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A Sol–Gel-Derived Acetylcholinesterase Microarray for Nanovolume Small-Molecule Screening

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A fluorimetric acetylcholinesterase (AChE) assay was developed and characterized both in solution and with the enzyme entrapped in sol–gel-derived silica. The assay is based on a disulfide–thiol interchange reaction between the intramolecularly quenched dimeric dye BODIPY FL L-cystine and thiocholine generated by the AChE-catalyzed hydrolysis of acetylthiocholine (ATCh), which results in a brightly fluorescent monomeric product owing to the cleavage of the disulfide-coupled form of the dye. The new assay was validated by comparison with the Ellman assay performed under parallel conditions and was used in both kinetic and end point assays. The assay was extended to the fabrication of functional AChE microarrays using contact pin-printing of sol–gel-derived silica. A total of 392 sol–gel formulations were screened for gelation times and 192 of these were further evaluated for array fabrication on four different surfaces using a factor analysis approach. Of these, 66 sol–gel/surface combinations produced robust microarrays, while 26 sol–gel/surface combinations were identified that could produce highly active AChE microarrays. The Z factor for the on-array assay using an optimal sol–gel/surface combination, which considers both signal variability and difference in signals between positive and negative controls, was determined to be 0.60, which is above the minimum level required for applicability to screening. By overprinting nanoliter volumes of solutions containing the dye, ATCh, and potential inhibitors, these microarrays could be used to screen two libraries of small molecules, one composed of newly synthesized alkaloids and another consisting of ~1000 known bioactive compounds, both as discrete compounds and mixtures thereof, for activity against AChE. IC₅₀ values were obtained on microarrays for compounds showing significant inhibitory activity, demonstrating the utility of arrays for quantitative inhibition assays.

Acetylcholinesterase (AChE) plays an important role in acetylcholine (ACh)-mediated neurotransmission, catalyzing the hydrolysis of the neurotransmitter ACh to inactive choline, thereby

releasing acetate.¹ While the role of AChE in neurotransmission was discovered almost a century ago,² it has recently regained attention due to its association with Alzheimer's disease (AD)³ and other neurodegenerative diseases⁴ characterized by low ACh levels owing to catabolism by AChE.⁵

There exist a variety of homogeneous assays for AChE activity that can be used in high-throughput screening for inhibitor identification.^{6–11} However, recent requirements for increased assay throughput and decreased reagent volumes have resulted in miniaturization of assays toward protein (and substrate) microarrays for detection of enzyme activity,^{12–25} including AChE

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microarrays,²⁶ using nanoliter-volume assays^{14,27} and highly parallel screening.²⁸

An emerging functional protein microarray platform involves the pin-printing of protein-doped silica sols onto functionalized surfaces.^{26,27,29–36} Advantages of this method include the applicability of sol–gel immobilization to a wide variety of proteins,^{37–39} the ability to use native proteins and to tune the silica matrix with various precursors and additives to optimize the activity of that protein,^{40,41} high protein loading,³⁰ the ability to co-entrap multiple protein components in a single array element,^{27,29,35} and amenability to nanovolume assays.^{14,27} However, while sol–gel-based protein microarrays show promise for high-throughput screening (HTS) to identify enzyme inhibitors, there are significant issues that must be addressed to fabricate high-density arrays that are suitable for HTS. First, the sol–gel composite material needs to have a long gelation time to allow for fabrication of uniform, high-density microarrays without gelation in the spotting pin. Second, the material must have good adhesion to the substrate and should not crack when overprinted with assay solutions. Third, the material must provide quantitative signals without interferences related to nonspecific binding of assay components. Lastly, the material must be able to retain the activity of the enzyme(s) under study. Given that there are numerous variables that can affect material properties (i.e., buffer type, concentration and pH, the nature and concentration of silica precursors, the presence of silane and/or polymer additives, slide surface chemistry, small-molecule additives, etc.), some groups have resorted to large-scale screening of sol–gel compositions (up to 100 000) to identify materials with suitable properties for array fabrication.^{36,42} However, in this work we show that a small directed screen can be performed on the basis of the systematic fulfillment of various criteria in a stepwise manner, such that the number of materials screened, and the associated time and cost, can be greatly reduced.

The second goal of the study was to develop a robust assay that was amenable to microarray-based small-molecule screening.

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There are a number of fluorogenic AChE assays available;^{7,9,26,43,44} however, these assays utilize excitation in either the near-UV or blue-violet visible region,^{7,43} which is not compatible with typical DNA microarray scanners, require secondary screens in order to eliminate off-target hits,⁴⁵ or require tight control of pH and ionic strength.²⁶ To overcome these issues, we have adapted the well-known Ellman assay,⁶ which monitors the hydrolysis of acetylthiocholine (ATCh) via the reaction of thiocholine with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), to a fluorescence-based platform, using a fluorogenic BODIPY dye in place of DTNB. BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) and derivatives thereof have exceptional spectral and photophysical stability and are not sensitive to pH.^{46,47} These probes are heavily autoquenched when in a dimeric form and show a significant increase in fluorescence when in the monomeric form.⁴⁸ The assay described in this work uses BODIPY FL L-cystine, a symmetric disulfide,⁴⁹ which has been employed previously to monitor thiohydrolase activity.⁵⁰ The assay is based on a sulfide–thiol interchange reaction,⁵¹ which in this case is between the intramolecularly quenched dimeric dye and thiocholine generated by the AChE-catalyzed hydrolysis of ATCh, which results in a brightly fluorescent (green) monomeric product owing to the cleavage of the disulfide-coupled form of the dye. The assay is compatible with the excitation/emission wavelengths of standard microarray readers and can be used in conjunction with overprinting of reagents onto AChE-doped sol–gel-derived microarrays, as shown in Figure 1. The microarray-based screening platform is demonstrated using two libraries of potential AChE inhibitors, one based on recently synthesized alkaloids⁵² and the other consisting of ~1000 known bioactive compounds. The results show that this method can be used to identify inhibitors of AChE and for secondary screening to determine inhibition constants.

EXPERIMENTAL SECTION

Reagents. AChE from *Electrophorus electricus* (EC 3.1.1.7), Dowex 50WX8-100 ion-exchange resin, acetylthiocholine iodide, tetramethylorthosilicate (TMOS), colloidal silica (LUDOX AM-30, 30 wt % suspension in water), paraoxon, anhydrous glycerol, sorbitol, trehalose, N^ε-acetyl-L-lysine, Triton X-100, polyvinyl alcohol (PVA, MW ~ 9000–10 000), polyethylene imine (PEI, MW 1300), and polyethylene glycol (PEG, MW 600) were purchased from SigmaAldrich (Oakville, ON). N-(3-Triethoxysilylpropyl) gluconamide (GLS), bis[(3-methyldimethoxysilyl)propyl]polypropylene oxide (MDSPPPO), 3-(aminopropyl)-

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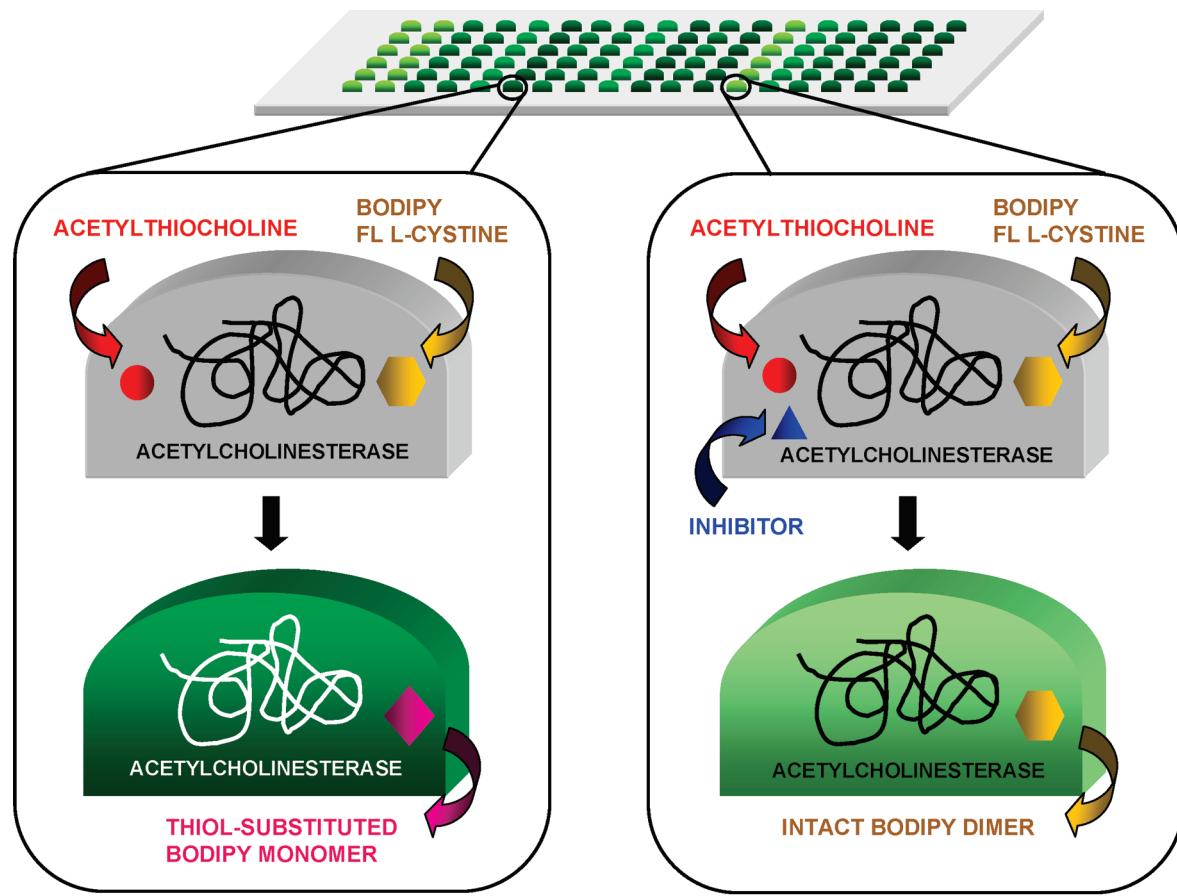


Figure 1. Scheme for on-array small-molecule screening for activity against AChE using BODIPY FL L-cystine.

triethoxysilane (APTES), methyltrimethoxysilane (MTMS), bis(triethoxysilyl)ethane (Bis-TEOS), and carboxyethylsilanetriol (Si-COOH) were from Gelest (Morrisville, PA). BODIPY FL L-cystine was obtained from Invitrogen (Burlington, ON). Sodium silicate (SS, 27 wt % SiO₂, 10 wt % NaOH) was purchased from Fisher Scientific (Ottawa, ON). Diglycerylsilane (DGS) was prepared as described previously from distilled TMOS and anhydrous glycerol.⁵³ Poly(methylmethacrylate) (PMMA) slides were obtained from Exakt Technologies (Oklahoma City, OK), while aldehyde-, epoxy-, and amine-derivatized slides and Stealth pins were from ArrayIt (Sunnyvale, CA). Alkaloid compounds evaluated for their inhibitory activity were isolated and/or synthesized as reported elsewhere,⁵² while compounds used for mixture screening were part of the bioactive subset of the Canadian Compound Collection maintained by the McMaster University High-Throughput Screening Laboratory. Water was filtered through a Milli-Q Synthesis A10 water purification system (ddH₂O). All other chemicals and solvents were of analytical grade and used without further purification.

Fluorimetric Enzyme Assays. Details of solution and solid-phase assays, including reagent volumes and concentrations and platereader settings, are provided in the Supporting Information, along with details on the determination of Michaelis constants in solution and sol-gel-derived materials.

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Materials Screening. Details of the procedures used to screen materials are provided in the Supporting Information. Briefly, two silane precursors (DGS and SS) at two concentrations and two buffers (HEPES and Tris) at two concentrations (25 and 50 mM) and 7 pH values (7.0–8.2) were first evaluated to find materials with gelation times over 2.5 h. A subset of these materials was then evaluated with three polymers (PEG, PEI, PVA) at two concentrations, six organosilanes (GLS, APTES, Bis-PEG, Bis-TEOS, PhDMS, Si-COOH), and five small-molecule additives (glycerol, trehalose, sorbitol, N^ε-acetyl-L-lysine, Triton X-100), each at a single concentration, to find sols that showed adequate gelation times to allow printing. Suitable sols were printed onto four types of functionalized slide surfaces (PMMA, aldehyde, amine, epoxy) and evaluated for printability (spot uniformity, no cracking, no spreading). Those materials that produced good arrays were then evaluated with entrapped AChE to identify materials that were amenable to overprinting of reagents and finally narrowed down to materials that produced high enzyme activity and good signal-to-background levels.

Microarray-Based Assays. Z' Analysis. A 48 × 25 element microarray (400 μm spots, 800 μm spacing) was printed with the optimal sol-gel-based material, with spotting solutions containing 200 U/mL of AChE (see Supporting Information for details on printing of microarrays). The spots were overprinted, using an SMP15XB Stealth pin, with 12.5 nL of 5% glycerol in 25 mM Tris, pH 7.0 containing 14 μM BODIPY FL L-cystine and either 300

μM ATCh (high control, HC) or no ATCh (low control, LC). Reactions were allowed to proceed for 30 min, after which the arrays were imaged using a NovaRay microarray imager (see Supporting Information for details). The spot intensities were quantified using ImageJ, and a total of 200 samples were used for LCs and HCs. The Z' factor was calculated for each assay using the formula⁵⁴

$$Z' = 1 - \frac{(3\sigma_L + 3\sigma_H)}{|\mu_H - \mu_L|} \quad (1)$$

where σ_L is the standard deviation (SD) of the LC, σ_H is the SD of the HC, μ_H is the average signal of the HC, and μ_L is the average signal of the LC.

Compound Screening and IC_{50} Determination on Microarrays. Arrays of 1200 spots ($400\ \mu\text{m}$ diameter) in a 48×25 spot pattern were printed onto amine-derivatized slides using spotting solutions consisting of 200 U/mL AChE in 1.4 SS/1.0 PVA/glycerol. The spots were aged for 1 h at room temperature and $\sim 80\%$ relative humidity inside the microarrayer chamber. After aging, the spots were then overprinted ($\sim 12.5\ \text{nL}$ per assay) with either 1% (v/v) DMSO or a solution of the test compound in DMSO (at a final concentration of $\sim 30\text{--}50\ \mu\text{M}$) in 5% (w/w) glycerol, 25 mM Tris buffer, pH 7.0. After incubation for 1 h, the arrays were overprinted again with $\sim 12.5\ \text{nL}$ of a solution containing 5% (w/w) glycerol in 25 mM Tris buffer, pH 7.0, with 300 μM ATCh, 14 μM BODIPY FL L-cystine, and either 1% (v/v) DMSO or the test compound at the concentration used in first overprinting, in order to minimize dilution effects. Each compound was tested in duplicate. The arrays were imaged and analyzed as described previously after a 30 min reaction time. Activities against AChE were determined using the following equation:

$$\% \text{ activity} = \frac{S_T - S_{NC}}{S_{PC} - S_{NC}} \times 100 \quad (2)$$

where S_T , S_{NC} , and S_{PC} are the signal intensities of the test compound, negative control (NC), and positive control (PC), respectively. Compounds that showed less than 25% activity in both tests were considered as “potential inhibitors”.

To measure the IC_{50} value of a potential inhibitor, 12×25 AChE arrays were printed and treated as described above, except that the arrays were exposed to different concentrations of the inhibitor solution straddling its expected IC_{50} value, with each compound concentration being printed in a 1×25 pattern over a single row of the microarray. After imaging and quantification, the average intensity values ($n = 25$) were determined and plotted against inhibitor concentration. Curve-fitting was performed using SigmaPlot 2000, and the IC_{50} value was determined by fitting data to a four-parameter Hill equation⁵⁵

$$B = B_0 + \left(\frac{B_{max}[I]^n}{IC_{50} + [I]^n} \right) \quad (3)$$

where B is the intensity at a given concentration of inhibitor $[I]$, B_0 and B_{max} are the minimum and maximum intensity values, respectively, IC_{50} is the inflection point of the isotherm, and n is the Hill number, which influences the pitch of the inflection and can be used to determine the number of binding sites per enzyme (assumed to be 1).

Screening of Mixtures and Deconvolution. To increase throughput in screening a ~ 1000 compound library to identify inhibitors of AChE, screening was performed using mixtures containing 20 compounds, with each compound at a concentration of $2\ \mu\text{M}$. As the PC, one mixture was spiked with the known inhibitor sanguinine. A mixture containing no AChE inhibitor was used as the NC. The AChE arrays were printed, treated, and analyzed as described above. Mixtures that showed less than 50% activity in duplicate assays were deconvoluted by screening the components individually, at a concentration of $5\ \mu\text{M}$ each, in order to identify the active compound responsible for the inhibitory action observed in the mixture.

RESULTS AND DISCUSSION

Assay Proof-of-Concept. Initial studies demonstrated that the BODIPY FL L-cystine reacts in a similar manner to DTNB, whereby it undergoes a sulfur exchange reaction with thiocholine to produce a large increase in signal (see Supporting Information, Figure S1). Its suitability for monitoring AChE activity was determined by measuring the increase in the fluorescence signal of PC samples over NC samples either containing the inhibitor galanthamine or without ATCh, at a pH of 7.0, which was determined to produce the lowest background signals from autohydrolysis of ATCh (see Supporting Information, Figure S2). The results of studies conducted both in solution and in silica using optimal concentrations of AChE, ATCh, and BODIPY were compared with those obtained using Ellman assays run under parallel conditions (see Supporting Information, Figure S3 and Table S1). For both types of assays, the rates of reaction of PC samples in silica were $\sim 20\%$ lower compared to their solution-based counterparts. The PC/NC ratio for the fluorimetric and Ellman assays were also lower in silica than in solution; however, the AChE-catalyzed reactions in silica were generally at least 3-fold faster than uncatalyzed reactions, providing a sufficient assay window to allow screening.

Enzyme Kinetics in Solution and Silica. Michaelis–Menten plots for determination of K_M and V_{max} values of AChE-catalyzed hydrolysis of ATCh were obtained for both solution-based and in-silica fluorescence assays, as shown in Figure S4 (Supporting Information). For the solution-based assay, K_M was found to be $153 \pm 22\ \mu\text{M}$, while the Ellman assay produced a value of $\sim 134 \pm 13\ \mu\text{M}$, with both values being in good agreement with the literature value ($140\ \mu\text{M}$).⁶ The apparent K_M for the fluorescence-based assay in silica was $319 \pm 51\ \mu\text{M}$, which agreed reasonably well with the corresponding Ellman assay performed in silica ($263 \pm 48\ \mu\text{M}$, data not shown). The apparent V_{max} in silica ($406 \pm 27\ \Delta\text{RFU}/\text{min}$) for the fluorescence assay was $\sim 20\%$ lower than that of the solution-based assay ($491 \pm 21\ \Delta\text{RFU}/\text{min}$). The same was true for the Ellman assay, where V_{max} for the solution-based and in-silica assays were $0.019 \pm 0.001\ \Delta\text{AU}/\text{min}$ and 0.017 ± 0.001 , respectively. Increases in K_M and decreases in V_{max} upon entrapment of enzymes in silica have previously been noted for a number of other entrapped

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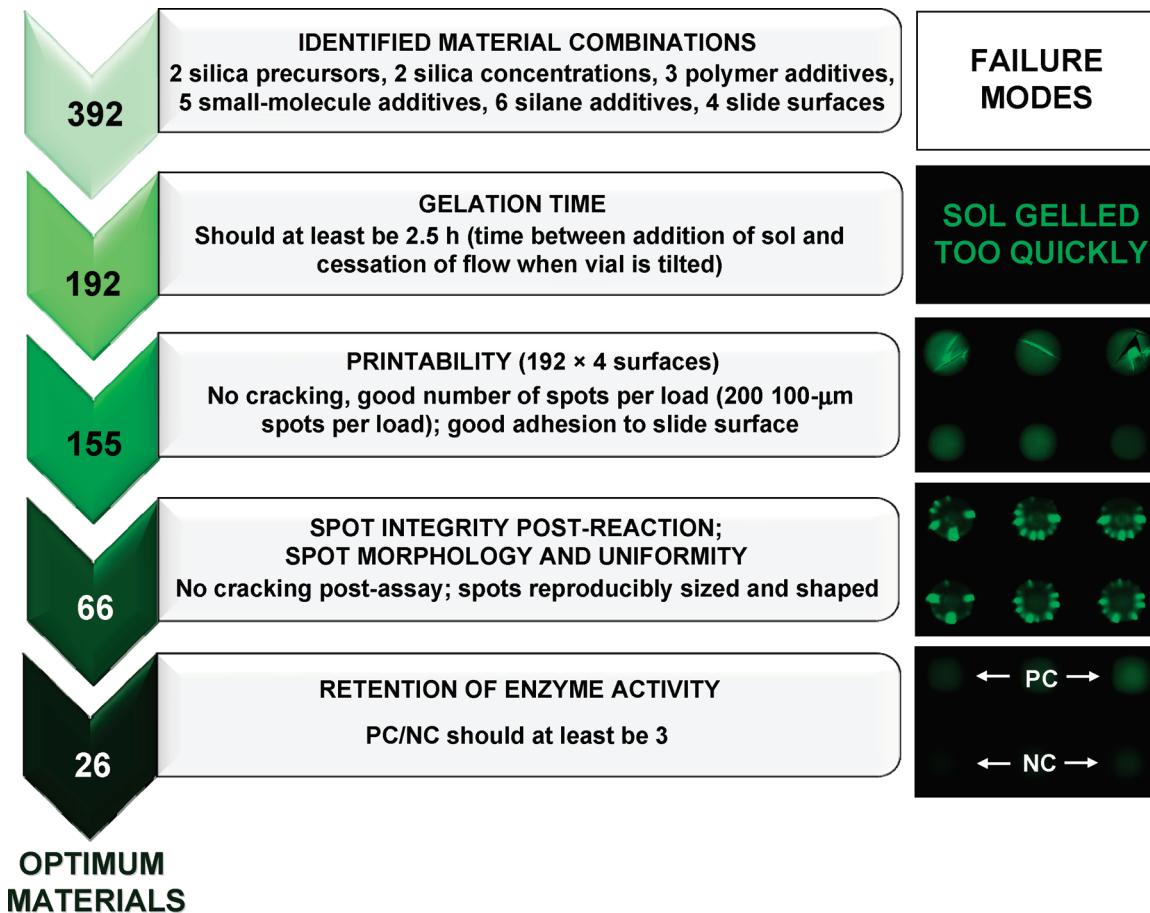


Figure 2. A directed material screening approach for identification of optimum materials for fabricating sol–gel-derived AChE microarrays.

enzymes⁵⁶ and reflect mass transfer limitations in the solid-phase assay; i.e., weaker apparent substrate–enzyme binding arises due to a slower rate of delivery of substrate to the enzyme, while lower reaction velocity results from retarded substrate and product diffusion through the pores of the gel matrix.⁵⁶

Materials Screening for Sol–Gel-Derived AChE Microarrays. A selection of materials was screened to find materials that (1) had long gelation times, (2) would allow printing of 200 spots from a single uptake of liquid without gelation in the pin, (3) showed good adhesion to the substrate, (4) did not crack when overprinted with assay solutions, (5) retained the activity of AChE, and (6) provided quantitative signals with minimal background. To minimize the number of compositions to be tested, a directed, small-scale screen was performed using materials that had previously been shown to be amenable to enzyme entrapment, with processing conditions restricted to those that would be expected to retain enzyme activity. Ultimately, this resulted in screening of two silane precursors (SS and DGS)^{27,57} at two concentrations, two buffers at two concentrations and seven pH values, three polymers at two concentrations, six organosilanes, and five small-molecule additives, with sols that showed adequate gelation times being printed onto four types of functionalized slide surfaces (PMMA, aldehyde, amine, epoxy). To further reduce the

total number of compositions tested, a preliminary factor analysis was performed using only two silane precursors at two concentrations, two buffers at two concentrations, and seven pH values (112 total combinations) to find materials with gelation times of 2.5 h or greater (this value being selected on the basis of preliminary studies showing that such gelation times allowed printing of 200 array elements, which is the maximum number per uptake). It was found that 25 mM HEPES buffer at pH 8.0 satisfied the gelation requirement (>2.5 h) when mixed 1:1 (v/v) with SS or DGS at the two silica concentrations. Thus, a total of four compositions were carried forward for studies involving polymer, organosilane, or small-molecule additives.

In the next stage, the four starting materials were mixed with the three polymers at two concentrations each, and from these studies only PEG- and PVA-doped materials were carried forward, as PEI-doped materials showed very rapid gelation times. The 16 total combinations (two silanes at two concentrations with two polymers at two concentrations) were then examined with each of the six organosilanes. From these studies, only GLS-doped materials showed acceptable gelation times; APTES-doped materials gelled too quickly, while the other organosilanes were not miscible and thus led to undesirable phase-separated materials. Finally, the five small-molecules additives were investigated for each of the 32 acceptable compositions (16 initial materials with or without GLS), and from this a total of 192 materials were identified that had suitably long gelation times to be carried forward for printability studies on the four slide surfaces. Thus,

(56) Besanger, T. R.; Chen, Y.; Deisingh, A. K.; Hodgson, R.; Jin, W.; Mayer, S.; Brook, M. A.; Brennan, J. D. *Anal. Chem.* 2003, 75, 2382–2391.

(57) Besanger, T. R.; Easwaramoorthy, B.; Brennan, J. D. *Anal. Chem.* 2004, 76, 6470–6475.

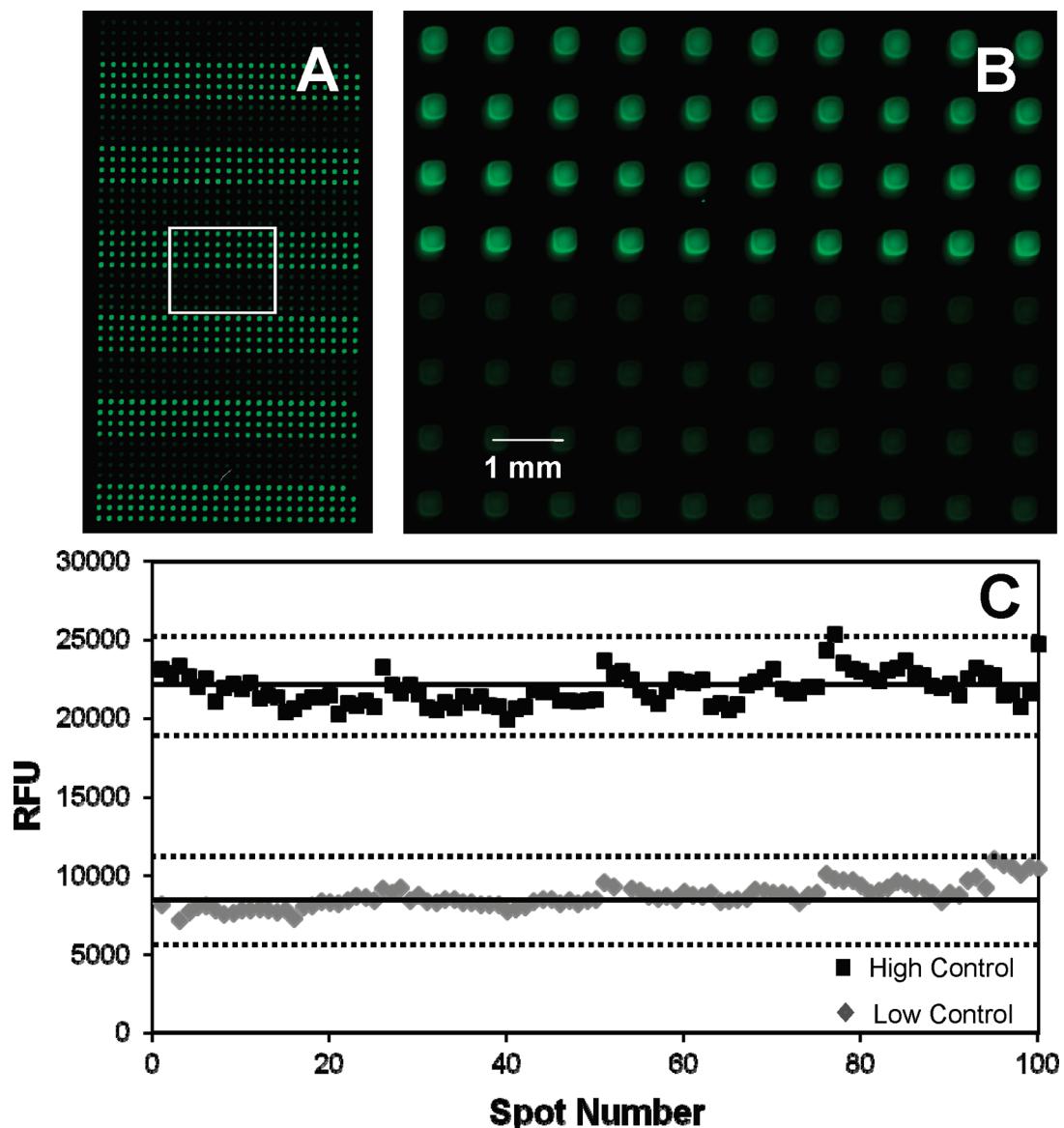


Figure 3. A section of AChE microarray showing HC (bright green) and LC (light green) spots (a black-green palette was applied as pseudocolor for clarity of presentation) (A), a magnified view of the boxed area to highlight spot morphology and alignment (B), and Z plot (C). The spots were pin-printed from solutions containing 200 U/mL AChE in 1.4 SS/1.0 PVA/glycerol and overprinted with 5% glycerol in 25 mM Tris, pH 7.0 containing 14 μ M BODIPY FL L-cystine, with or without 300 μ M ATCh. Solid lines indicate the mean of the replicates, while dashed lines correspond to 3SD.

of a total of \sim 20 000 potential compositions, it was possible to evaluate only 392 individual compositions and find \sim 50% of the materials to have suitable properties for further study.

The 192 materials were printed onto each of the four slide surfaces, aged at 4 °C for a minimum of 24 h, and then imaged by optical microscopy and assessed for cracking, phase separation, spreading, and the number of spots printed. A total of 155 material/surface combinations (out of a total of 768) allowed printing of 200 array elements in a single uptake and thus were considered “printable”. Amine and epoxy surfaces were the best for supporting sol–gel microarrays, while aldehyde and PMMA surfaces usually resulted in inconsistent printing (i.e., missing spots), irregular spot shapes or sizes, or rapid depletion of material from the pin (low spot numbers printed). At this stage, AChE was already incorporated in the spots, and the spots were evaluated for good morphology, signal reproducibility, and integrity after overprinting of reagents, the latter being indicative of resistance to hydration stress. A total of 66

material/surface combinations passed this step (see Supporting Information, Table S2 for a list of these combinations), all of which were also found to be able to maintain enzyme activity, as evidenced by PC/NC ratios greater than 1. Using a minimum PC/NC ratio of 3, in order to ensure a robust assay, narrowed the list down to 26 candidates. Interestingly, the list consisted mostly of DGS-based formulations printed on amine surfaces; however, for the purpose of fabricating AChE microarrays, an SS-based formulation (1.4 SS/1.0 PVA/glycerol) printed onto an amine-derivatized slide was chosen, as this material had the highest PC/NC ratio and produced spots with excellent spot-to-spot reproducibility. The screening data is summarized in Figure 2, showing the attrition of materials upon moving through the stages of the materials screening and providing images that are representative of failure modes observed at different stages.

Figure 3A shows a section of a typical AChE microarray prepared with the optimal material after performing a fluorimetric AChE activity assay, along with a magnified view of the area boxed

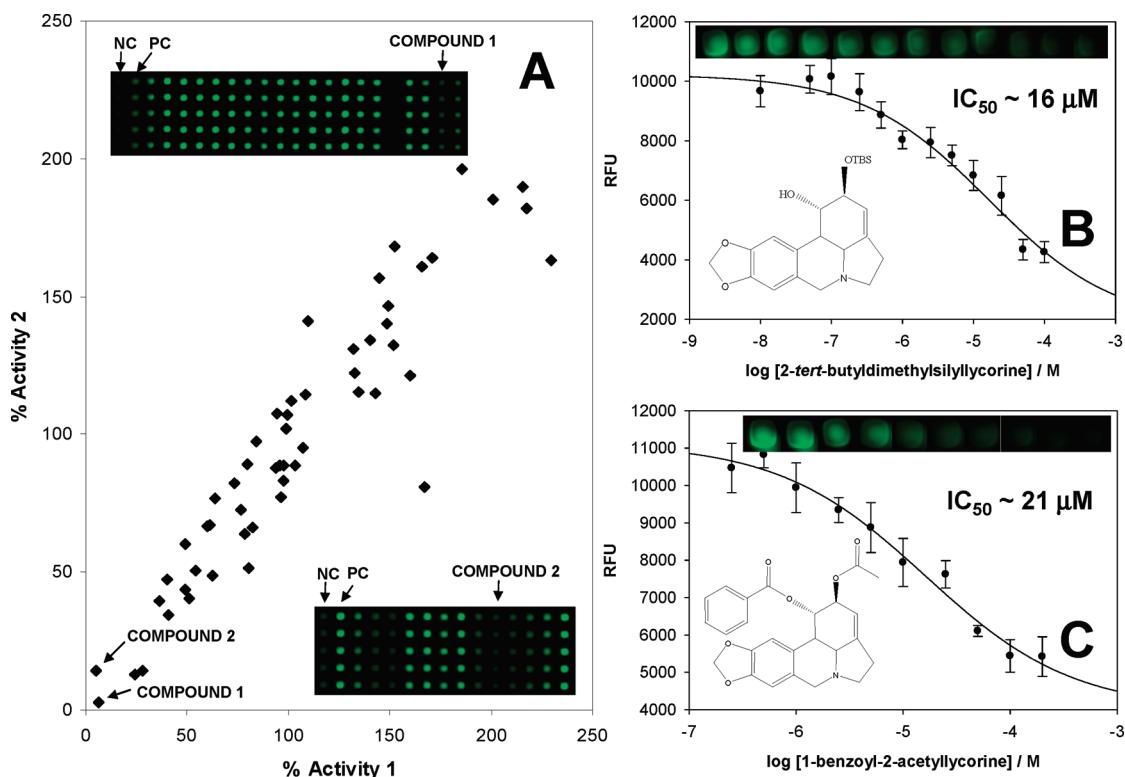


Figure 4. Duplicate plot for on-array screening of synthetic analogs of Amaryllidaceae alkaloids (A) and IC₅₀ plots of identified potential inhibitors marked as compound **1** (B) and compound **2** (C). Representative spots are shown to illustrate differences in signal proportional to inhibitor concentration.

by the white rectangle in Figure 3B. The microspots had uniform size (~400 μm in diameter and 16 μm in thickness, on the average) and shape and adhered well to the surface, presumably because of electrostatic interactions between the negatively charged silanol sites in the entrapment matrix and the positively charged amine-derivatized slide surface. No visible cracking could be observed in the microspots after overprinting, attesting to the ability of the material to withstand hydration stress. Additionally, a difference in signal between PC and NC was readily apparent, indicating that the material could support enzyme activity. The optimal material could also maintain AChE stability during storage. Arrays that were stored at 4 °C and then tested after 4 weeks and 10 weeks showed 91% and 81% activity, respectively, in comparison with one that was tested immediately after printing.

Detection of AChE Activity and Inhibition on Arrays. Initial optimization studies performed on the microarray included assessment of dye, enzyme, and substrate concentrations (see Supporting Information, Figures S5–S7) and reaction times. Once optimal conditions were determined, the reliability and reproducibility of the assay was assessed via the Z' test, which gives a measure of the robustness of the assay based on differences in the signal and SDs for HC and LC data, with a value of 0.5 generally regarded as the minimum required for applicability to screening. On the basis of assessment of 100 HCs and LCs, the mean and SD for HC and LC samples were $21\,951 \pm 1018$ RFU and 8761 ± 753 RFU, respectively (Figure 3C). The Z' factor was calculated to be 0.60, resulting in an “excellent assay” ranking, according to the system of Zhang et al.⁵⁸

(58) Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. *J. Biomol. Screen.* **1999**, *4*, 67–73.

Inhibition of AChE activity was tested by comparing signals from samples incubated with high levels (~30–50 μM) of known AChE inhibitors, exemplified by paraoxon, galanthamine, and sanguinine, to that obtained from a sample to which no inhibitor was added, prior to overprinting with BODIPY FL L-cystine and ATCh. Samples that were incubated with inhibitors had markedly lower fluorescence than the uninhibited sample. Moreover, the overall array-to-array variation in response using different concentrations of inhibitors was within 16%, highlighting the potential of the method for screening of AChE inhibitors (see Supporting Information, Figure S8 and Figures 4 and 5).

Screening for Inhibitory Activity. The Amaryllidaceae family is known to be a rich source of alkaloids with diverse biological activities⁵⁹ and have yielded the AChE inhibitors galanthamine (from *Galanthus nivalis*⁶⁰), and sanguinine (from *Eucharis grandiflora*⁶¹). To determine whether other compounds that are similarly active against AChE could be found from the same family, the AChE microarray was screened against a small library of compounds, consisting mostly of synthetic analogs of Amaryllidaceae alkaloids. Each compound was tested twice at a concentration of ~30–50 μM following the inhibition assay described above in order to produce the duplicate plot shown in Figure 4A. The data fall on a diagonal, which is indicative of the data being highly reproducible between assays. The data demonstrate that a significant number of compounds (9 of 62) show <50% activity

(59) Weniger, B.; Italiano, L.; Beck, J. P.; Bastida, J.; Bergonon, S.; Codina, C.; Lobstein, A.; Anton, R. *Planta Med.* **1995**, *61*, 77–79.

(60) Svedberg, M. M.; Bednar, I.; Nordberg, A. *Neuropharmacology* **2004**, *47*, 558–571.

(61) Lopez, S.; Bastida, J.; Viladomat, F.; Codina, C. *Life Sci.* **2002**, *71*, 2521–2529.

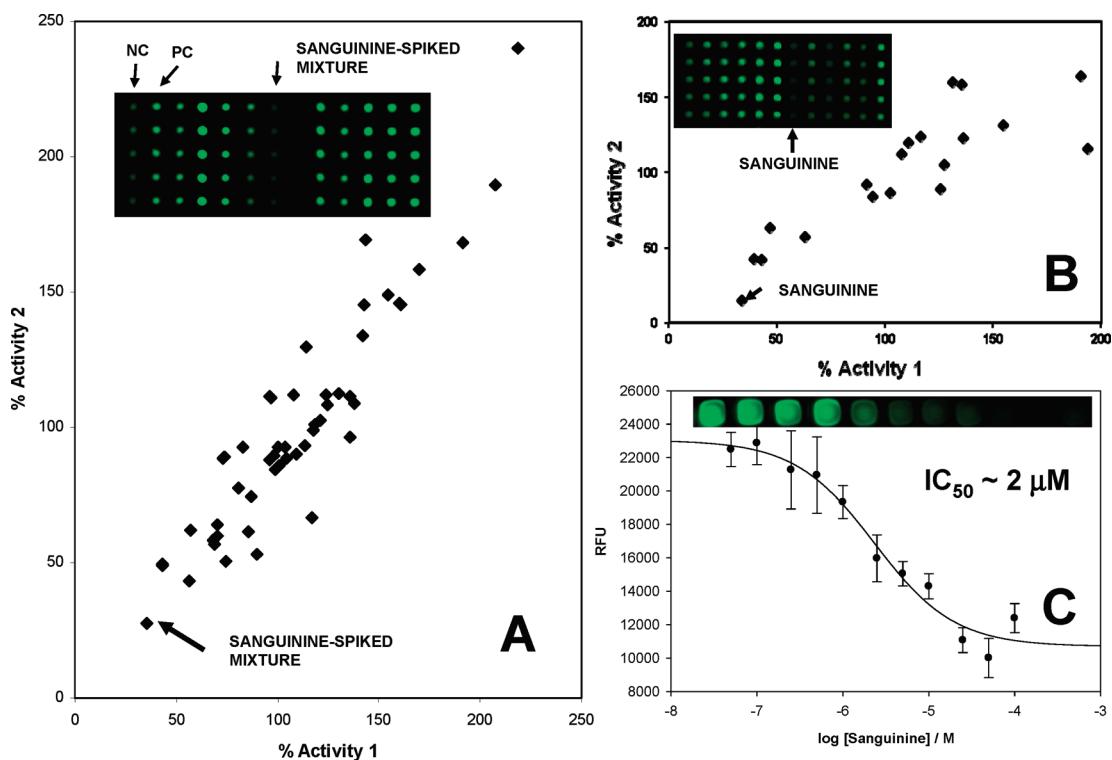


Figure 5. Duplicate plots for on-array screening of mixtures of druglike compounds (A), the components of sanguinine-spiked mixture (B), and IC₅₀ plot of sanguinine, with representative spots shown to illustrate differences in signal proportional to inhibitor concentration (C).

in both assays, attesting to the importance of the Amaryllidaceae family of compounds as inhibitors of AChE. Interestingly, most of the compounds that exhibited such inhibitory activity were found to possess a lycorine-type framework. These findings are in good agreement with the results of a previous AChE screen of Amaryllidaceae alkaloids having different ring types,⁶² in which lycorine-type compounds were found to be the most active. Two of the compounds that showed significant inhibition (i.e., <20% residual activity in both assays) were identified as 2-*tert*-butyldimethylsilyllycorine and 1-benzoyl-2-acetyllycorine. These same compounds were included among those identified as potential AChE inhibitors in plate-based screening using Ellman assay, from which structure–activity data has recently been reported.⁵²

The AChE microarray was also evaluated for its utility in quantitative assays of inhibitor potency, as determined by IC₅₀ values. The microspots were incubated with solutions of the potential inhibitors at varying concentrations straddling their expected IC₅₀ values based on the Ellman assay.⁵² The levels of fluorescence signals from the microspots were found to relate inversely to the concentration of the inhibitor with which they were incubated previously, allowing for the determination of IC₅₀ values, as shown in Figure 4B,C. The calculated K_i values for 2-*tert*-butyldimethylsilyllycorine and 1-benzoyl-2-acetyllycorine were 2.6 and 3.9 μM, respectively, which were in reasonable agreement with the values obtained using Ellman assay (0.9 μM and 1.0 μM, respectively).⁵²

Mixtures of druglike compounds were also screened for possible activity against AChE, with each mixture comprised of 20 compounds. These mixtures were used for primary screening,

followed by deconvolution in order to identify the active component. In this way, the assay throughput could be significantly increased. Using only 52 mixtures, a total of 1040 compounds were screened rapidly. Each of these mixtures was tested twice, producing the duplicate plot shown in Figure 5A. As was the case for the lycorines, the data in the duplicate plot was highly reproducible. However, in this case, none of these mixtures yielded results that suggested the presence of a potent inhibitor from among the compounds in this library, with the exception of one mixture that was spiked with sanguinine, which showed <50% activity for both assays. Two other mixtures showed <50% activity in at least one assay and suggested that weaker inhibitors may also be present in these mixtures. When the components of the most potent mixture were tested individually, sanguinine was confirmed to be responsible for the inhibitory action observed in the mixture (Figure 5B). Again, determination of the IC₅₀ value could be performed on an array (Figure 5C). These results clearly underscore the potential of this technique for rapid, high-throughput screening of small molecules.

Overall, the new AChE assay in the microarray format enables qualitative screening for inhibitory activity, as well as quantitative determination of potency of action. It also affords significant time and cost savings. Although three printing cycles (one to print and two to overprint) are required to fabricate and assay the arrays, each cycle entails only a few minutes to complete (e.g., 5 min to print or to overprint on 100 microspots, including pin washing and drying, per pin). Use of multiple pins (up to 48 per printhead) could vastly increase the throughput, allowing for parallel processing of a far higher number of samples than is possible in plate-based assays on a similar time scale. Each array element utilizes only nanoliter volumes of reagents (6.9 nL to print, 12.5 nL to

(62) Elgorashi, E. E.; Stafford, G. I.; Van Staden, J. *Planta Med.* 2004, 70, 260–262.

overprint), drastically reducing total compound consumption and permitting runs of more analytical replicates for better reliability of results. With the low cost associated with array fabrication and running assays, as well as the short processing time involved, the AChE microarrays can be employed as cost-effective, single-use devices, thus making for a facile platform for high-throughput screening.

CONCLUSIONS

A novel fluorimetric AChE activity assay utilizing a thiol-reactive, fluorogenic dye has been developed and utilized in conjunction with sol–gel-derived AChE microarrays to allow high-throughput, nanovolume screening of small molecules. The new assay does not rely on changes in pH to produce a signal, and thus it is less sensitive to buffer concentration and ionic strength than current fluorimetric assays. The new assay follows Michaelis–Menten kinetics and provides K_M values that are comparable to the values determined using the more conventional Ellman assay. The microarray-based assay showed excellent reproducibility ($Z = 0.60$) and could be used quantitatively to measure both activity and inhibition of AChE and to assess the inhibition constant of hits identified in primary screens. We also demonstrated that it is possible to rapidly detect inhibitors present in

compound mixtures, which increased the throughput of the primary screen while reducing reagent consumption. Future work will include the development of higher throughput assay formats based on increasing the number of immobilized enzymes per array and the number of sample delivery pins to increase the parallelization of inhibitor delivery.

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SUPPORTING INFORMATION AVAILABLE

Additional material is available as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org> free of charge.

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