scale of the separation, the detection limit is compromised. Various capillary lengths (27 cm, 37 cm, 47 cm and 57 cm) were used to determine the ideal length for the performing the assay. While the results are not illustrated, it was found that the 37 cm capillary gave the best results.

The effect of the internal diameter of the capillary on the separation and detection of the complex from the NN382-insulin was also investigated. It was found that changing the internal diameter of the capillary greatly effected the separation and detection of the immunocomplex. The width of the laser spot is estimated to be a little over 50 μm. It was found that using a 50 μm I.D. capillary allowed for resolution and detection of the immunocomplex. The laser is slightly out of focus when a 50 μm I.D. capillary is used, resulting in a loss of sensitivity. In the past, we have achieved mass detection limits of 100 zmol for NN382-labeled peptides using 75 μm I.D. capillaries [20]. We were only able to obtain a mass detection limit of 7.7 amol (1.1 n*M*) for the noncompetitive assay. This suggests that the laser is not entirely focused on the capillary. It also suggests that the immunocomplex dissociates over the time scale of the separation.

3.3. Optimization of separation parameters

The separation parameters were optimized by independently varying the separation voltage, buffer strength, pH and ramp time. The best results were obtained with an applied voltage of 25 kV, 4 min ramp time, 50 m*M* phosphate and 25 m*M* potassium sulfate buffer, at pH 7.0. The use of high voltage and high ionic strength buffer resulted in Joule heating, as confirmed by an Ohm's law plot. Alternative buffers, such as tricine, borate, Tris and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), were tried in an effort to reduce the current generated, however, none of these buffers were able to sufficiently resolve the immunocomplex from NN382-insulin.

The effect of voltage ramp time is an electrophoretic property that is rarely mentioned. In the experiments, we found that increasing the ramp time had the effect of increasing the peak area of the complex by as much as an order of magnitude (Fig. 5). Consequently, the detection limit of the assay was improved by an order of magnitude. Despite the fact that the sample barely experiences the full voltage before migrating past the detection window, a 4 min ramp time at 25 kV provided the best results. It is possible that when the voltage is applied over a short time period, the injected sample plug may experience localized heating, causing the complex to dissociate.

3.4. Effect of incubation time

In all experiments NN382-insulin and insulin antibody were incubated for 5 min at room temperature. In order to determine if 5 min was a sufficient incubation time period, NN382-insulin and insulin antibody mixtures were incubated for 10, 20, 40 and

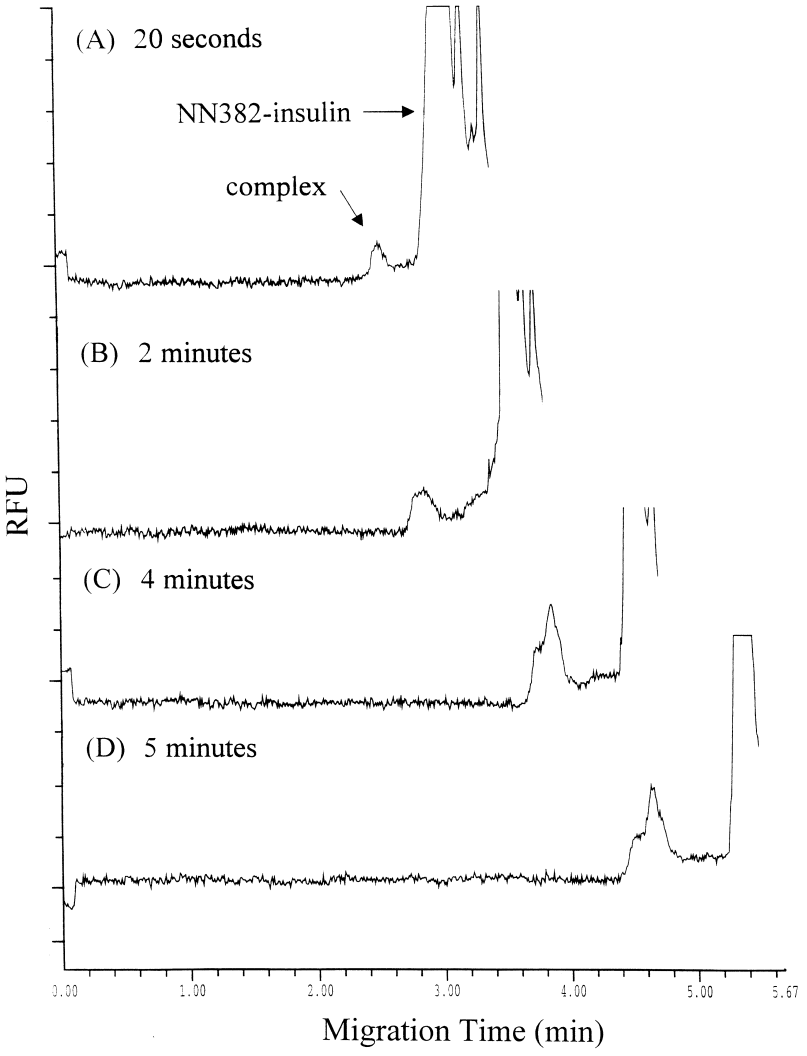

Fig. 5. Comparison of electropherograms of noncompetitive CEIA with different ramp times. Conditions as in Fig. 2.

60 min. The area of the complex peak did not increase with longer incubation periods. It was therefore unnecessary to incubate the mixture for longer than 5 min.

4. Conclusion

Noncompetitive CEIA with NIR-LIF detection is possible. The method developed represents the first CEIA with NIR detection. The detection limit of the assay shows no improvement over what has been achieved through the use of visible lasers [5,6,21,22]. However, detection limits of 400 zmol for NN382-insulin were obtained, suggesting that the 7.7 amol detection limit of the immunocomplex may arise from dissociation of the complex over the time scale of the separation. The work presented demonstrates that the NIR dye NN382 may be used as a tracer in the separation of an antigen from its immunocomplex.

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