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Rapid and Facile Microwave-Assisted Surface Chemistry for Functionalized Microarray Slides

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This report describes a rapid and facile method for surface functionalization and ligand patterning of glass slides based on microwave-assisted synthesis and a microarraying robot. The optimized reaction enables surface modification 42-times faster than conventional techniques and includes a carboxylated self-assembled monolayer, polyethylene glycol linkers of varying length, and stable amide bonds to small molecule, peptide, or protein ligands to be screened for binding to living cells. Customized slide racks that permit functionalization of 100 slides at a time to produce a cost-efficient, highly reproducible batch process. Ligand spots can be positioned on the glass slides precisely using a microarraying robot, and spot size adjusted for any desired application. Using this system, live cell binding to a variety of ligands is demonstrate and PEG linker length is optimized. Taken together, the technology we describe should enable high-throughput screening of disease-specific ligands that bind to living cells.

1. Introduction

The functionalization of inorganic solid substrates plays an important role in drug discovery, biomaterial applications, and the field of biotechnology.[1–4] Inorganic solid substrates are also ideal for cell based high-throughput screening systems capable of selecting disease-specific targeting ligands having the potential for high performance in vivo.[5–8] Ligand-patterned microarrays are an attractive system for screening and have been used to discover interactions of targeting ligands with various cells using highly parallel experimental methods and analyses.[9–12]

Various coupling reactions have been developed to immobilize ligands onto glass surfaces covalently, including formation of silyl ethers,[13] 1,3-dipolar cycloaddition,[14] Diels–Alder reactions,[15] and Michael additions onto epoxide-, isocyanate-, or alkyne-derivatized surfaces;[16] however, these conventional chemical syntheses are insufficient for obtaining fast reactions with high yields, often delaying the high-throughput process due to complicated and redundant chemical steps for substrate preparation. In addition to ligand imprinting, straightforward and efficient functionalization techniques that include high-density reactive chemical groups, high conjugation yields, low nonspecific adsorption, and homogeneous surface characteristics are needed.

Over the last decade, microwave (MW) irradiation has become an alternative to conventional methods in the field of organic chemistry, offering enhanced speed, reproducibility, and scalability.[17,18] MW irradiation utilizes the ability of electric charges present in liquid, or a conducting ion in a solid, to transform electromagnetic energy into heat, resulting in reaction rate acceleration. Recently, Garcia et al. have described the use of a MW-assisted method for grafting a silica layer onto solid substrates, and carbon surfaces also have been derivatized using MW-based surface modification.[19–21] Also, Haensch et al. have reported thermal and chemical stability of organic monolayers under MW irradiation.[22]

In addition to substrate modifications, a selective, sensitive, and reliable high-throughput screening method is essential for analyzing large numbers of compounds rapidly. Stockwell and colleagues have developed a cell-based assay format for high-throughput screens of small molecule interactions with biological systems.[23] This assay format is capable of detecting post-translational and biosynthetic events without the use of engineered cell lines or radioactivity.

In this study, we hypothesized that MW-assisted reactions could be exploited to develop derivatized glass slides that were optimal in terms of surface chemistry, linker length, and performance when used in high-throughput screens of ligand binding to living cells.

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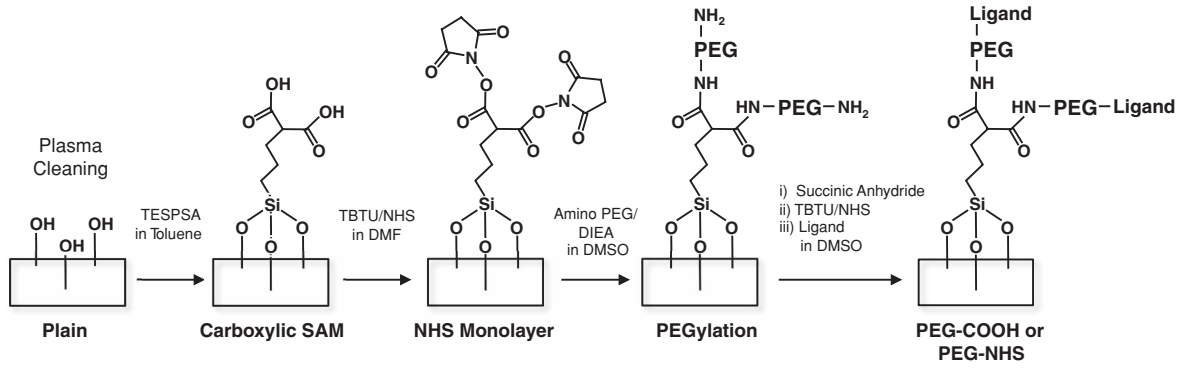
DOI: 10.1002/adfm.201102033

A.



B.

Surface Modification of Glass Slide	MW Reaction Power (W)	MW Reaction Time	Conventional Reaction Time	Solvent	Contact Angle (°)	Stability at RT
Carboxylic SAM	210	15 min	3 hr	Toluene	36 ± 2	> month
NHS Monolayer	70	40 s	3 hr	DMF	42 ± 2	> week*
PEGylation	70	60 s	> 3 hr	DMSO	35 ± 2	> month
PEG-COOH	70	60 s	1 hr	DMF	35 ± 2	> month
PEG-NHS	70	40 s	3 hr	DMF	35 ± 2	> week*
Total Reaction Time		18.4 min	> 13 hr			

*Stability at room temperature under vacuum after nitrogen purging.

Figure 1. Preparation of functionalized microarray glass slides: A) Chemical steps for surface functionalization and ligand conjugation. B) Rate enhancement of MW-assisted reactions for glass surface functionalization. MW = microwave; NHS = *N*-hydroxysuccinimide; PEG = polyethylene glycol; SAM = self-assembled monolayer.

2. Results and Discussion

2.1. Rapid Preparation of Functionalized Slides

Figure 1a summarizes the optimized series of steps for preparing microarray slides bearing functional groups, such as amine, carboxylic acid, and *N*-hydroxysuccinimide (NHS) ester, conjugated covalently through a polyethylene glycol (PEG) linker using MW-assisted synthesis. In summary, the functionalized surface was prepared by (1) forming a carboxyl-terminated self-assembled monolayer (SAM), (2) NHS ester substitution (3) amide-based grafting of an optimal length PEG linker, and (4) converting to a carboxyl or NHS ester-terminated surface for ligand conjugation. A series of sequential chemical reactions were optimized beforehand by conventional solution chemistry (Figure 1b and Figure S1). MW-assisted surface preparation was then optimized and compared to the best conventional reaction conditions (Figure 1B and Table S1).

2.1.1. Optimization of MW-Assisted SAM Fabrication

Surface oxidation status is one of the key factors influencing the silane reaction yield;[24,25] therefore, plain glass slides were treated using an oxygen plasma etching system to enrich the hydroxyl groups and strip out most of the organic contaminants (i.e., hydrocarbon). A highly oxidized and homogeneous surface was achieved after a 15 min plasma treatment (<5° surface contact angle by a water drop). To prepare slides cost-efficiently and in large batches, polyoxymethylene (Delrin) was chosen as the rack material because of its resistance to radiofrequency (RF) plasma irradiation, MW irradiation, and pressure (**Figure 2**a). A total of 200 slides could be processed per single plasma cycle by fitting 4 racks in the plasma chamber. For the MW reactions, 2 racks holding 100 slides were placed into a custom bath previously filled with solvent and reactants. Because commercially available MW reactors are used only for small scale reactions, we used a household MW oven equipped with a power control inverter. Obviously, the household MW oven is not designed for laboratory usage, and technical difficulties relating to non-uniform heating and mixing issues remain unsolved. To avoid these problems, we carefully controlled the batch process and evaluated the quality of the individual slides by measuring the surface contact angle in each step (Figure 2).

The water contact angle of the carboxylic SAM slides prepared using conventional reaction methods was approximately 36 ± 2°. Fabrication of the silane monolayer was challenging due to its self-condensation property, and could be facilitated by increasing the thermal agitation, concentration, and reaction time. A moderate reaction condition is desirable for silanization because MW irradiation creates an extreme thermal environment resulting in highly condensed products with an inhomogeneous surface. To control solvent temperature under MW

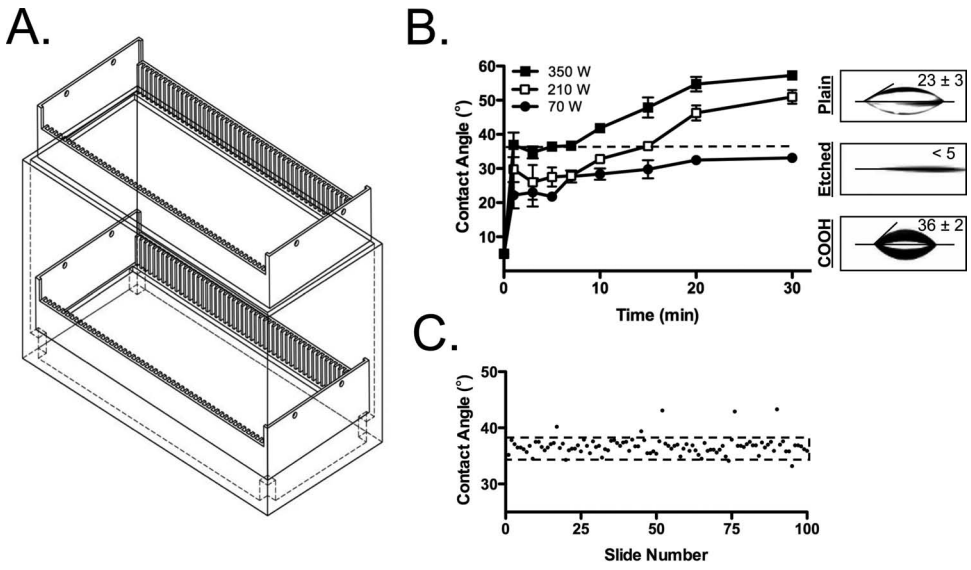


Figure 2. Large-scale preparation of slides using slotted racks and bath: A) The rack is designed to hold 50 microscope slides, while the bath can hold 2 racks. The racks and bath are made of polyoxymethylene (Delrin) to withstand plasma-assisted cleaning and MW-assisted chemistry. B) The water contact angle measurement (mean ± SD) as function of microwave power. Red dashed line is at the desired 36° contact angle. C) Contact angle measurement of carboxylic SAM slides produced by the large-scaled microwave reaction. Red dashed lines indicate 36° ± 2°.

irradiation, toluene was selected because toluene is nonpolar (dielectric constant = 2.4) and absorbs microwaves slowly compared to polar solvents. Using toluene for silane fabrication, we could control the entire solvent temperature slowly and homogeneously, which significantly reduced localized overheating in the reaction mixture. To determine an optimum condition for silane fabrication, the contact angle of glass slides was monitored in terms of MW power, reaction time, and silane concentration (Figure 2b and Table S1). Proper monolayer fabrication under MW irradiation was obtained when the reaction mixture reached 72 °C to 75 °C, resulting in a contact angle of 36° (red dotted line in Figure 2b). The contact angle increased over time regardless of MW power, indicating formation of a multilayer (Figure 2b). Although higher MW power (i.e., 350 W) resulted in a more rapid reaction, localized overheating in the mixture caused inhomogeneous multilayers with inconsistent contact angle values (data not shown). In contrast, lower MW power (i.e., 70 W) delayed the reaction and could not attain the proper contact angel value during the given time. Consequently, we found 210 W MW power to be the best condition with reliable time reduction (12-fold faster than conventional reactions). In addition, we observed that MW-assisted silanization was more concentration-sensitive than the conventional method (Table S1); therefore, the lowest silane content (0.1%) was preferred. We selected 0.1% 3-(triethoxysilyl)propylsuccinic anhydride (TESPSA) in toluene with MW irradiation at 210 W for 15 min as the optimum condition based on consistency and fidelity. From this setup, we could obtain 94 qualified products out of 100 slides (Figure 2c) within 20 min. This method does not require complicated chemical and machinery set-up and is ideally suited for a large-scaled manufacturing system of SAM slides in a laboratory setting. As a result, MW irradiation dramatically accelerated overall microarray fabrication, and was more than 42-fold faster than conventional reaction methods (18.4 min vs. 13 h; Figure 1b).

2.1.2. Surface Functionalization and Ligand Conjugation

The carboxylic SAM surface was utilized for grafting a PEG layer that could expose chemically reactive functional groups. Haensch et al. reported that the use of SAMs is limited for further MW-assisted synthesis due to their thermal and chemical stability.[22] Because the ordering of the monolayer and the integrity of the end groups could be affected by continuous MW irradiation over 30 min, PEGylation and additional derivatization on the TESPSA slides should be processed with minimal exposure to MW irradiation. We decreased MW power to a minimal level (70 W) and used polar solvents, such as DMSO and DMF. The use of polar solvents, which provide efficient energy transfer from the electromagnetic field, has the potential to increase reaction speed by a factor of 10 to 1000 with a lower MW power setting. The irradiation time was carefully tested in 20 s intervals to prevent thermal decomposition of the functional groups (i.e., NHS ester, Figure S2). The reaction in each step was completed in one minute, and the reaction rate was enhanced by more than 300-fold (Figure 1b). To evaluate the surface functionality, various fluorophores were conjugated to the PEG-NHS surface prepared by either conventional or MW-assisted methods. An intensity profile of Alexa-Fluor555 spots (red channel in the RGB images) was printed using a contact-type robotic microarrayer (**Figure 3**a). The dye molecules were specifically conjugated through the NHS groups and no signal difference between the 2 preparation methods was observed. The spot-to-spot and slide-to-slide homogeneity of surface functionalization was evaluated by measuring the fluorescent intensities of multiple Alexa Fluor 555 spots (Table S2). A total of 150 spots from 5 different PEG-NHS slides were analyzed. The variation among slides was 4.01% to 5.57%, which suggests high precision of the functionalization process.

The ultimate goal of our study was to fabricate ligand-patterned substrates in a rapid and efficient manner for screening live cell binding. We selected various ligands containing single or

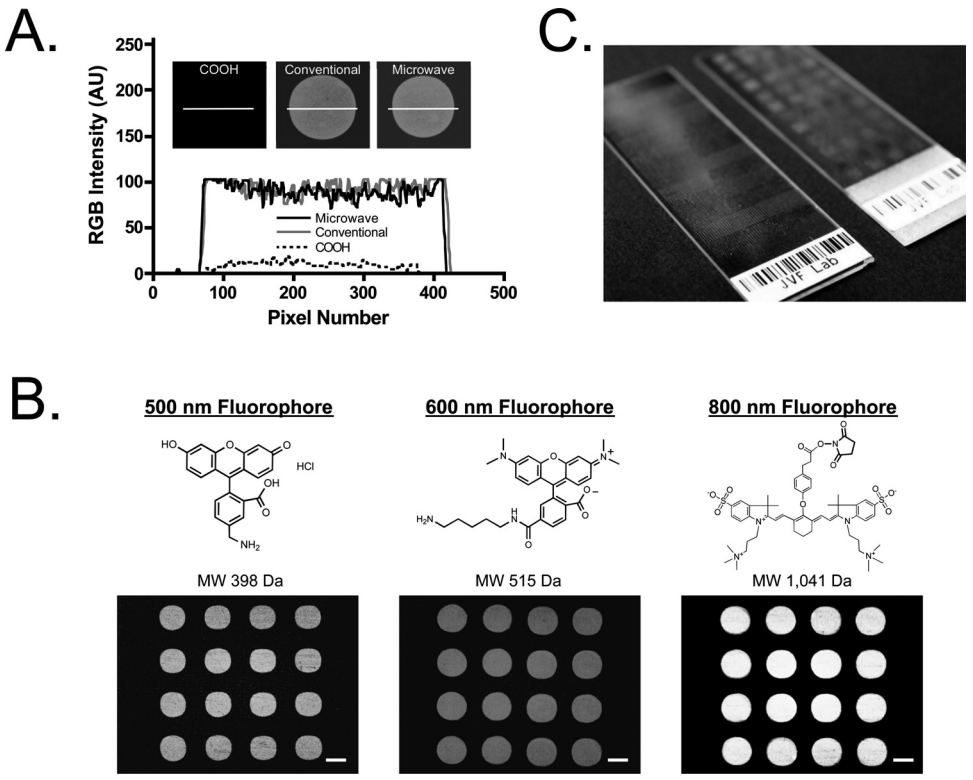


Figure 3. Preparation and characterization of functionalized microarray glass slides: A) Intensity profile analysis of fluorescence spots on functionalized glass slides prepared by conventional reaction or microwave reaction. B) Fluorescent dye conjugation using the PEG-NHS slide (5-AMF and Alexafluor555) or PEG-amine slide (ZW800-1 NHS ester) functionalized by the microwave reaction. Scale bars = 200 µm. C) Image of ligand-patterned slides after robotic microarraying of functionalized slides.

multiple reactive primary amines for conjugation to the functionalized glass surface (i.e., NHS ester). As shown in Figure 1a, the amine-reactive PEG-NHS slide was prepared to create one-step amide-bond formation through the conventional NHS reaction. Previously, we developed an automated, high-throughput sample spotting technique using a microarraying robot to generate up to 5,076 chemical spots on to a single glass slide.[26] To conjugate a series of ligands covalently, we re-optimized the spotting buffer to include 70% glycerol in DMSO supplemented with diisopropylethylamine (DIEA) for aprotic conditions or 70% glycerol in PBS (pH 8) for protic conditions, resulting in homogeneous 300-µm diameter spots. Accurately aligned fluorescent spots were formed with an excellent signal-to-background ratio (Figure 3b). Chemical conjugation directed toward either the NHS-functionalized surface (tested using 5-AMF and amino AlexaFluor555) or amine-functionalized surface (tested using ZW800-1) was achieved (Figure S2). From this setup, the array of ligand patterns varied from a 4 × 4 (16 spots) geometry up to a 108 × 47 (5,076 spots) geometry and could be generated on the functionalized slides without complicated patterning processes (Figure 3c).

2.2. Optimization of Cell Based Screening

2.2.1. Cell Adhesion Assay

The M21 human melanoma cell line was labeled with a new 700-nm near-infrared (NIR) fluorophore ESNF-10, whose chemical structure and optical properties are shown in **Figure 4**a. Intrinsic hydrophobicity (Log D at pH 7.4 = 3.56) and the positive charge distribution caused the molecules to accumulate into intracellular membranes rapidly (Figure 4b). We selected various ligands such as a carbohydrate-specific protein (wheat germ agglutinin; WGA), an integrin-specific small molecule (cyclo Arg-Gly-Asp-d-Tyr-Lys; cRGDyK), and positively charged polymers (polyallyamine; PAAm and poly-L-Lysine; PLL). These ligands were spotted on the PEG-NHS slides under protic condition for WGA (70% glycerol/PBS, pH 8) and aprotic condition for other ligands (70% glycerol/DMSO/DIEA, pH >9). After washing away unbound ligands, ESNF-10-loaded cells were panned over the ligand-patterned slide to identify live cell-specific binding. The slides were incubated for 1 h at 37 °C and gently washed with DMEM. Each ligand spot clearly exhibited specific cellular surface binding, with up to 230 cells per spot (Figure 4b). This screening method permitted a precise characterization of assay sensitivity.

2.2.2. Validation of Optimal Linker Space

The most challenging issue in high-throughput live cell screening is nonspecific background adsorption of cells. Many efforts have been made to solve this issue by providing linker space between the ligand and the slide surface or by inactivating the glass slide surface with chemicals.[27,28] To investigate the effect of linker length, we tested various amino PEGs ranging in size from 1 kDa (22 ethylene glycol repeating units) to

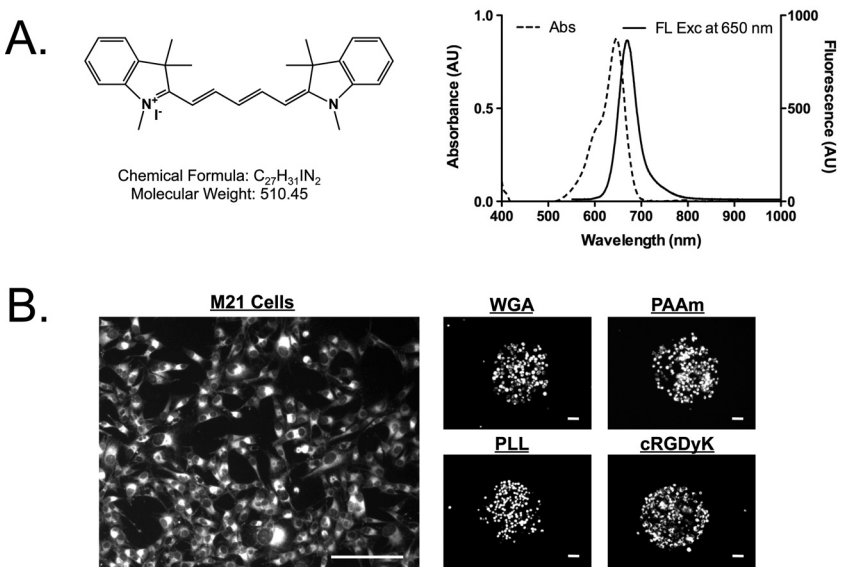


Figure 4. Live cell adhesion to the glass Surface and versatility of ligand conjugation: A) Chemical structure and optical properties of the self-accumulating NIR fluorophore ESNF-10 used for live cell staining. B) ESNF-10-labeled (i.e., 700 nm fluorescent) M21 human melanoma cells adhered to tissue culture plastic (left) and after live cell panning (right) on slides conjugated with wheat germ agglutinin (WGA), polyallyamine (PAAm), poly-L-Lysine (PLL), or the integrin binding peptide cyclic RGD (cRGDyK). Scale bars = 50 µm.

35 kDa (800 ethylene glycol repeating units) on the silanized glass surface. In a 4 × 4 grid of 300 µm spots, amino AlexaFluor555 or PAAm were spotted onto the PEG-NHS surface, and ESNF-10 loaded M21 cells were panned over the slides for 1 h. Signal uniformity of AlexaFluor555 was observed from every PEG slide indicating an identical yield of ligand conjugation regardless of linker length (**Figure 5**). However, we found PEG 4 kDa and PEG 6 kDa to be the most robust binding layer for living cells with significantly reduced nonspecific adhesion and improved cell accessibility because of balanced hydrophobicity and hydrogen bonds.[29] PAAm conjugated directly to the TESPSA surface (no PEG) showed much lower nonspecific cell binding than 1 kDa PEG-PAAm. We believe that this is due to the high hydrophobicity of short chain PEG (i.e., a wax in bulk form at RT) and the proximity of PAAm to the glass surface. Shorter PEGs (<2 kDa) behave as a hydrophobic straight anchor on the surface, which generated strong nonspecific cell adsorption.[30] In contrast, PEGs with a molecular weight larger than 6 kDa presumably reptate, and entangle intramolecularly,

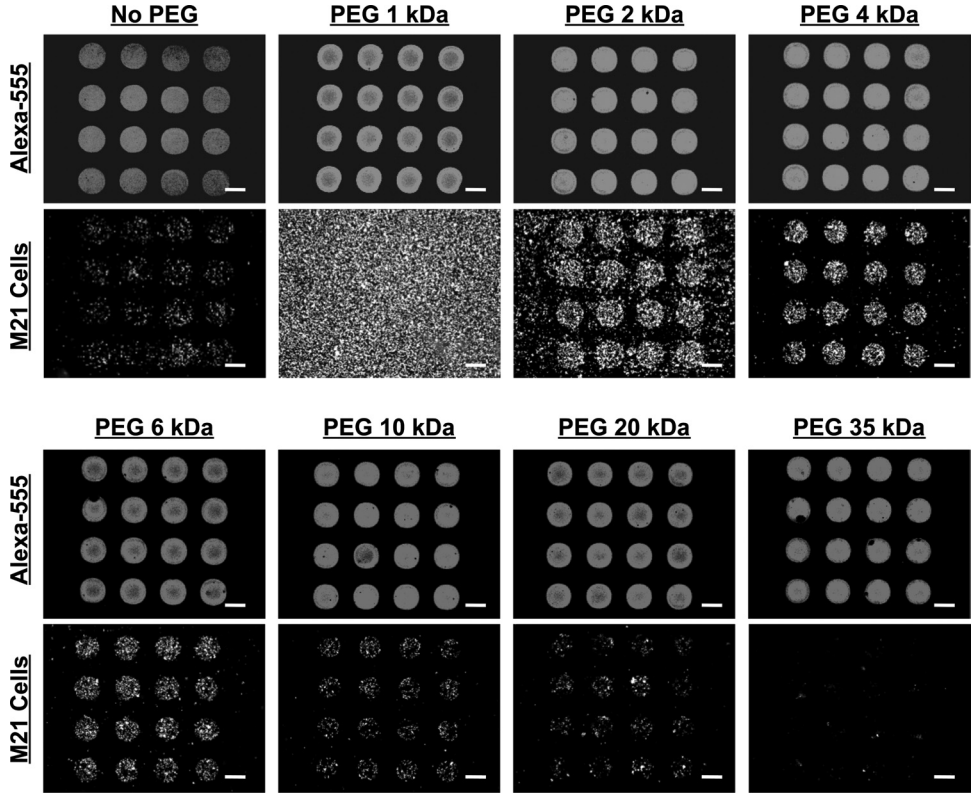


Figure 5. Effect of PEG linker length on ligand spotting and non-specific live cell binding: Glass slides having varying PEG-bisamine linkers of lengths 1 kDa, 2 kDa, 4 kDa, 6 kDa, 10 kDa, 20 kDa, or 35 kDa were prepared. Polyallyamine (PAAm) or Alexa-555 (600 nm fluorescence) were conjugated covalently to the PEG. Live M21 cells labeled with ESNF-10 were panned over the PAAm slides for 1 h. Scale bars = 200 µm.

which resulted in an inhibitory effect of cell-ligand interactions by steric hindrance.

3. Conclusion

In this study, we developed an integrated system capable of screening over 5,000 potential cell binding ligands incorporated into 300-μm sized circular spots on functionalized glass slides. Using MW irradiation, we obtained a complete chemical reaction in 18.4 min and produced a batch reaction of 100 slides with a 94% yield of identical surface functionality using a conventional MW oven. Highly dense and flexible ligand spots were arrayed by a microarraying robot on a PEG-linked surface providing adequate space for up to 230 living cell interactions per spot. By combining MW-assisted chemical reactions and robotic chemistry printing techniques, we provided rapid and facile synthesis of functionalized microarrays and high-throughput screening of ligand-specific live cell binding.

4. Experimental Section

Materials: All chemicals and solvents were of American Chemical Society grade or higher. Plain glass microscope slides (75 mm × 25 mm), 3-(triethoxysilyl)propylsuccinic anhydride (TESPSA), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), NHS, diisopropylethylamine (DIEA), succinic anhydride, and wheat germ agglutinin (WGA) were purchased from Fisher Scientific (Pittsburgh, PA). Different lengths of PEG, 3 kDa poly-L-lysine (PLL), and 60 kDa polyallylamine (PAAm) were purchased from Sigma-Aldrich (St Louis, MO). PEG-bisamine was prepared by converting hydroxyl groups as previously reported.[31,32] cRGDyk peptide was purchased from AnaSpec, Inc. (Fremont, CA). 5-AMF and amino AlexaFluor555 were purchased from Invitrogen (Carlsbad, CA) and Cy5.5 mono NHS ester was obtained from GE Healthcare (Piscataway, NJ). ZW800-1 was synthesized as reported previously.[33] The detailed synthesis of ESNF-10 will be reported elsewhere (Henary et al., manuscript in preparation). All other chemicals were commercially available unless otherwise noted. Slotted glass slide racks designed to hold up to 50 slides and a rack bath were made of polyoxymethylene (Delrin) with the assistance of LAE Technologies, Inc. (Barrie, Canada).

MW-assisted Surface Chemistry: Precleaned glass slides were processed in an oxygen plasma system (Plasma Etch, Inc., Carson City, NV) with 125 W/50 kHz RF power under 0.2 Torr for 15 min. Optimization of subsequent reactions was carried out using a household microwave oven (Sharp R-202EW, Mahwah, NJ). First, carboxylic SAM[34] was fabricated on the oxidized surface by MW-assisted synthesis using 0.1% TESPSA (v:v) in anhydrous toluene. The power of the MW was adjusted to 70, 210, or 350 W, and the reaction time varied between 1 and 30 min. Upon completion of the specified reaction time, slides were removed from the silane solution and vigorously rinsed with toluene and methanol. Two carboxyl groups were induced from one anhydride by dipping the slides in boiled water for 1 min. After drying with a stream of nitrogen, the silane monolayer on the slides was baked in a drying oven for 20 min at 110 °C. Carboxylic acid moieties were converted to reactive NHS esters in order to graft a PEG-bis(amine) layer to act as a linker. To investigate the effect of linker length on interactions between printed ligands and living cells, PEGs with different lengths (1, 2, 4, 6, 10, 20, and 35 kDa) were introduced. A mixture of 100-mM TBTU, 200-mM DIEA, and 100-mM NHS in DMF was added to the slides and reacted at 70 W MW power for 40 s. Next, the mixture was rinsed with ethanol and dried under a nitrogen stream. The activated slides were then reacted with a 10-mM solution of PEG-bis(amine) in DMSO (pH 9.0 ~ 10.0, adjusted by DIEA) at 70 W MW power for 60 s. After rinsing vigorously with DMSO and ethanol, and drying under a nitrogen stream, the amine-terminated PEG slides were placed into DMF containing 180-mM succinic anhydride and 100-mM DIEA and reacted at 70 W MW power for 60 s. The carboxyl-terminated PEG slides were converted to amine-reactive (NHS) esters by incubating in 100-mM TBTU, 200-mM DIEA and 100-mM NHS in DMF at 70 W MW power for 40 s, rinsed with ethanol and dried under a nitrogen stream. The slides were stored in a vacuum desiccator at –20 °C for up to several months without noticeable loss of activity.

Microarray Printing of Ligand Arrays: A contact type microarraying robot (OmniGrid Accent, DigiLab, Inc., Holliston, MA) mounted with SMP 11 pins (ArryIt, Telechem, Sunnyvale, CA) was used to print the ligands onto the functionalized surface. Ligand solutions were prepared as 1-mM stock solution in 70% glycerol either in DMSO (for aprotic conditions) or in phosphate buffered saline (PBS), pH 8 (for protic conditions), with 20 μL of each ligand distributed in the wells of a 384-well plate. The microarraying robot was instructed to pick up a small amount of sample solution (≥250 nL) from the 384-well plate and repetitively deliver approximately 3 to 4 nL to defined locations placed 500 to 800-μm apart on the slides. The spot size was adjusted to 300 ± 10 μm in diameter by controlling pin-contacting time. To verify surface functionality, 5-AMF and AlexaFluor555 were printed on PEG-NHS functionalized slides, and ZW800-1 NHS was printed on PEG-amine functionalized slides. After incubating for 3 h at room temperature, the slides were rinsed with ethanol and dried under a nitrogen stream.

Cell Adhesion Assay: The human melanoma cell line M21 (ATCC, Manassas, VA) was grown in a standard tissue culture dish (100 mm × 20 mm) in Dulbecco's minimum essential medium (DMEM), supplemented with 10% fetal bovine serum and 100 units/mL penicillin under 5% CO_2, 37 °C. When the cells reached 70% to 80% confluency, ESNF-10 dye (2×10^6 cells $nmol^{-1}$) was added to the dish and incubated for 20 min at 37 °C. The cells were trypsinized and seeded onto the ligand-presenting surface at a density of 2×10^5 cells cm^{-2} in DMEM/FBS solution. After 1 h incubation at 37 °C under 5% CO_2, the slide was gently washed with DMEM/FBS. Cell adhesion was examined under a fluorescent microscope.

Instrumentation: Contact angles were determined by the static sessile drop method using a Mouse-X (SurfaceTech Co. Ltd., Seoul, South Korea) equipped with a CCD camera. Deionized water drops were placed at least 3 locations on the surface in an ambient environment, and the obtained contact angles were averaged from the points. Fluorescence images were obtained using a Nikon TE-300 epifluorescence microscopy equipped with a 100 W mercury light source. Standard and custom filter sets (Nikon) were comprised of the following excitation/dichroic/emission filter wavelengths: blue (340–360 nm/400 nm/430–490 nm), green (460–500 nm/505 nm/505–560 nm), red (525–555 nm/565 nm/590–650 nm), and NIR (725–775 nm/785 nm/790–830 nm), respectively. For in vitro optical property measurement, online fiberoptic HR2000 absorbance (200–1000 nm) and USB2000FL fluorescence (350–1000 nm) spectrometers (Ocean Optics, Dunedin, FL) were used. NIR excitation was provided by a 665-nm laser diode (Opcom Inc., Xiamen, China) set to 5 mW and coupled through a 300-mm core diameter, NA 0.22 fiber (Fiberguide Industries, Stirling, NJ). The fluorescence intensity of each sample spot was analyzed using IPLab 3.6 software (Nikon) and ImageJ (NIH, Bethesda, MD). Data plotting was performed using Prism (GraphPad Software, Inc, San Diego, CA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

We thank Marsha Paul (Caliper Life Science, Hopkinton, MA), Alex Allardyce (ChemAxon, Budapest, Hungary), Rene Schena (ArrayIt

Corp., Sunnyvale, CA), Colin Johnson (LAE Technologies, Barrie, Canada), and Charles McKinnon (DigiLab, Ann Arbor, MI) for technical support and many helpful discussions. We also thank,, Jong Seo Yoon, and Tejas Gajera for assistance with development of the Robotic Chemistry System. We thank Lindsey Gendall for editing, and Linda Keys and Eugenia Trabucchi for administrative assistance. This study was supported by a grant from the Nehemias Gorin Foundation and the following grants from the National Institutes of Health: NCI BRP grant #R01-CA-115296 (JVF), NIBIB grant #R01-EB-010022 (JVF and HSC), NIBIB grant #R01-EB-011523 (HSC and JVF), and NCI grant #F32- CA-132332 (KAN).

Received: August 26, 2011
Revised: October 25, 2011
Published online: December 21, 2011

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