

Video Article

A Guided Materials Screening Approach for Developing Quantitative Sol-gel Derived Protein Microarrays

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Abstract

Microarrays have found use in the development of high-throughput assays for new materials and discovery of small-molecule drug leads. Herein we describe a guided material screening approach to identify sol-gel based materials that are suitable for producing three-dimensional protein microarrays. The approach first identifies materials that can be printed as microarrays, narrows down the number of materials by identifying those that are compatible with a given enzyme assay, and then hones in on optimal materials based on retention of maximum enzyme activity. This approach is applied to develop microarrays suitable for two different enzyme assays, one using acetylcholinesterase and the other using a set of four key kinases involved in cancer. In each case, it was possible to produce microarrays that could be used for quantitative small-molecule screening assays and production of dose-dependent inhibitor response curves. Importantly, the ability to screen many materials produced information on the types of materials that best suited both microarray production and retention of enzyme activity. The materials data provide insight into basic material requirements necessary for tailoring optimal, high-density sol-gel derived microarrays.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50689/>

Introduction

Microarrays have gained popularity within the scientific community as a method for increasing assay throughput. Since the development of microarray technology to assess gene expression¹ in the mid-1990s, microarrays have found use in the development of high-throughput assays to identify protein-protein and protein-small molecule interactions, and to find new materials with unique properties.²⁻⁵ More recently, microarrays have been developed wherein specific materials are used to immobilize functional proteins, producing a three-dimensional microarray element within which proteins are entrapped, allowing for facile measurement of enzymatic activity and inhibition on the microarray itself using a suitable fluorescence assay that is coupled to substrate turnover.⁵⁻¹⁰ Importantly, such microarrays can be designed to include all necessary components to screen samples and controls together in a highly paralleled fashion.^{5,b}

Early examples of protein microarrays were typically prepared using standard methods for protein immobilization onto solid supports, such as covalent attachment,¹¹ affinity capture³ and physical adsorption.¹² While these methods of bio-immobilization allow for increased sample concentration and accelerated reaction kinetics required for assay miniaturization, they each suffer drawbacks. In general, all cause a reduction in native biomolecule functionality owing to chemical modification of the surface, hindered access to active sites, or improper orientation due to non-specific immobilization. Thus, all methods result in lower assay sensitivity despite an increase in biomolecule deposition, a response that likely arises due to the need to artificially bind biomolecules to a surface.

An emerging approach for the production of functional biomolecule microarrays is through pin-printing of protein-doped silica sols onto solid supports, which are typically either functionalized glass slides or individual wells of microwell plates. The sol-gel process itself takes place in an aqueous environment at room temperature, and it is the liquid precursor that is printed and then gels to entrap biomolecules within the 3D matrix, allowing for high protein loading¹³ as well as entrapment of multiple components within the same microarray element.^{13,14} Tailoring of the sol-gel derived material can be done through careful selection of different silica precursors, as well as by altering the aqueous component through use of different buffers (pH, ionic strength), and inclusion of various additives (polymers, small molecules) to achieve an optimal material, the specific nature of which depends on the biomolecule that is entrapped.¹⁰

A potential limitation associated with developing sol-gel derived protein microarrays via pin-printing is the need to identify sol-gel based composite materials that can be printed without gelling in the pin or showing undesirable properties (irreproducible spot sizes, cracking, poor adhesion, incompatibility with assay components, poor protein activity) once printed on a surface.⁵ Simultaneous optimization of all of these

parameters precludes an approach wherein materials can be designed *de novo*, or examined slowly in a serial manner. On the other hand, random screening of many thousand or tens of thousands of materials is neither time- nor cost-effective.

In this article, we describe a directed screening approach that allows rapid identification of suitable materials for production of protein microarrays without the need to randomly screen large numbers of materials. Using a guided approach, materials suitable for microarray printing are first identified, followed by a series of small-scale screens to identify optimal sol-gel derived material combinations that can be printed reproducibly, without cracking and are compatible with a given assay. Finally, optimal materials are identified based on retention of enzyme activity and performance in a final small-molecule screening assay. In this way, optimal materials can be identified from many thousand candidates using only a few hundred assay steps. We demonstrate this approach for fabrication of both high-density acetylcholinesterase and multikinase microarrays and the use of such microarrays for small-molecule screening.

Protocol

1. Preparation of Additive Solutions

1. Prepare 25 and 50 mM Tris(hydroxymethyl)aminomethane (Tris base, M_r 121.14 g/mol) and HEPES (M_r 238.3 g/mol) at pH 7.0, 7.2, 7.4, 7.6, 7.8, 8.0 and 8.2 in 50 ml centrifuge tubes. Adjust the pH using 1 N HCl and NaOH, respectively. Store at room temperature and replace after 3 months.
2. Prepare 24% (w/v) poly(vinyl alcohol) (PVA): weigh 2.4 g of PVA (M_w 9,000 - 10,000, 80% hydrolyzed) and add to 10 ml of deionized-distilled water (ddH₂O), dissolve the polymer pellets by vortexing. Use the 24% (w/v) solution to prepare equal volumes of 16% (w/v), 12% (w/v) and 8% (w/v) PVA solutions. Degas solutions by sonication before use. Store at 4 °C for up to 1 month.
3. Prepare 24% (w/v) polyethylene imine (PEI): add 4.8 ml of PEI (M_w 1,300, 50% (w/w) in H₂O) to 5.2 ml of ddH₂O. Use the 24% (w/v) solution to prepare equal volumes of 16% (w/v), 12% (w/v) and 8% (w/v) PEI solutions. Degas solutions by sonication before use. Store at 4 °C for up to 1 month.
4. Prepare 60% (w/v) polyethylene glycol (PEG): weigh 6 g of PEG (M_w 600) and add to 10 ml of ddH₂O, dissolve the polymer pellets by vortexing. Use serial dilution to prepare an equal volume of 30% (w/v) PEG solution. Degas solutions by sonication before use. Store at 4 °C for up to 1 month.
5. Prepare 24% (v/v) N-(3-triethoxysilylpropyl) gluconamide (GLS): add 480 μ l of GLS (50% in ethanol - stock GLS may require the use of a water bath to warm and dissolve solution if crystalline) to 520 μ l of 95% (v/v) ethanol and mix for 20 min by sonication. Use the 24% (v/v) solution to make an equal volume of 16% (v/v) GLS solution. Make fresh solutions for use per day.
6. Prepare 24% (v/v) methyltrimethoxysilane (MTMS): add 240 μ l of MTMS to 760 μ l of existing acidified ddH₂O (pH 2.0, 1 N HCl) and mix for 20 min by sonification. Use the 24% (v/v) solution to make an equal volume of 16% (v/v) MTMS solution. Make fresh solutions for use per day.
7. Prepare 24% (v/v) bis[(3-methyldimethoxysilyl)propyl]polypropylene oxide (MDSPPPO): add 240 μ l of MDSPPPO to 760 μ l of existing acidified ddH₂O (pH 2.0, 1 N HCl) and mix for 20 min by sonification. Use the 24% (v/v) solution to make an equal volume of 16% (v/v) MDSPPPO solution. Make fresh solutions for use per day.
8. Prepare 24% (v/v) 3-(aminopropyl)-triethoxysilane (APTES): add 240 μ l of APTES to 760 μ l of existing acidified ddH₂O (pH 2.0, 1 N HCl) and mix for 20 min by sonification. Use the 24% (v/v) solution to make an equal volume of 16% (v/v) APTES solution. Make fresh solutions for use per day.
9. Prepare 24% (v/v) bis(triethoxysilyl)ethane (bis-TEOS): add 240 μ l of bis-TEOS to 760 μ l of existing acidified ddH₂O (pH 2.0, 1 N HCl) and mix for 20 min by sonification. Use the 24% (v/v) solution to make an equal volume of 16% (v/v) bis-TEOS solution. Make fresh solutions for use per day.
10. Prepare 24% (v/v) carboxyethylsilanetriol (Si-COOH): add 960 μ l of Si-COOH (25% in ddH₂O) to 40 μ l of existing acidified ddH₂O (pH 2.0, 1 N HCl) and mix for 20 min by sonification. Use the 24% (v/v) solution to make an equal volume of 16% (v/v) Si-COOH solution. Make fresh solutions for use per day.
11. Separately prepare 3 mM N_ε-acetyl-L-lysine, D-sorbitol, α - α -trehalose (M_r 188.22 g/mol, 182.17 g/mol, and 378.33 g/mol, respectively) and 1.5 mM Triton X-100 (average M_w 625 g/mol) in ddH₂O. Volumes can vary depending on how much is required; make fresh solutions for use per day.
12. Prepare 60% (w/w) glycerol: add 2.4 g of anhydrous glycerol to 1.6 g of ddH₂O, mix by vortex. Degas solution by sonication before use. Store at 4 °C for up to 1 month.
13. Prepare 30% (w/w) glycerol: add 1.6 g of anhydrous glycerol to 2.4 g of ddH₂O, mix by vortex. Degas solution by sonication before use. Store at 4 °C for up to 1 month.

2. Preparation of Silica Sols

Following the procedures below, the respective sols, when kept on ice, can be used up to 1 hr after addition of water. Sols used beyond 1 hr result in decreased/inconsistent material gelation times.

1. Preparation of a sodium-silicate (SS) based sol
 1. Weigh out 120 g of ion exchange resin into a 500 ml plastic beaker.
 2. Add 150 ml of 0.1 N HCl and stir for 1 hr using a 2-inch magnetic stir bar.
 3. Filter the solution using a Büchner funnel connected to vacuum.
 4. Slowly add ddH₂O to wash the filtered resin until the resulting filtrate is clear. This takes approximately 100 ml of ddH₂O.
 5. Collect and store the prepared resin in a plastic container at room temperature for up to 1 month.
 6. Weigh out 2.59 g of sodium silicate (SS, 27% (w/w) SiO₂, 10% (w/w) NaOH) into a 50 ml plastic beaker.
 7. Add 10 ml of ddH₂O to the SS. Swirl gently by hand to mix the solution.
 8. Weigh out 5.60 g of prepared resin into a separate 50 ml plastic beaker. Add to the sodium silicate solution and mix for 2 min using a one-inch magnetic stir bar.

9. Filter the mixture solution with a Büchner funnel connected to an aspirator attached to a water faucet.
 10. Use a 10 ml plastic syringe equipped with a 0.2 μm membrane filter to filter the sol solution. This yields a sol with 5.6% (w/w) silica referred to as SS in further text. Keep the sol on ice when not in use.
 11. $\frac{1}{2}$ SS: add 1 ml of prepared SS sol to 1 ml of ddH₂O, mix by vortex. Keep the sol on ice when not in use.
2. Preparation of a diglycerylsilane (DGS) based sol
 1. Synthesize DGS as described elsewhere.^{15,16} Store crystalline DGS in a desiccator at room temperature for up to 6 months.
 2. Weigh out roughly 1 g of crystalline DGS.
 3. Use a mortar and pestle to grind the DGS to a fine powder. Complete this step as quickly as possible to prevent moisture absorption from the air.
 4. Carefully transfer the finely ground DGS to an empty 20-ml scintillation vial, record the mass (to a thousandth of a gram).
 5. Add ddH₂O to yield a 0.5 g/ml DGS.
 6. Sonicate the hydrolyzed DGS on ice for 20 min, mix by vortex for 5 sec every 5 min. During humid days, complete dissolution of DGS may require adding 10 μl of 1 N HCl. This should be added to the solution before sonication.
 7. Use a 3-ml plastic syringe equipped with a 0.2- μm membrane filter to filter the sol solution, removing fine particles. This yields a sol with 5.0% (w/w) silica referred to as DGS in further text. Keep the sol on ice when not in use.
 8. $\frac{1}{2}$ DGS: add 1 ml of prepared DGS sol to 1 ml of ddH₂O, mix by vortex. Keep the sol on ice when not in use.

3. Pre-screening to Identify Printable Materials

A generalized scheme showing the different stages of the guided factor analysis used to identify printable materials is shown in **Figure 1**. A minimum total material volume of 100 μl is recommended. Use of smaller volumes makes visualizing material gelation difficult.

1. Stage 1: buffer and silane
 1. Add 50 μl of buffer (25 and 50 mM Tris or HEPES at pH 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2) to a 2 ml glass scintillation vial.
 2. Add 50 μl of the appropriate sol (DGS, $\frac{1}{2}$ DGS, SS, $\frac{1}{2}$ SS) and vortex mixture in an on-and-off fashion for 10 sec by moving the vial on and off the vortex mixer.
 3. Set materials to rest at room temperature, monitoring the time of material gelation. The gelation time is defined as the point at which the material stops flowing freely upon rotating the vial to a 45° angle.
 4. Exclude material combinations with gelation times less than 2.5 hr from subsequent material screening stages.
2. Stage 2: buffer, silane and polymer
 1. In a 2-ml glass scintillation vial, add 3.13 μl of either 16% (w/v) or 8% (w/v) PVA/PEI solution or 6.3 μl of 30% (w/v) or 60% (w/v) PEG solution.
 2. Add 50 mM HEPES (pH 8.0) to bring the combined volume to 50 μl , mix the solution by vortex.
 3. Add 50 μl of the appropriate sol (DGS, $\frac{1}{2}$ DGS, SS, $\frac{1}{2}$ SS), mix in the fashion described in step 3.1.2.
 4. Exclude material combinations with gelation times (as defined in step 3.1.3) less than 2.5 hr from subsequent material screening stages.
3. Stage 3: buffer, silane, polymer and organosilane
 1. In a 2-ml glass scintillation vial, add 3.13 μl of either 16% (w/v) or 8% (w/v) PVA solution or 6.3 μl of 30% (w/v) or 60% (w/v) PEG solution.
 2. Add 3.13 μl of 16% (w/v) organosilane (GLS, MTMS, MDSPPO, bis-TEOS, Si-COOH or APTES).
 3. Add 50 mM HEPES (pH 8.0) to bring the combined volume to 50 μl , mix the solution by vortex.
 4. Add 50 μl of the appropriate sol (DGS, $\frac{1}{2}$ DGS, SS, $\frac{1}{2}$ SS) and mix in the fashion described in step 3.1.2. Exclude material combinations with gelation times (as defined in step 3.1.3) less than 2.5 hr from subsequent material screening stages.
4. Stage 4: buffer, silane, polymer, organosilane and small-molecule additive
 1. In a 2-ml glass scintillation vial, add 2.08 μl of either 24% (w/v) or 12% (w/v) PVA solution or 6.3 μl of 30% (w/v) or 60% (w/v) PEG solution.
 2. Add 2.08 μl of 24% (w/v) organosilane (GLS, Si-COOH).
 3. Add 2.08 μl of the 3 mM small-molecule solution (*N*_ε-acetyl-L-lysine, D-sorbitol, α - α -trehalose) or 1.5 mM (Triton X-100).
 4. Add 50 mM HEPES (pH 8.0) to bring the combined volume to 50 μl , mix the solution by vortex.
 5. Add 50 μl of the appropriate sol (DGS, $\frac{1}{2}$ DGS, SS, $\frac{1}{2}$ SS) and mix in the fashion described in step 3.1.2.
 6. Exclude material combinations with gelation times (as defined in step 3.1.3) less than 2.5 hr from subsequent material printing stages.

4. Preparation of Printable Protein-doped Materials

The materials identified at the end of step 3.4.6 are carried forward in printability studies, with the appropriate enzyme now being included in the sol. In all cases outlined below, aqueous solution containing all components except for the silane is prepared in a microwell, followed by the addition of the sol just prior to printing. To accommodate the use of a 384-microtiter plate, a total solution volume of 50 μl is used instead of 100 μl .

1. Acetylcholinesterase (AChE) microarray
 1. Prepare a 2 kU/ml solution of AChE in 50 mM HEPES (pH 8.0) following the lot specific enzyme activity (units of activity per mg) information provided by the manufacturer.
 2. Aliquot 2 kU/ml enzyme stock solution into 5 μl fractions using 500- μl microcentrifuge tubes and store at -20 °C. 2 kU/ml AChE stock solutions remain stable for up to 4 months when stored at -20 °C.

3. Prepare base materials by combining half the volumes of corresponding polymers, organosilanes and small-molecule additives identified through step 3.4.6 in a 384-well microtiter plate.
 4. Add 5 μ l of 2 kU/ml AChE in 50 mM HEPES (pH 8.0).
 5. Using 50 mM HEPES (pH 8.0), bring the combined well volume to 25 μ l mix by pipetting the solution up and down several times.
2. Multikinase microarray
 1. Prepare 100 ng/ μ l kinase solutions (p38 α , MAPK2, EGFR, GSK-3 β) in ddH₂O following the activity (U/ml or U/mg) and quantity information provided by the manufacturer. Store kinase solutions in aliquots of 2 μ l at -80 °C.
 2. Prepare kinase substrates (MBP, p(E₄Y), GSM) at 5 mg/ml, 50 mg/ml or 300 μ M in ddH₂O, following the quantity information provided by the manufacturer. Store substrates in aliquots of 2.5 μ l at -80 °C.
 3. Prepare base materials by combining half the volumes of corresponding polymers, organosilanes and small-molecule additives identified through step 3.4.6 in a 384-well microtiter plate.
 4. To each well containing the base materials, add 2.5 μ l kinase and 2 μ l of its respective substrate (p38 α and MBP, MAPK2 and MBP, EGFR and p(E₄Y), GSK-3 β and GSM) as prepared in steps 4.2.1 and 4.2.2, respectively.
 5. Using 50 mM HEPES (pH 7.4), bring the combined well volume to 25 μ l. Mix by pipetting.

5. Microarray Formation

This section explains the detailed procedure for printing materials on a single slide surface. To print on multiple modified slide surfaces (amine, epoxy, aldehyde and PMMA), this procedure is repeated 4 times.

1. Form microarrays by using a contact pin-printing robot equipped with an XYZ stage. Use slotted sheath pins of 100 μ m diameter to deposit the protein-doped sols.
2. Set humidity within the printing chamber to 80-90%. Humidity <80% may result in sample evaporation and inconsistencies in printing, due to the small deposition volumes.
3. Sonicate pins in ddH₂O for 15 min prior to printing and dry under a stream of nitrogen. Use a pipe cleaner to remove residual moisture from within the pin holder and carefully place the pin in the print head. Failure to remove residual moisture may hinder the pin from moving freely with the holder, resulting in missed spots.
4. Control array patterns through the Chip Writer Pro (CWP) program. For each sample well within the 384-well source plate, 200 spots (maximum number of spots per uptake using the SMP3 sheath pin) are printed onto a surface-modified glass slide.
5. Commence the printing process through the software. Set travel in the XY direction to 10 mm/s and sample approach speed (Z direction) to 2 mm/s with a 2.5 sec sample loading time.
6. Pause the run through CWP. Lower the pin into the sample and add 25 μ l of the respective sol (DGS, 1/2DGS, SS, 1/2SS) to the well by pipette. Mix the solution using an up-and-down pipetting motion repeated 50x. When mixing by pipetting, minimize the amount of air incorporated into the solution. Air bubbles prevent complete loading of sample within the pin.
7. Commence the printing process through CWP, and print the sample onto one slide surface. Pause the printing process before loading sample from the subsequent source plate well.
8. Remove the printing pin using a magnet and place a pipe cleaner in the print head to prevent moisture buildup in the print head.
9. Rinse the printing pin with ddH₂O, and sonicate in clean ddH₂O for 30 sec.
10. Dry the printing pin under a stream of nitrogen and remove the pipe cleaner, placing the pin back into the print head.
11. Commence printing by following steps 5.6 - 5.10, for all samples remaining within the source plate. Up to 12,000 spots of 100 μ m diameter can be deposited onto a single slide.
12. This method can also be applied to printing materials onto the bottom of wells within a 96-well microplate. Following the CWP calibration file, recalibrate the printing pin to ensure the distance travelled up between spot deposition is above the height of the microwell plate. This allows the pin to print consistently from well to well in a linear fashion without damaging the pin.
13. Age the array for a minimum of 30 min and up to 24 hr within the printing chamber at 80-90% humidity following completion of the entire printing experiment.

6. Acetylcholinesterase Activity Assay

1. Preparing positive control (PC) samples
 1. Prepare 1 mM acetylthiocholine iodide (ATCh: M_r 289.18 g/mol) in 4% glycerol, 25 mM Tris (pH 7.0) in a 1.5 ml microcentrifuge tube. ATCh needs to be prepared fresh before each use and using a buffer at pH 7.0 as higher pH buffers cause rapid autohydrolysis, producing false positives. The final volume can be varied depending on the number of samples (25 μ l per sample).
 2. Add 0.14 μ l of 5 mM bodipy-FI-L-cystine to 25 μ l of 1 mM ATCh in the well of a 384-microtiter plate.
 3. Add 24.86 μ l 4% glycerol, 25 mM Tris (pH 7.0); mix by pipetting carefully to avoid bubble formation in the solution. Potential enzyme inhibitors can be incorporated into the PC solution before bringing the volume to 50 μ l with buffer.
2. Preparing negative control (NC) samples
 1. Add 0.14 μ l of 5 mM bodipy-FI-L-cystine to 49.86 μ l of 4% glycerol, 25 mM Tris (pH 6.5) in the well of a 384-microtiter plate. Mix by pipetting.
3. Overprinting PC and NC solutions
 1. Overprint the aged microarrays following steps 5.1 - 5.10 (ignoring step 5.6) of the protocol. Use slotted sheath pins of 235 μ m diameter to deposit the PC and NC overprinting solutions. This ensures the solution covers the previously deposited spot entirely.
 2. Age arrays at 80-90% humidity for 1 hr at room temperature. Due to the autohydrolysis of ATCh, longer incubation times may result in false positives or increased enzyme activity.

7. Kinase Activity Assay

1. Prepare 500 μM adenosine 5'-triphosphate (ATP) solution using stock ATP (100 mM) stored at $-20\text{ }^{\circ}\text{C}$. Make fresh solution for each experiment and adjust the volume to experimental need: each printing solution requires 5 μl .
2. Prepare a 100 mM magnesium chloride (MgCl_2 ; M_r 95.21 g/mol) stock solution in ddH_2O .
3. Positive control (PC)
 1. Add 2.5 μl of 100 mM MgCl_2 and 5 μl 500 μM ATP to the well of a 384-microtiter plate.
 2. Bring the total well volume to 50 μl with 50 mM HEPES (pH 7.4); mix by pipetting. Potential enzyme inhibitors can be incorporated into the PC solution before bringing the volume to 50 μl with buffer.
4. Negative control (NC)
 1. Add 2.5 μl of 100 mM MgCl_2 and 5 μl ddH_2O to the well of a 384-microtiter plate.
 2. Bring the total well volume to 50 μl with 50 mM HEPES (pH 7.4) and mix by pipetting.
5. Overprinting PC and NC assay cofactors
 1. Follow step 6.3.1 of the protocol.
 2. Age arrays at 80-90% humidity for 2 hr at room temperature.
6. Slide staining
 1. Place printed microarray slides individually in a Petri dish with enough dye (from kit) to cover the entire slide. Shake moderately (~ 200 rpm) for 45 min using a plate shaker.
 2. Remove the slide using forceps and place into a clean Petri dish with wash buffer (from kit). Shake moderately (~ 200 rpm) for 45 min using a plate shaker.
 3. Remove the slide using forceps and spin dry using a conventional desktop microarray microcentrifuge equipped with a slide holder.

8. Microarray Imaging and Analysis

1. Imaging

Note that the imaging method will be specific to the type of array reader available. For these studies, an Alpha Innotech NovaRay imager with a white light source and CCD detection system equipped with a 478 ± 17 nm excitation and 538 ± 21 nm emission filter for the AChE microarrays, and 530 ± 40 nm excitation and 614 ± 62 nm emission filter for kinase microarrays was used. Confocal laser scanning systems can also be used for reading the arrays, though the settings will be specific to the instrument.

 1. Place slide into the slide holder with the spots facing up.
 2. Set the number of preview sections (a minimum of 2 is recommended, one on either end of the microscope slide), resolution (a minimum of 4 μm is recommended) and exposure (auto is recommended) within the imaging software.
 3. Acquire slide image and save as a ".tiff" file.
2. Analysis
 1. Open acquired slide image in ImageJ64.
 2. Click the oval selection tool and measure the signal intensity of each spot using the measure option under the analyze tab. When selecting the area to measure, select a region slightly larger than the observed spot and of consistent size among spots to reduce ImageJ subjectivity.
 3. Average the intensity from 25 similar PC spots, divided by the average intensity of 25 similar NC spots to obtain PC/NC ratios for the individual material compositions.

Representative Results

By performing a guided factor analysis for the material screen, we were able to minimize the number of materials tested from $\sim 20,000$ to a few hundred that had gelation times suitable for printing. By applying a strict guideline requiring material gelation time of 2.5 hr or greater, materials likely to clog the printing pins or produce irreproducible arrays were never printed. The printable materials identified to have sufficient (>2.5 hr) gelation times were printed onto 4 different functionalized glass slide surfaces. In order to be considered "printable", the maximum number of spots per uptake volume of the pin had to be printed ($\text{SMP}_3 = 200$). Spots were also assessed for spot morphology to ensure no cracking or undesirable phase separation had occurred using simple brightfield microscopy as shown in **Figure 2**.

From this stage of identified printable materials, microarrays were produced with AChE and kinases incorporated into the buffered aqueous component. Materials that were compatible with the assay procedure (including potential overprinting and washing or staining steps) were identified by observing retention of microarray spots (no cracking, loss of spots or unusual fluorescence patterns) and a positive control (PC) to negative control (NC) ratio greater than 1 as observed through image. As this was roughly 50% of the materials, a greater PC/NC ratio of 3 was used to define optimal materials with retention of protein activity. Through this method, 26 sol-gel derived materials containing AChE and 2 materials containing kinases satisfied the >3 PC/NC criteria. **Figure 3** and **Figure 4** show a graphical breakdown of the 5 guided material screen steps for the identification of optimal AChE and kinase microarrays, respectively.

The assays could also be validated through the generation of a Z' score.¹⁷ This was done using the material that produced the highest PC/NC ratio. **Figure 5** shows the Z' plot obtained by comparing the signal generated from 200 spots, 100 PC and 100 NC after overprinting the indicator dye and substrate on the AChE array. The AChE and the kinase arrays resulted in the respective Z' scores of 0.60 and 0.67, indicative of an excellent assay. However, it should be noted that before assay validation, on-array enzyme, dye, substrate and cofactor concentrations had to

be optimized by overprinting a range of concentrations of each component and selecting the concentration that produced the highest signal, as described in detail elsewhere.⁵

To validate the assays, quantitative inhibition data were obtained using known and unknown AChE inhibitors, with results performed in duplicate and used to produce duplicate plots (Figure 6A) and inhibition curves (Figures 6B and 6C). Spots were first overprinted with mixtures of known biologically active small-molecule inhibitors then with dye and substrate, and control mixtures containing either known inhibitors or no inhibitor were included. Duplicate plots were generated to assess enzyme activity, and any mixtures that resulted in less than 25% enzyme activity were considered positive for inhibition. Individual compounds from such mixtures were then tested in duplicate to identify the specific small molecule(s) responsible for inhibition. Once identified, these small molecules were used to generate quantitative inhibition curves to determine IC₅₀ values and inhibition constants.

Similar qualitative results were obtained using the multikinase array with a common kinase inhibitor, staurosporine. Figure 7A and 7B show the microarray image and indicate that the signal intensities after overprinting and staining the multikinase array are as expected for a negative control (- ATP), positive control (+ ATP) and known inhibitor (+ ATP + inh). To demonstrate the ability to obtain quantitative inhibition data from the microarrays, a concentration dependent inhibition assay was done for a single kinase. As shown in Figures 8A and 8B, signal intensity decreases as inhibitor concentration increases, and the response follows the expected concentration dependent inhibition curve for the p38α/MBP kinase/substrate system.

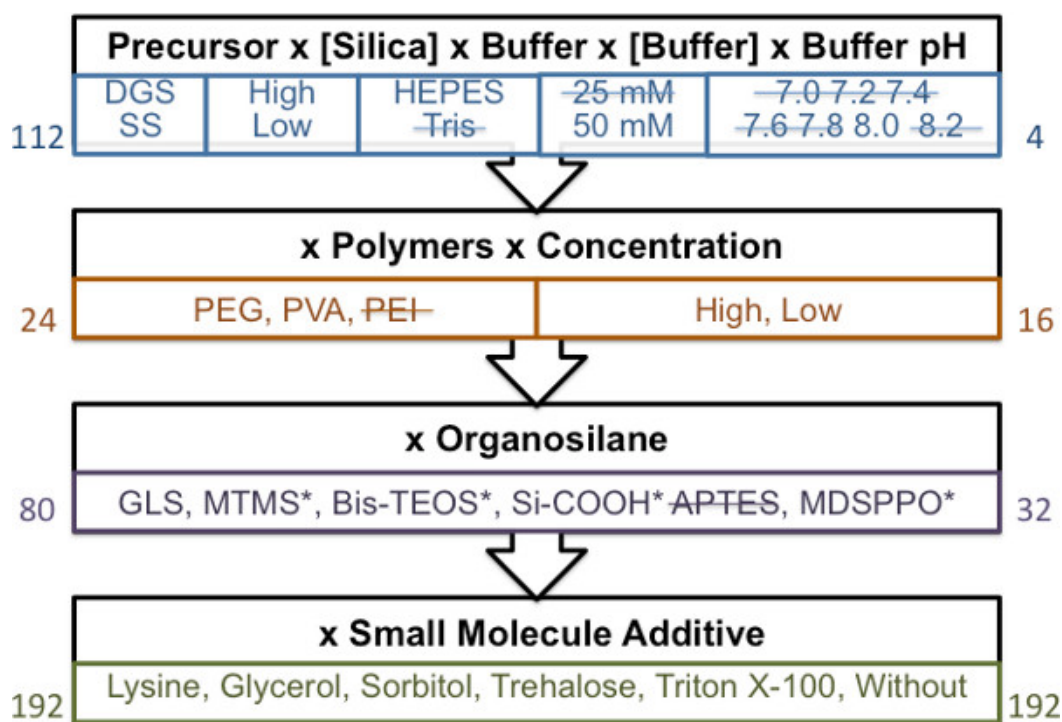


Figure 1. General schematic for the guided materials screening approach. Each block represents a step of the screen in sequential order. Numbers on the left represent the total number of materials prepared for analysis. Using a gelation time greater than 2.5 hr (materials with gelation times less than 2.5 hr are indicated by the strikethrough), the number of materials that passed each stage and carried forward during the material screen are indicated by the number on the right. *Represents materials with less than optimal phase separation.

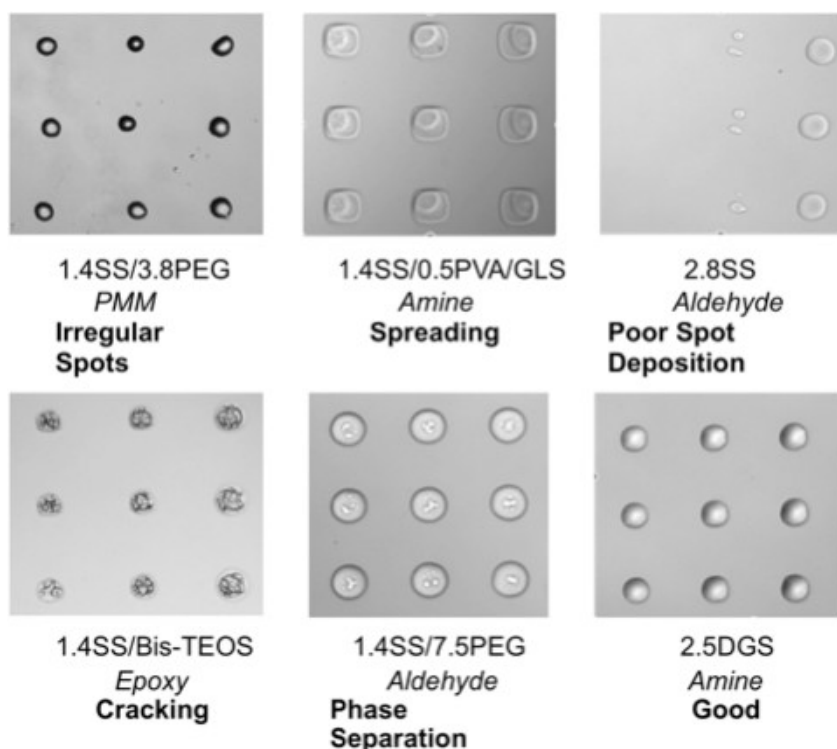


Figure 2. Optical images showing various failure modes of materials at the printability step of the screen. An image of a "good" material (second row, third column) is also shown for comparison. Reprinted with permission from reference ⁸, copyright 2013 American Chemical Society.

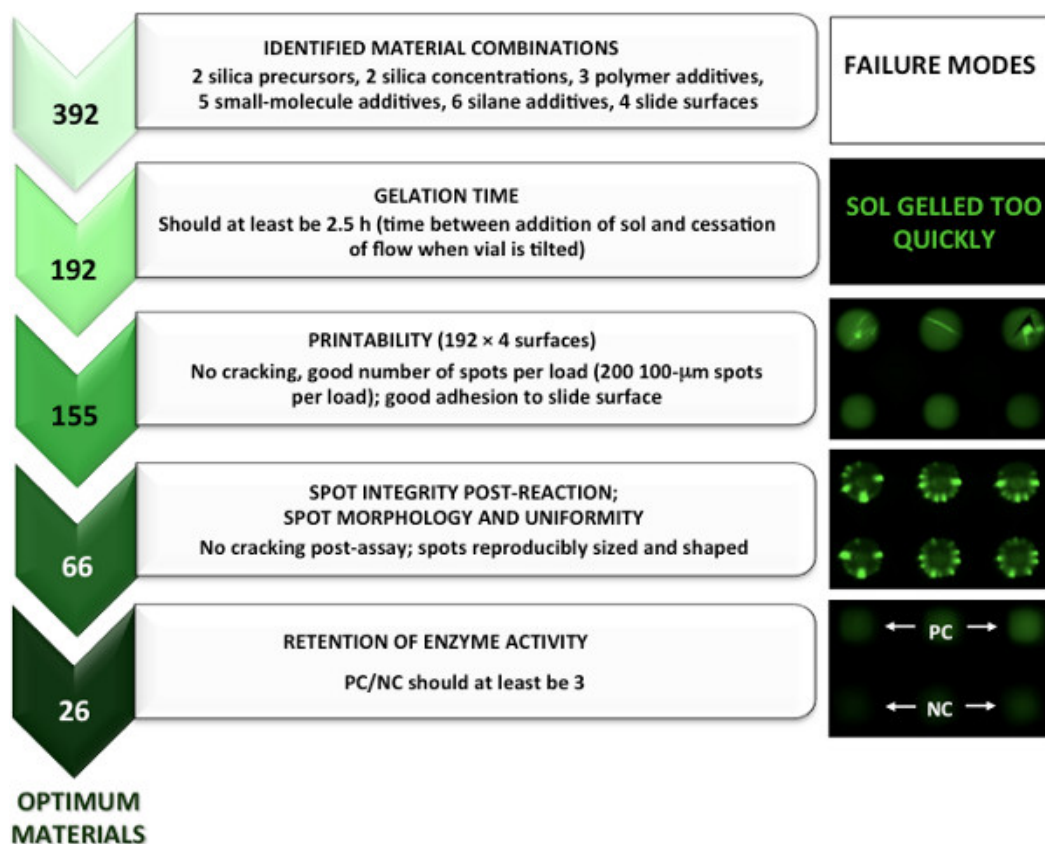
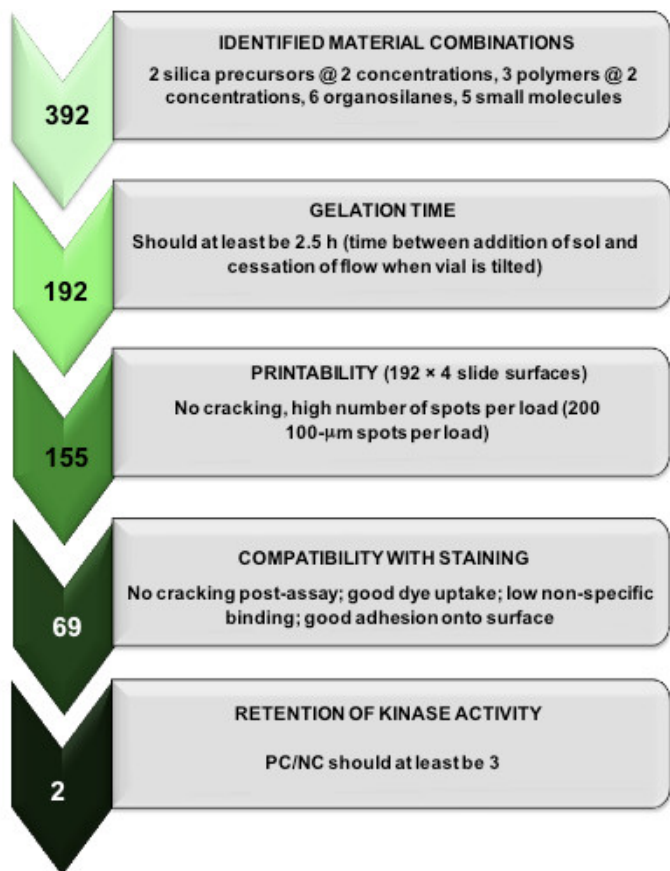


Figure 3. A directed material screening approach for identification of optimal materials for fabricating sol-gel-derived AChE microarrays. Reprinted with permission from reference ⁵, copyright 2013 American Chemical Society.



OPTIMUM MATERIALS

Figure 4. A directed materials screening approach for identification of optimal materials for fabricating sol-gel-derived kinase microarrays. Reprinted with permission from reference ⁸, copyright 2013 American Chemical Society.

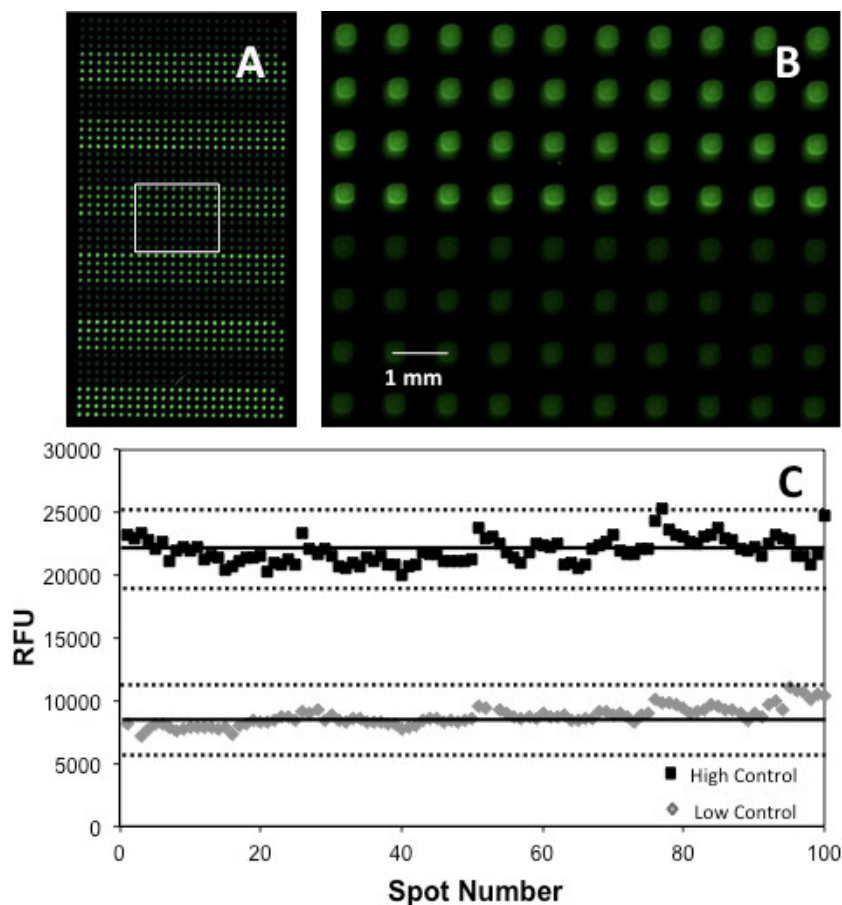


Figure 5. (A) A section of AChE microarray showing HC (bright green) and LC (light green) spots (a black-green palette was applied as pseudocolor for clarity of presentation); (B) a magnified view of the boxed area to highlight spot morphology and alignment; and (C) a Z' plot. Solid lines indicate the mean of the replicates, while dashed lines correspond to 3SD. Reprinted with permission from reference ⁵, copyright 2013 American Chemical Society.

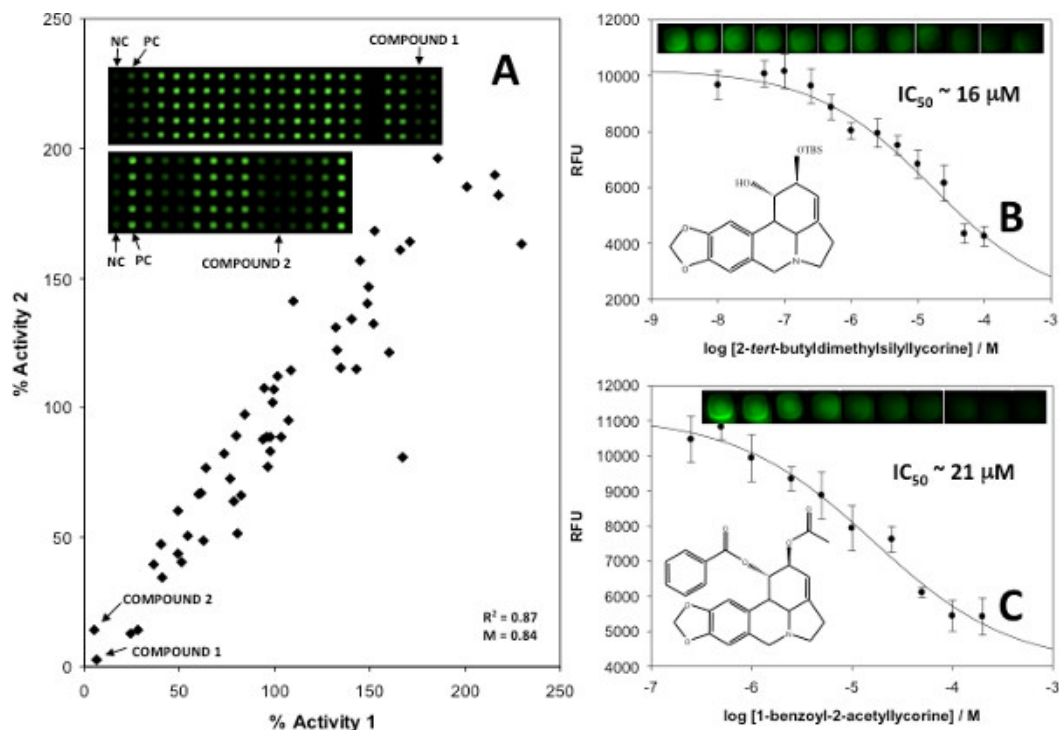


Figure 6. (A) Duplicate plot for on-array screening of synthetic analogs of Amaryllidaceae alkaloids; (B) IC_{50} plots of identified potential inhibitors marked as compounds 1 and (C) compound 2, with error bars representing one standard deviation of the mean from 25 replicates. Representative spots are shown to illustrate differences in signal proportional to inhibitor concentrations. Reprinted with permission from reference ⁵, copyright 2013 American Chemical Society. [Click here to view larger figure.](#)

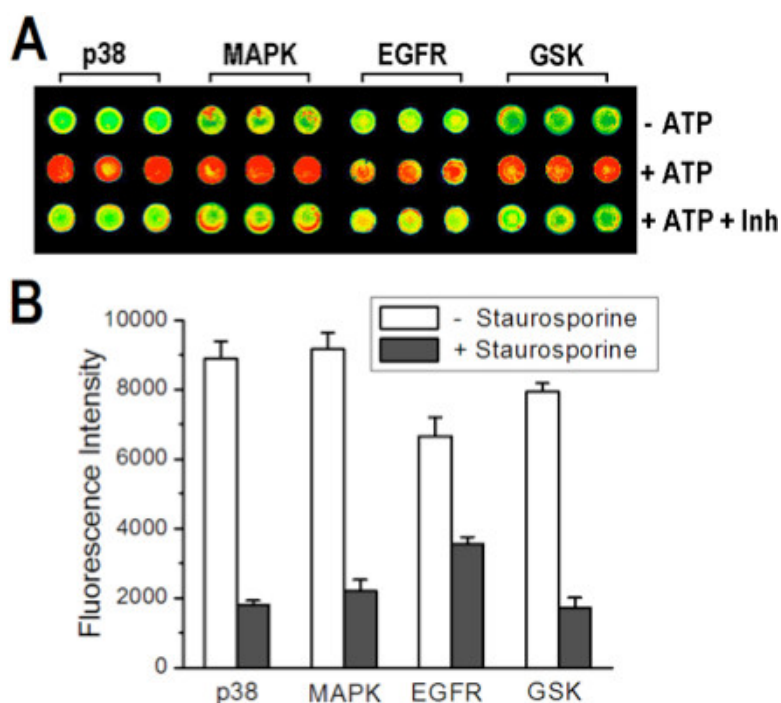


Figure 7. On-array assay of four kinases using 1.4SS/1.0PVA for entrapment and printed onto an amine-derivatized slide. (A) An image of a section of microarray in which spots with kinases co-entrapped with their respective substrates were overprinted with buffer (NC, top row), or solutions containing ATP (PC, middle row) or ATP + staurosporine (bottom row). (B) Bar graphs comparing signal intensities between inhibited and uninhibited reactions, after subtraction of background signals and error bars representing one standard deviation of the mean from 25 replicates. Reprinted with permission from reference ⁸, copyright 2013 American Chemical Society.

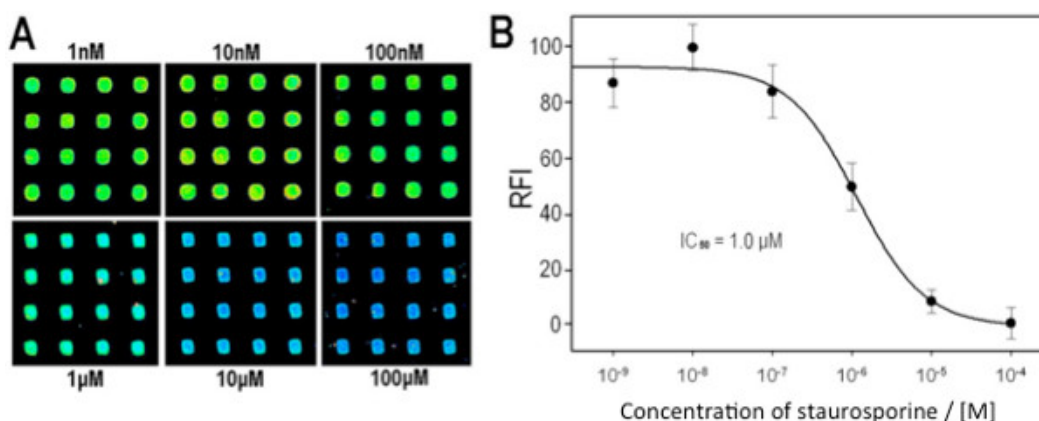


Figure 8. Inhibition assay on a p38a/MBP microarray. (A) Sections of microarrays showing representative spots overspotted with varying concentrations of staurosporine, as indicated (the images were obtained by a single scan of the same slide; composite image is shown for clarity). (B) IC₅₀ curve generated from the analyzed array images. The intensity obtained at 100 mM was subtracted from all images; all other intensities were normalized by setting the intensity obtained at 10 nM to a value of 100% activity. Reprinted with permission from reference ⁸, copyright 2013 American Chemical Society.

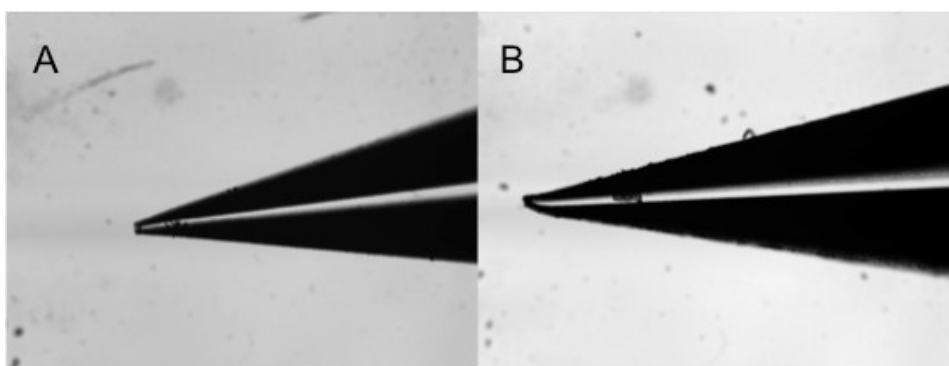


Figure 9. Images of microscopic stealth pin used for contact pin-printing showing various imperfections: (A) clogged, (B) bent.

Discussion

The methodology described here was selected as the most suitable for identifying printable sol-gel derived materials with a contact printer, producing a time- and cost-effective procedure for rapidly identifying optimal materials without having to screen large numbers of materials. From a total of ~20,000 potential materials, it was possible to identify ~200 materials that were suitable for printing on the basis of gelation time alone. This significantly reduced the number of materials needed to be prepared for subsequent printing trials. These printable materials were then printed onto 4 slide surfaces for a total of 768 material-slide combinations. On average, 50 spots/replicates of one material can be printed in ~3 min, including sample loading, spot deposition and pin cleaning. Of those, 155 materials, or roughly 20%, allowed for printing the maximum number of spots per solution uptake and produced reproducible spot sizes. It should be noted that of the 4 slide surfaces tested, materials printed better in the order: amine > epoxy > aldehyde > PMMA; PMMA slides did not produce useful arrays for any materials. This was likely attributed to the polarity of the surface coating. Comparing the aforementioned slide surfaces, the more polar amine and epoxy were better suited for the aqueous sols compared to the PMMA slides. Furthermore, of the tested surfaces, the amine coated slides provide a potential positively charged surface for the deposited anionic sol to bond. We suspect, the silica nanoparticles at the interface between the slide and the sol interact along the surface. Both the epoxy and the aldehyde slide surfaces lack the same initial charge-based interaction. To ensure optimal spot deposition it is highly recommended to use pre-coated slides from a supplier such as Arrayit. In-house coating produces inconsistent surfaces that lead to poor spot reproducibility¹³ and, in some cases, may lead to quantification problems.¹⁸ Of equal importance, temperature and humidity affect the "printability" of the materials. While no detailed studies on the effects related to temperature were carried out, printing was always carried out at room temperature (23±3 °C). Humidity (greater than 80%) was also controlled within the print chamber to prevent irregular shape deposition due to small deposition volumes (0.7-2.3 nL) and evaporation.

While the material screen was guided towards identifying optimal sol-gel derived materials specifically for printing of AChE and kinases, a small set of materials were identified that worked for both types of proteins. Indeed, both of the materials that were identified for kinase microarray fabrication were based on SS+PVA+glycerol, and both materials were also identified within the 26 materials selected for AChE microarrays. These "optimal" materials may offer a generic starting point for developing further protein-doped sol-gel based microarrays, and small screens centered around these compositions may identify even better materials for microarray fabrication. A second point to note is the importance of the enzyme used. In the case of AChE (a rather robust enzyme), 26 (or roughly 40%) of the original 66 materials identified as assay-compatible retained the activity of entrapped AChE. However, for the more delicate kinases, only 2 of the 69 assay-compatible compositions, or roughly 3% of the materials, were able to retain the activity of all kinases. While sufficient numbers of different enzymes have not been studied to make conclusive statements, it appears that optimizing array fabrication with relatively unstable enzymes may lead to identification of materials that can entrap a wide range of proteins to allow multiplexed microarray fabrication.

Independent of the chosen protein, the major cut-off factor for identifying printable materials was the need for a long material gelation times (>2.5 hr). When developing SS based sol-gel materials, it is very important to ensure that, following ion exchange and filtration, the sol is at about pH 4. Sols with a lower initial pH may result in materials with a lower-than-neutral pH, which can affect enzyme activity.¹⁹ Adjusting the amount of Dowex (ion exchange resin) to SS can alter the final pH of the sol. When a new batch of the resin is prepared the ratio of resin to SS needs to be adjusted so as to produce sols at about pH 4 following the procedure in section 2 of the protocol.

Similarly, the preparation of crystalline DGS is often a source of error associated with material failure when using DGS based sols for the biomolecule entrapment. Although not reported here in detail, great care needs to be taken during the synthesis of crystalline DGS, in particular the need to avoid the presence of water during the synthesis, which can produce polyglycerated silicates rather than monomeric DGS. Also, due to the hygroscopic nature of DGS, the crystalline sample needs to be stored desiccated and used within 6 months after synthesis. Crystalline DGS older than 6 months may not dissolve fully (owing to partially condensed polyglyceryl silicate material) even with sonication in an acidic environment. Incomplete DGS dissolution produces sols with unknown and uncontrollable silica content and thus, less robust materials.

An important point to note with contact printing is the quality of the pins. Damaged or mishandled pins (**Figure 9**) will never produce reproducible arrays independent of the material being printed. It is recommended to check pin quality using a dissection microscope to ensure broken or clogged pins are not used. Careful handling ensures long life for the pins. Free movement of the pin is also important. In cases where moisture is trapped in the print head between the head and the pin, the pin will not seat correctly and thus will not make proper contact with the surface, resulting in a lack of deposition of material.

In conclusion, we have provided a detailed, multistep screening approach for developing high-density protein doped pin-printed microarrays. The screening involves optimization of material properties (gelation time and printability) to allow printing of materials, followed by more focused screening to identify materials that are compatible with a given assay and able to retain enzyme activity. This guided material screening approach can be applied to additional microarray formats to reduce time and cost associated with producing efficient high-density microarrays.

Disclosures

We have nothing to disclose.

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