Adaptation of Nanoarrays for the Study of α-Synuclein Aggregation - Preliminary Results

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ABSTRACT

In previous publications we have shown that we can perform enzymatic reactions in nanoarrays by means of a microarray-reader based on a conventional microscope. In this publication we report on a modification of this system in order to monitor the aggregation kinetics of the natively unfolded protein α-synuclein. We describe the motivation for this development, the problems associated with the miniaturization of the aggregation assay, and the validation of our modifications.

Keywords: Nanoarrays, High Throughput Screening, Evaporation Control, α-Synuclein, Thioflavin T, Quantitative Fluorescence Microscopy

1. INTRODUCTION

A large number[1,2] of proteins show either partially disordered regions or lack an ordered secondary structure under physiological condition. These natively unstructured proteins are classified into two groups: 1) proteins that are flexible but still possess a secondary structure, e.g. molten globules; and 2) proteins that behave essentially as random coils. The term "natively unfolded" has been employed for proteins of the second class. The primary structure of a protein determines its secondary structure. More than 15,000 proteins share a pronounced degree of structural disorder and these are mostly rich in certain amino acids such as glutamic acid, lysine, and glycine. Natively unfolded proteins characteristically exhibit a high net-charge and low hydrophobicity, as well as the tendency to form depositions associated with several neurodegenerative disorders. Examples are Parkinson's disease (PD), Alzheimer's disease, dementia with Lewy body, and multiple system atrophy (MSA). The lack of an ordered secondary structure also potentiates low affinity interactions with other molecules. Thus, the regulation of many cellular processes may be controlled by the transition from a disordered to an ordered state of a participating protein.

α-Synuclein[1,3] is a member of the class of natively unfolded proteins and constitutes the major component of Lewy bodies, fibrillar cytoplasmic inclusions found in cells of the *substantia nigra* in the brain of individuals suffering from PD. α-Synuclein is a 14 kDa (140 amino acids) pre-synaptic protein abundant in various regions in the brain but its natural functions are still a matter of speculation. α-Synuclein lacks a well-defined secondary structure under physiological conditions but exhibits well demarcated domains, some of which adopt alternative conformations under different conditions. An interesting recent finding [4,5] is the existence of distinct intramolecular interactions that appear to establish a native state of autoinhibition with respect to aggregation, a process triggered by mutation, low pH, high temperature and the binding of numerous ligands. The β-sheet conformation is characteristic of the resulting amyloid structures, including the fibrils pathognomonic of the dopaminergic neurons affected in PD.

Thioflavin T[6,7,8]. (ThioT) is a classical stain for amyloid depositions in brains from Alzheimer's disease infected individuals. It is also in widespread use in kinetic studies of fibril formation *in vitro*. Unbound ThioT has an absorption peak at 350 nm and an emission peak at 430 nm. When bound to aggregated α-synuclein, particularly fibrils, the absorption peak shifts to 450 nm and the emission peak to 485 nm; the intensity also increases dramatically. Whereas

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ThioT binds rapidly and specifically to the anti-parallel β-sheet fibrils, it does not interact with the monomeric form or oligomeric intermediates.

The development of rational therapeutic strategies requires an understanding of the underlying mechanisms and factors mediating oligomerization and fibrillization. However, it is difficult to explore this multidimensional reaction scheme because of the characteristically long lag times and short transition times for these reactions [4]. The aggregation kinetics of α-synuclein are usually determined in milliliter cuvettes or in 96-well microtiter plates. We are interested in scaling down the assay volume to eight nanoliter "cuvettes" that we term nanowells, implying a ~500,000-fold reduction in the overall assay volume. The miniaturization leads to numerous improvements:

- a dramatic reduction in the amounts of α-synuclein and all other compounds necessary for the assay;
- a significant reduction in the physical space required for the measurement instrumentation;
- the ability to perform a large number of tests in parallel through the array of nanowells, and;
- a reduction in the time required for the biochemical reactions in which the rate-limiting process is diffusion.

Our read-out platform is based on a conventional microscope system. In this paper we discuss the issues involved in miniaturizing the aggregation assay.

2. BACKGROUND

In previous publications, we reported on a miniaturized system designed to monitor enzymatic reactions[9-13]. This system is based on nanoarrays with volumes of 6.3 and 8 nanoliters depending on the shape of the wells; see Table 1.

Table 1: Well sizes used for enzyme reactions studies and α-synuclein experiments

Depth [μm]	**Square well 400 x 400 μm²**	**Round well 400 μm² (diameter)**
50	8.0 nL	6.3 nL

The wells are etched into silicon and have a top layer of silicon nitride. The nanoarray chip itself has a physical size of 2 × 1 cm² and two arrays of 5 × 5 wells have been implemented per chip[14].

The read-out system is based on a conventional, epi-illumination Zeiss Axioskop microscope equipped with a scientific grade CCD camera[15,16]. The system uses a relatively high numerical aperture objective: 20× / 0.75 FLUAR Zeiss. With the 20× magnification, an entire 400 μm-wide well occupies one image, while the 0.75 NA permits the capture of a significant amount of the light emitted by the fluorescent ThioT. The trinocular tube with the eyepieces and the relay optics have been replaced with an inverted 5× / 0.25 FLUAR Zeiss objective (Figure 1). This inverted lens concentrates the measured light onto the smallest possible area of the CCD sensor, producing a higher signal-to-noise ratio (SNR) in the final digital image than if the light had been distributed over a larger number of CCD pixels[14]. The 100 W mercury-arc light source (USH-102D, Ushio Inc., Tokyo, Japan) and the associated optics are configured for Köhler illumination. The CCD camera is a Princeton Versarray 512B Back-illuminated CCD camera with 512 × 512 pixels (Princeton Instruments, Trenton, New Jersey, USA). Each pixel has a size of 24 μm × 24 μm.

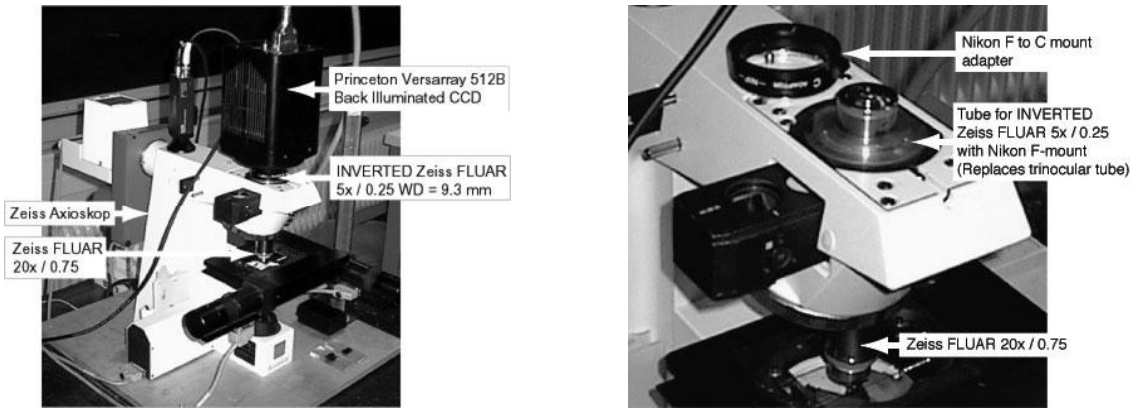

Figure 1: The microscope based read-out system is shown: a Zeiss Axioskop using a 20 x / 0.75 objective, an inverted 5 x / 0.25 objective and a Princeton Versarray CCD camera.

3. EVAPORATION ISSUES

Aggregation assays[17,18] with α-synuclein can require substantial amounts of time; an experiment taking three or more days is not unusual. Nanoliter quantities of fluid, however, can evaporate in a few seconds. In order to prevent evaporation of the liquid in the wells, we house the nanoarray chips in a transparent, plastic chamber that provides one opening for the 20× microscope objective, two openings for forced air transport (input and output), and electrical ports for temperature and humidity measurements and controls. Within this chamber we provide a feedback-controlled temperature and humidity environment for the nanoarrays used in α-synuclein experiments as well as other experiments.

Temperature control of the chip is straightforward and accomplished with a PE120 heating/cooling stage and a PE94 temperature controller (Linkam, Tadworth, UK). The control of the humidity, however, is a more complicated issue. Distilled water is held in a small plastic reservoir. The humidity inside the experimental chamber is measured and the data controls a *nebulizer*, a device that turns liquids into a fine mist. The mist is then transported by forced air to the chamber to achieve the desired humidity. Figure 2 shows the front view of the chamber plus connection tubes.

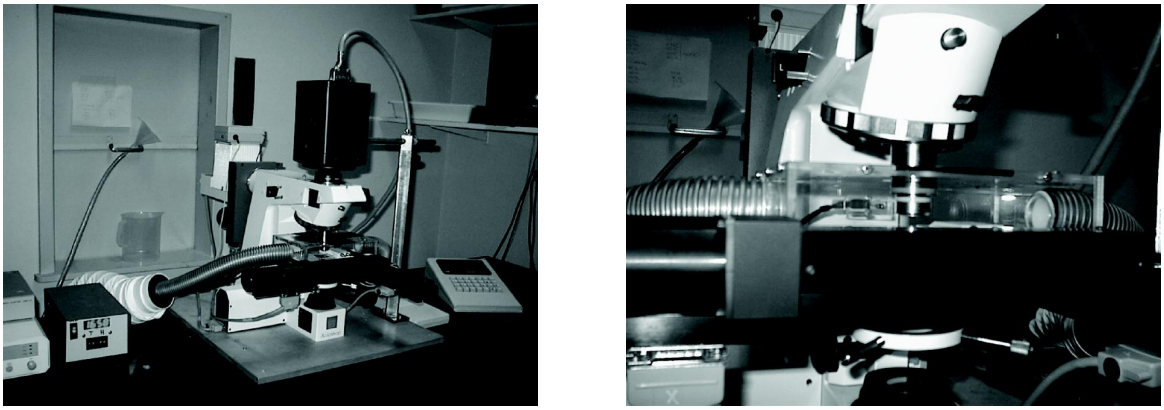

Figure 2: Front view of the self-made humidifying chamber build for an upright Zeiss Axioskop microscope.

Figure 3 depicts the plastic container holding the smaller water reservoir. The mist is produced using a miniature nebulizer DK-24 (Conrad Electronic GmbH, Hirschau, Germany). Distilled water is atomized by means of an ultrasonic resonator at a rate of 250 mL per hour. Because of the vibrations associated with the nebulizer, it cannot be placed upon the same table as the microscope. A rigid plastic tube with a large section removed (see Figure 3) is mounted

horizontally above the water tank and goes through the larger container to the outside. There it is connected to two 100 mm diameter flexible tubes. The large hole in the rigid tube allows the mist to be directed to the experimental chamber by a 100 mm inline fan. The whole system is closed.

An ordinary hair dryer connected to the larger container is used to heat the circulating air. This allows a higher humidity uptake. The inline fan, the hair dryer and the nebulizer are controlled by PAC2 phase-angle power controllers (United Automation, Southport, UK) that use analog control voltages to regulate the devices.

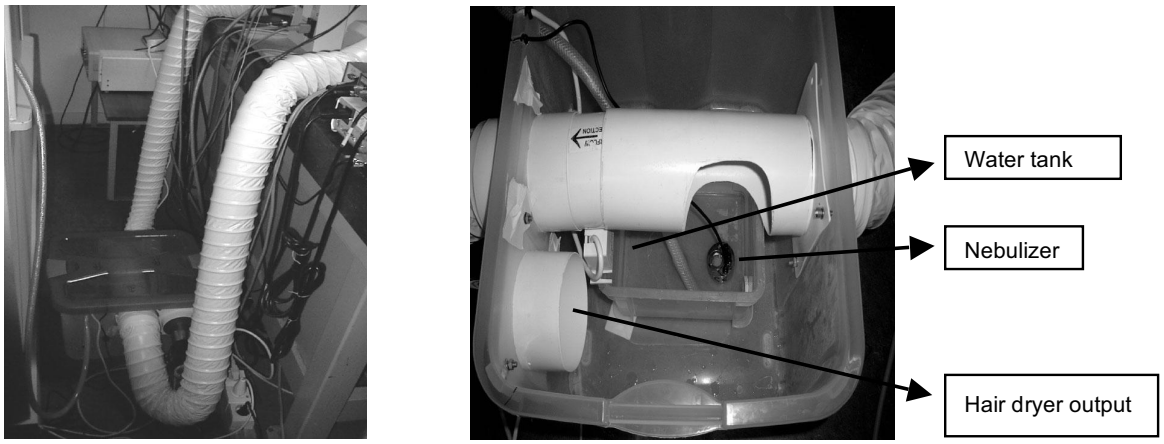

Figure 3: Water reservoir with mist generator and air control.

The humidifying chamber is equipped with a PIC16F873 microcontroller (Microchip Technology Inc., Chandler, Arizona, USA) that utilizes a humidity and temperature sensor SHT-71 (Sensirion, Zürich, Switzerland). This sensor (Figure 4) consists of a single chip that includes a calibrated digital output. The chip can be used for temperatures between –40°C and 120°C and relative humidities between 0% and 100%. The SHT-71 is factory calibrated with calibration coefficients programmed into the OTP (one-time programmable) memory of the sensor. As shown in Figure 5, the desired values for temperature and relative humidity are set from 00 to 99 by means of BCD switches. A dual DAC is used to generate the control voltages. The PIC16F873 chip reads all settings, calculates the temperature, humidity and the dew point in the humidifying chamber and controls the nebulizer, the fan and the hair dryer.

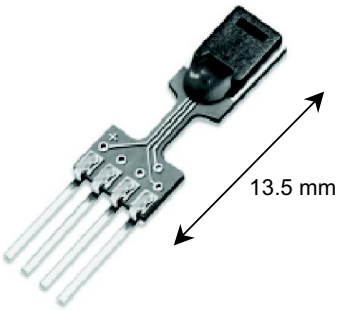

Figure 4: SHT71 humidity and temperature sensor

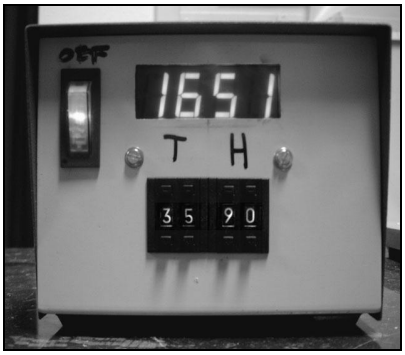

Figure 5: Control box for the required temperature and humidity

4. VALIDATION

Evaporation: The humidifying chamber reduces the evaporation rate of the liquid inside the sealed wells of the nanoarray. The wells are filled with the solution by means of a sliding motion of a Pyrex coverslip on top of the 5×5 array, which pushes the liquid into the wells. Subsequently, the edges of the coverslip are sealed by means of a colorless nail polish. Figure 6 depicts the progress curve of evaporation in a controlled and uncontrolled environment over a period of time for a 25 well array.

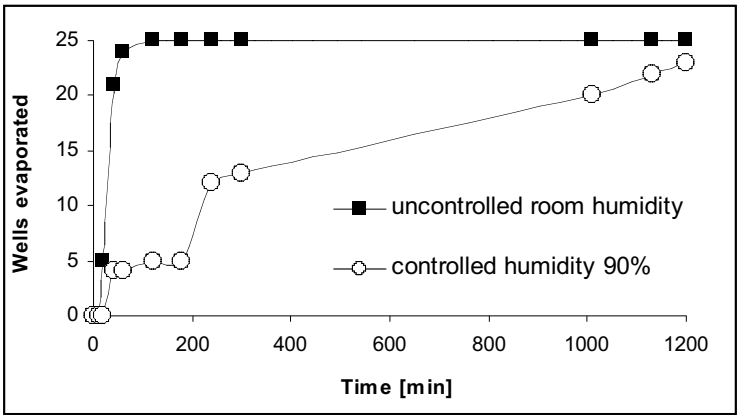

Figure 6: The number of evaporated wells as a function of time in an uncontrolled and controlled humidity environment.

In an environment with no control of humidity, 50 percent of the wells were empty after 30 minutes. This time period was extended by a factor of eight, i.e. to four hours, by using feedback control to maintain 90% humidity. Preliminary experiments have been performed with bovine serum albumin (data not shown) as a coating for the nanoarray and the coverslip in order to reduce evaporation even further. This protein alters the surface properties of both materials in such a way that hydrophilicity is increased. The filling of the wells improves accordingly, preventing the formation of air bubbles and subsequent evaporation of the liquid.

Temperature Control: For biological measurements it is important to work at controlled, constant temperatures. Most aggregation studies of α-synuclein are performed at 37° C. Due to the poor conductance of the silicon nanoarrays it is necessary to know the actual temperature inside a well. We used a Sanwa DMM digital multimeter RD700 and K-AD sensor coupler (Sanwa Electric Instrument Ltd, Tokyo, Japan) in order to measure the temperature inside the wells of the nanoarray. Figure 7 shows the calibration curve of the reference temperature as given by the PE94 controller and the actual well temperature as measured by the multimeter. This curve indicates clearly that a reference temperature of 46° C corresponds to an actual temperature of 37° C inside the wells.

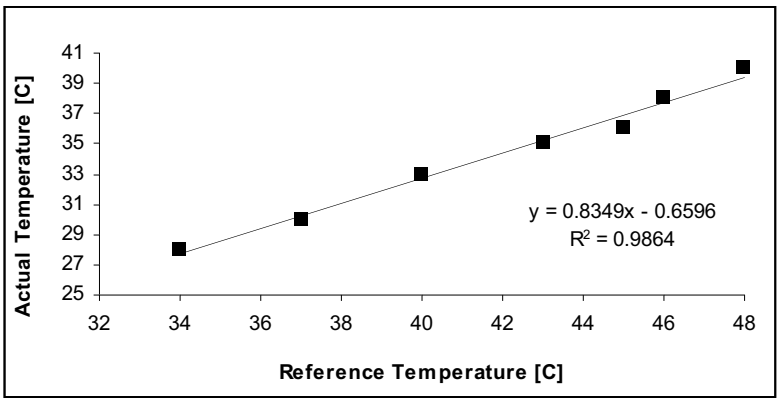

Figure 7: Actual measured temperature inside a well of a nanoarray and its reference temperature.

Fluorescence: ThioT was measured in the free (unbound) state and bound to α-synuclein fibrils in two independent experiments to examine the intensity as a function of 1) concentration and 2) integration time.

For the first experiment different concentrations of the native dye were used. The camera integration time was 80 ms and the humidity within the humidifying chamber was 90%. A Zeiss 01 filter set was used. All solutions were made with MilliQ distilled water, which also served as the control. Figure 8 shows the calibration curve for unbound thioflavin T. The minimum detectable signal is drawn as a horizontal dashed line and represents a signal level that is 3σ above the background signal level of the blank (control) solution. The lower detection limit with the Köhler illumination configuration was 100 µM thioflavin T.

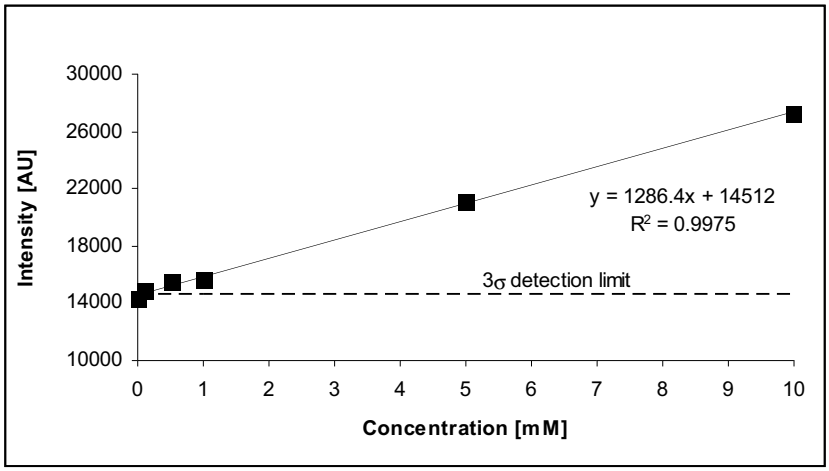

Figure 8: The fluorescence intensity of free ThioT as a function of its concentration.

In a second experiment a solution of 10 µm ThioT and 45 µm α-synuclein fibrils (both final concentrations) was used in order to determine the relation of the intensity of the complex of ThioT and α-synuclein fibrils as a function of the integration time of the CCD camera. The array was filled and sealed as explained above. The humidity was 95%. An Omega filter set XF114-2 (Omega® Optical Inc., Brattleboro, USA) was used in order to detect the shifted emission peak of 485 nm. The read out rate of the CCD camera was 1 MHz and the electronic gain was set to medium. The camera was cooled to –40° C.

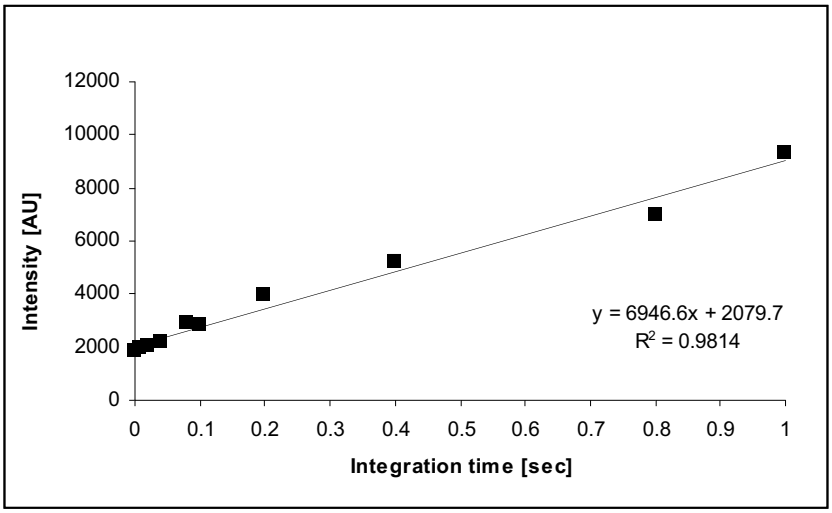

Figure 9: The fluorescence intensity of ThioT and α-synuclein fibrils as a function of the integration time The scale is not the same as that of Figure 8.

5. SUMMARY AND CONCLUSIONS

Our nanoarray system based on a conventional microscope is capable of measuring the weak fluorescence signals that we expect from the fluorophore ThioT. The minimum detection limit for free ThioT is 100 μM. For the detection of thioflavin T with α-synuclein fibrils a minimum integration time of 80 ms should be applied. Evaporation of the solution in the wells is significantly reduced through the use of a self-made humidifying chamber. Due to this innovation approximately 50 % of the wells are still filled after a time period of four hours. In experiments that we have performed with nanoarrays coated with bovine serum albumin this time is increased even further. The temperature inside the wells of the nanoarray can be controlled within an accuracy of ±1° C. We are continuing to improve this nanoarray instrumentation so that the study of aggregation kinetics of α-synuclein can be implemented in the miniaturized assay.

6. ACKNOWLEDGEMENTS

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