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A versatile approach to high-throughput microarrays using thiol-ene chemistry

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Microarray technology has become extremely useful in expediting the investigation of large libraries of materials in a variety of biomedical applications, such as in DNA chips, protein and cellular microarrays. In the development of cellular microarrays, traditional high-throughput printing strategies on stiff, glass substrates and non-covalent attachment methods are limiting. We have developed a facile strategy to fabricate multifunctional high-throughput microarrays embedded at the surface of a hydrogel substrate using thiol-ene chemistry. This user-friendly method provides a platform for the immobilization of a combination of bioactive and diagnostic molecules, such as peptides and dyes, at the surface of poly(ethylene glycol)-based hydrogels. The robust and orthogonal nature of thiol-ene chemistry allows for a range of covalent attachment strategies in a fast and reliable manner, and two complementary strategies for the attachment of active molecules are demonstrated.

The use of high-throughput microarrays is increasingly the method of choice for the effective screening of libraries of biomolecules, such as DNA, proteins, peptides and so on[1–4]. As a result, cellular microarrays are used frequently to explore interactions between cells and a wide range of molecules of either biological or synthetic origin[5]. The ability to screen combinations of surface-bound molecules is important because the extracellular environment contains a diverse mixture of matrix proteins, which carry a wealth of insoluble signals that direct cell functions[6,7]. By extracting the signalling motifs and embedding them on inert substrates, it is possible to understand the effects of isolated peptide sequences on a variety of cell types at the molecular level. Unfortunately, traditional strategies of serial screening are time-consuming and require the preparation of individual samples for each peptide sequence[8–12]. Additionally, many high-throughput microarray systems either do not bind the analyte of interest covalently, as is the case with most proteins, or involve many steps to conjugate biomolecules using a single conjugation strategy[13–16]. It is therefore of interest to develop strategies that exploit automated tools in combination with robust, efficient and orthogonal chemical concepts[10,11] to develop modular bioassays.

In recognizing the need for high-throughput technology in lab-on-chip applications, a variety of techniques have been developed to fabricate cellular microarrays. Photolithography, which is commonly used in microfabrication strategies, has been adapted to form micron-sized arrays of various peptides, proteins and synthetic polymers[6,17,18]. Kiessling and co-workers fabricated photopatterned arrays of self-assembled monolayers with alkanethiol functional peptides to identify sequences that promote stem-cell growth[19]. Alternative methods use inkjet printing to construct libraries of biomolecule arrays[4,20–26]. However, the majority of these approaches use arrays printed on stiff substrates, which may affect cellular functions and responses compared with those of soft polymer substrates[27,28]. Interestingly, Marquette and co-workers designed microarrays on polymer slides by using an automated piezoelectric arrayer to print series of modified DNA molecules, which were then transferred to a polydimethylsiloxane (PDMS) layer[29]. This method forms high-throughput arrays of active molecules covalently bound to a soft polymer backing. However, the curing of PDMS requires relatively high temperatures over an extended period of time, a process that may degrade many biomolecules, and the hydrophobicity of PDMS may lead to biofouling.

To overcome these issues, we sought to use thiol-ene chemistry to fabricate microarrays covalently attached at the surface of hydrogel substrates based on poly(ethylene glycol) (PEG). These hydrogel microarrays offer a wealth of advantages, such as chemical robustness, tunable mechanical properties and, more significantly, the ability to incorporate orthogonal reactive groups by exploiting the inherent reactivity of pendant thiols (cysteine residues) or alkenes, and thus offer an array of chemical handles for functionalization[30]. Herein, we demonstrate the development of high-throughput spotted microarrays that contain (i) thiol-functional peptides that direct cell adhesion, and (ii) functional groups for the orthogonal coupling of biomolecules and fluorophores that are not thiol and alkene functional (Fig. 1). This design allows the incorporation of a variety of analytes with either minimal or no modification of the biomolecules and/or dye of interest on a soft, biomimetic hydrogel substrate.

Results and discussion

The general procedure used to fabricate the hydrogel microarrays is shown in Fig. 1, and involves the initial printing of the molecules of interest on a glass slide by an automated microarrayer. A liquid PEG pre-polymer mixture is then poured onto the printed array and cured with an ultraviolet lamp ($\lambda = 365$ nm, 4.6 mW cm^{-2}) for two minutes. Significantly, the viscosity of the pre-polymer mixture, combined with the very rapid cross-linking reaction, does not dissolve or perturb the printed molecules and/or domains and maintains the integrity of the array. An additional advantage of this system is that the mild reaction conditions are

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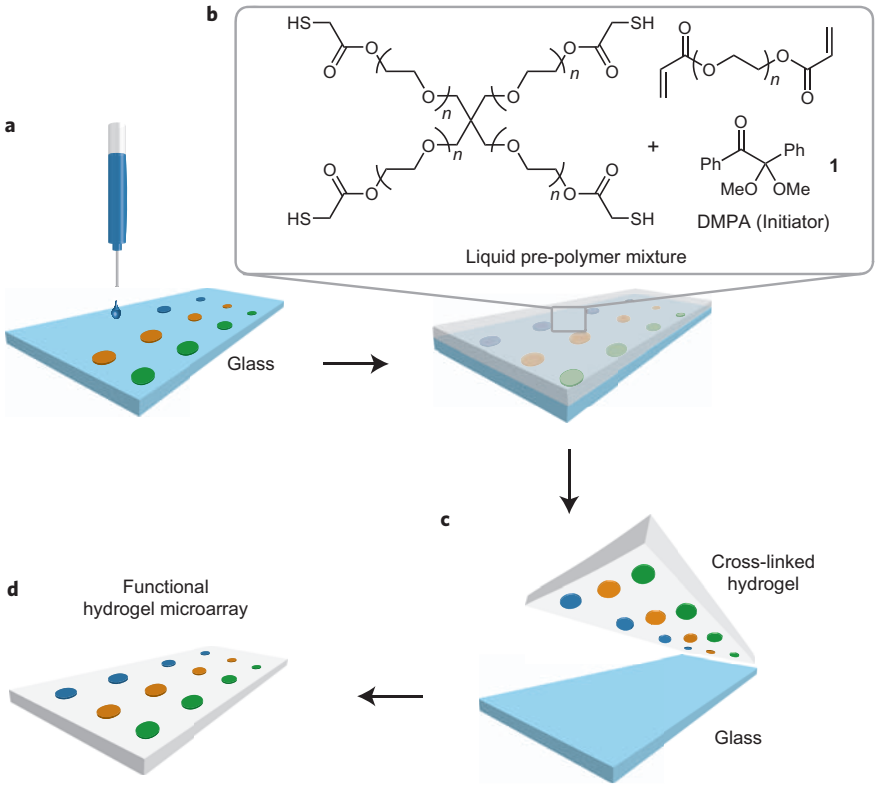

Figure 1 | General procedure for the fabrication of hydrogel microarrays using thiol-ene chemistry. a–c, Using an automated system, microarrays were printed on a glass slide (**a**), embedded under a thiol-ene prepolymer solution (a 9:8 wt/wt mixture of a four-arm PEG with thiols at the end-groups ($M_n = 2$ kg mol^{-1}), a bis-acrylated PEG ($M_n = 700$ g mol^{-1}) and ~0.1 wt% of the photoinitiator **1**) (**b**) and cured for two minutes under 365 nm light (**c**). **d**, Peeling from the glass slide exposes the functional microarray. DMPA = 2,2-dimethoxy-2-phenylacetophenone.

suitable for incorporating a wide range of biomolecules of interest, including systems that are temperature sensitive, such as oligo- and polypeptides. In turn, the mechanical integrity of these materials is illustrated by the simple peeling of the composite structure (films about 0.5 mm thick) from the glass slide to expose the face with the printed array, all without affecting the activity of the arrays, even after multiple handling and chemical treatment steps[31].

In selecting the chemistry and materials to produce the microarrays, a number of physical and chemical features were considered. PEG was chosen as the main component of the matrix material, as it has been shown to be resistant to cell and protein attachment, and therefore localizes cell interactions only at the printed functional spots that mediate cell adhesion[32]. Additionally, these materials can be swollen with water to 55 weight percent (wt%), and thus maintain a soft, hydrated environment suitable for *in vitro* and *in vivo* applications. Finally, the use of thiol-ene chemistry, a reaction that is known to perform well under benign reaction conditions (tolerance to oxygen and water), allows for a rich selection of functionalized molecules to be immobilized at the surface of the hydrogels.[31,33–36]

As a user-friendly platform, two main printing strategies were developed to demonstrate the ability to introduce a wide array of molecules that contain different functionalities simultaneously. Direct thiol-ene functionalization involves the printing of (bio)molecules of interest, for which the only requirement is the presence of a functional group reactive to thiol-ene chemistry that allows covalent attachment and localization at the surface of the PEG matrix (Fig. 2a)[33]. This method is amenable for many peptides through the thiol groups found on the cysteine amino acid or through the common allyl ester group that protects amino acids[37]. The second method, orthogonal postfunctionalization, requires the direct printing of a heterobifunctional linker that can both react with the PEG matrix and, through different coupling chemistries, allow for the secondary functionalization of molecules that do not readily have thiol and alkene functionality (Fig. 2b)[34,35].

To demonstrate the benefits of the direct thiol-ene functionalization of biomolecules for cell cultures, four different peptide systems were printed on a single hydrogel sample to assay cell adhesion. By adding a cysteine residue (thiol-containing amino acid) to a peptide sequence that is well-known to mediate cell adhesion, RGD, the CRGDS (cysteine-arginine-glycine-aspartic acid-serine) peptide can be attached covalently to the defined array areas of the hydrogel matrix[38,39]. As a negative control, the same cysteine-containing peptide was employed, but RGD was replaced with a non-binding RGE sequence, which led to CRGES[10] (E = glutamic acid). In a similar fashion, the RGD sequence was retained, but the cysteine residue was replaced by a glycine to give GRGDS, which would not be expected to undergo reaction with the hydrogel and therefore this physically adsorbed peptide would detach from the surface. Finally, an array of the same PEG pre-polymer mixture without any added peptides was printed to illustrate the lack of substrate activity.

Uniform and reproducible spots were printed from peptide solutions that had a concentration of at least 100 mg ml^{-1}, with 50 wt% of the solute being thiol-functionalized PEG (number averaged molecular weight ~2 kg mol^{-1}, Fig. 1). After drying, the thiol-ene PEG pre-polymer mixture was then layered over the dry spots and cured between glass slides. The integrity of the pattern transfer was characterized by optical microscopy and X-ray photoelectron spectroscopy, and profilometry depicted a relatively smooth surface over the printed spots on the hydrogel microarray (see Supplementary Figs S1–S3).

The hydrogel arrays were washed rigorously by immersion in water with agitation for one day and sterilized using an antibiotic

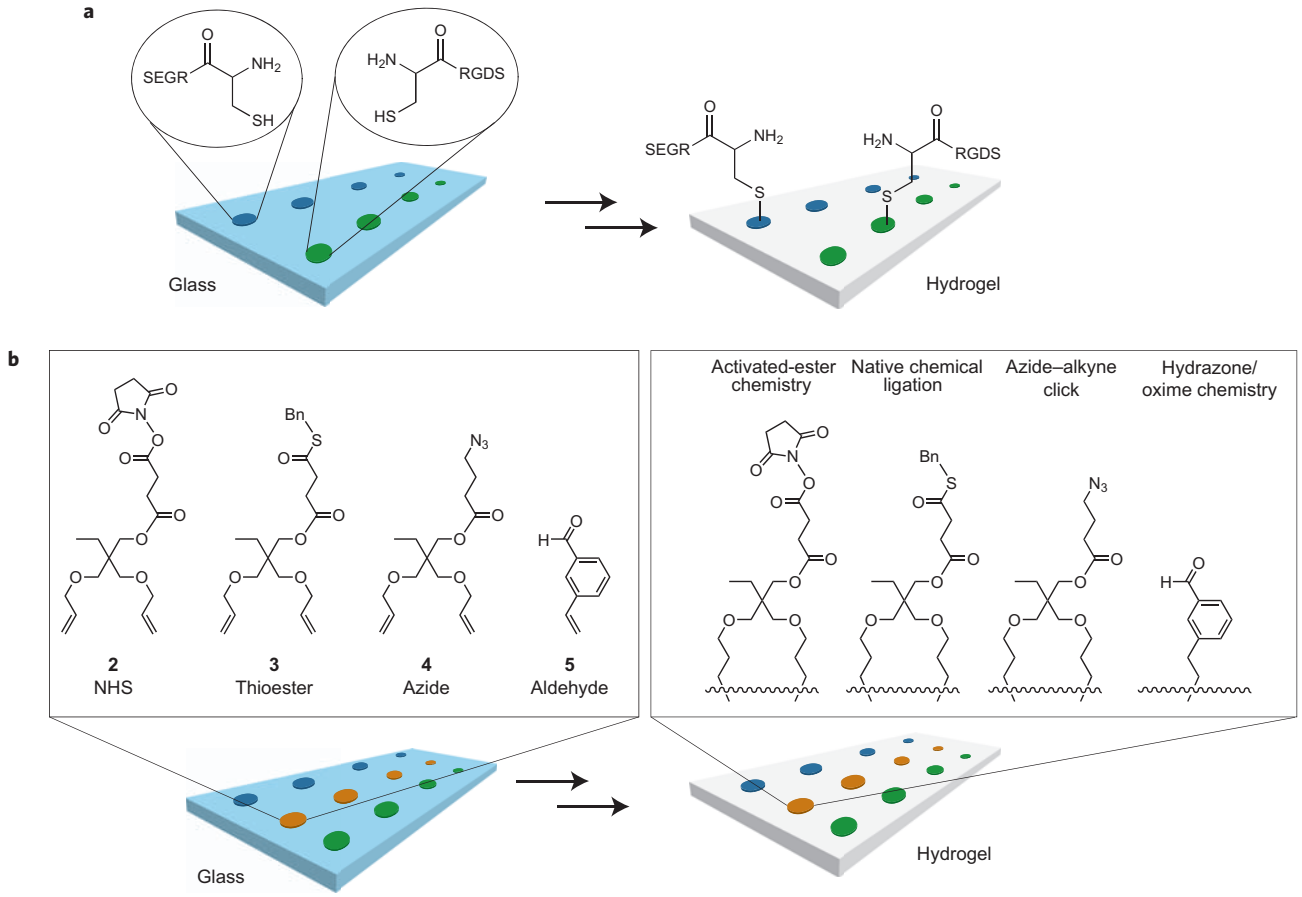

Figure 2 | Thiol- and alkene-functional hydrogel microarrays. a, The direct surface functionalization of hydrogel-supported microarrays by printing thiol- or alkene-functional biomolecules. Thiol-containing peptides with the amino acid sequence CRGDS and CRGES are shown. **b**, Postfunctionalizing microarrays of heterobifunctional crosslinkers through orthogonal handles for additional click transformations. **2**–**5** contain alkene functionality for incorporation into the thiol-ene hydrogel microarray in addition to orthogonal functional groups for postfunctionalization strategies. Bn = benzyl.

solution, after which fibroblast cells were seeded and cultured over three days. As shown in the optical microscope images (Fig. 3), the cells only attached to the CRGDS functionalized spots with no attachment to any of the negative controls. Significantly, cells initially attached to the GRGDS spots up to three hours postseeding (see Supplementary Fig. S4); however, the lack of covalent attachment between GRGDS and the matrix allowed the peptide to be solubilized and removed from the array surface over time, which led to detachment of the cells (Fig. 3b). In contrast, although the CRGES peptide was covalently attached to the surface of the matrix, the incorrect sequence did not mediate cell adhesion, as with the control matrix-only arrays that contained no peptide (Fig. 3c,d, respectively). From these experiments it can be concluded that direct peptide functionalization leads to the generation of a stable, defined array of peptides on the surface of a hydrogel matrix, for which the only requirement is the presence of thiol-ene reactive components (that is, cysteine residues).

Although the direct printing approach is a powerful technique for the fabrication of PEG-supported peptide arrays, to broaden the scope of the hydrogel microarrays we sought to develop a post-functionalization method that would allow the attachment, under mild conditions, of a wider selection of molecules that do not contain thiol and alkene groups, such as many proteins and dyes. The postfunctionalization strategy also provides a method to incorporate light-sensitive materials, such as many dyes that readily photobleach (for example, fluorescein). Such a strategy takes advantage of the tolerance of thiol-ene reactions to a variety of functional groups, which allows orthogonal systems to be incorporated during the curing process, and thus provides a substrate for further chemistry on the microarrays. To incorporate these functional groups, heterobifunctional cross-linkers, such as the *N*-hydroxysuccinimide (NHS) ester with terminal allyl groups (**2**) was evaluated (Fig. 2b). In this example, the allyl groups reacted readily with the thiols of the matrix, which resulted in the introduction of NHS functionalities to the surface of the microarray. The NHS reactive group was therefore localized specifically to the surface spots, and so provided a platform for further functionalization with any target molecule that contained a primary amine (peptides, proteins, dyes and so on).

This general heterobifunctional cross-linker strategy can also be applied to other systems, such as alkene–azide, alkene–aldehyde or alkene–thioester combinations, which allows for further modifications with alkynes, hydrazides and/or hydroxylamines and *N*-terminal cysteines, respectively. The specific heterobifunctional systems were chosen not only for their ease of synthesis (Fig. 4a), but also for their compatibility with the printing process (all are liquids). Starting from the commercially available trimethylolpropane diallyl ether (**6**), three cross-linkers were synthesized. Compound **4** was synthesized in one step by esterification with 4-azidobutanoic anhydride. Compounds **2** and **3** were synthesized in two steps, and in both cases the first step resulted in compound **7** through the ring opening of succinic anhydride with **6**. Subsequent coupling of **7** with NHS and benzyl mercaptan afforded compounds **2** and **3**, respectively. The aldehyde-functional compound (**5**) is commercially available and required no further purification. After direct functionalization of the hydrogels with the heterobifunctional compounds through the alkenes, the

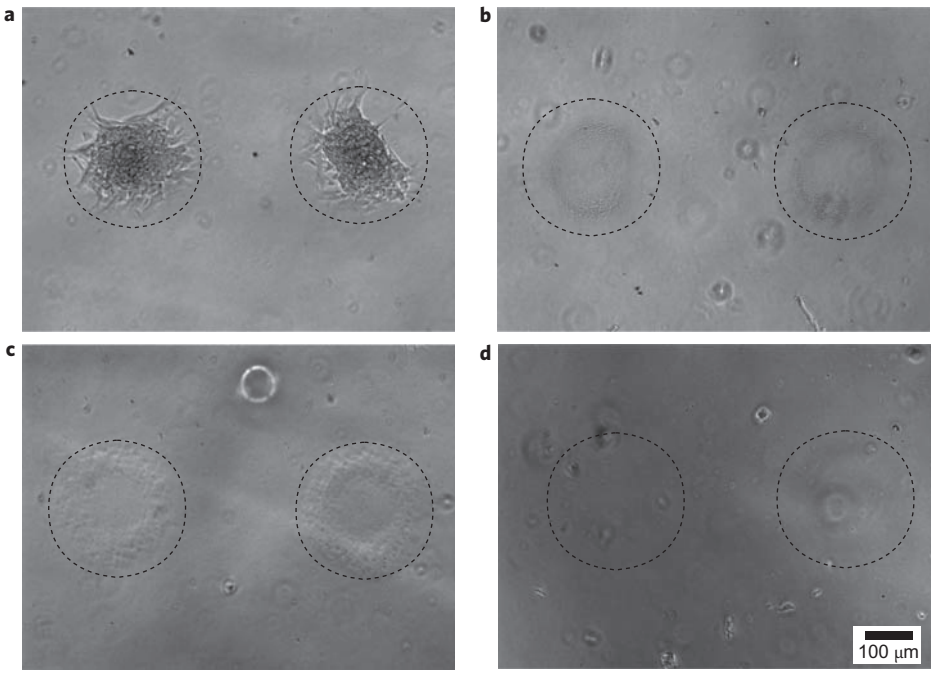

Figure 3 | Direct coupling of cell-adhesive peptides to microarrays. a–d, Brightfield microscope images of NIH3T3 fibroblast cells 72 hours postseeding on hydrogel microarrays functionalized with CRGDS (**a**), GRGDS (**b**), CRGES (**c**) and with polymer only (**d**). Cells only adhere and grow to confluency where the –RGD– adhesive peptide is bound covalently to the hydrogel microarray as in **a** (CRGDS). The scale bar applies to **a–d**.

orthogonal postfunctionalizations were carried out with their corresponding click-functional counterparts.

As a test vehicle, a range of functional dyes was selected for conjugation to the printed microarrays and characterized through fluorescence microscopy. For example, rhodamine that bore a primary amine (rhodamine-NH_2, **8**) was incubated with the NHS microarrays in a pH 8.6 buffer solution, and the site-specific attachment of the dye to the printed areas on the microarrays monitored by optical techniques. Analogously, fluorescein–alkyne **9** was coupled to the azide microarrays under Cu(I) click conditions[30], *N*-terminal cysteine-functional fluorescein (**10**) was coupled to the thioester microarray in pH 7.4 buffer[40] and Cascade Blue–$NHNH_2$ (**11**) was coupled to the aldehyde microarray in a pH 5 buffer solution[41]. In all cases, the orthogonal functional groups remained in the printed spots after the thiol-ene cross-linking reaction, and site-specific conjugation with the underlying microarray spots was clear (Fig. 4).

In the thioester conjugation strategy the cysteine was first coupled to the thioester through the thiol group, but the presence of the vicinal amine led to the transamidation product[40]. To demonstrate that the *N*-terminus cysteine residue is essential for the coupling, and that primary amines are not sufficient, **8** was incubated with the thioester printed arrays as a single compound in solution, and in a two-compound solution to compete with the fluorescein–cysteine dye **10**. In the former case, coupling was not observed, and in the latter experiment only **10** was observed to bind to the hydrogels (see Supplementary Fig. S5). Of significance, no modification of these postfunctionalization techniques was necessary for *N*-terminal cysteine or amine functional biomolecules. Such techniques can also be used for cases in which non-natural functional groups have been added to peptides or proteins, and so ensure a high degree of site-specific ligation of these biomolecules[41–43].

To demonstrate the potential of this strategy it was important to be able to vary and quantify the introduced functional groups as well as to demonstrate true orthogonality in a multicomponent–multifunctional system. The ability to tune the concentration of the dyes and/or biomolecules on the surface was established by printing a series of NHS cross-linker **2** spots of increasing concentration (1–20 volume percent) on a single microarray followed by incubation with **8**. As expected, with increasing concentration of the cross-linker, the fluorescence increased in a linear fashion (see Supplementary Fig. S6). To evaluate the extent of the reaction, the conversion of the NHS functional groups at the surface was determined using a competition experiment with a non-fluorescent primary amine (lysine) followed by reaction with **8**. In all cases, there was minimal to no fluorescence on the microarray from the dyes, which shows that an essentially quantitative reaction of the NHS groups had occurred under the optimized conditions developed to functionalize the microarrays. These results demonstrate the highly efficient nature of the functionalization reaction as well as an ability to control the level of surface groups, which provides a unique strategy to control the concentration of the biomolecules and/or dye at the surface of the microarray (see Supplementary Fig. S7).

The multicomponent and multifunctional orthogonality of this process to thiol-ene chemistry was then demonstrated by performing multiple reactions on a single microarray without out any cross-reactivity. To test this strategy, three different dyes (fluorescein, Cascade Blue and rhodamine) were attached sequentially to a single microarray using orthogonal conjugation chemistry with each dye localized at the specific and predetermined array positions (Fig. 5). The initial fabrication of the microarray using thiol-ene chemistry provided a means to print the thiol-containing compound **10** directly in the first row, and the second and third rows contained aldehyde **5** and NHS ester **2** functional compounds, respectively. Sequential postfunctionalizations of the NHS ester and aldehyde with **8** and **11**, respectively, yielded the site-specific localization of the three dyes using a combination of the direct printing and postfunctionalization strategies and exploiting the orthogonality of the chemistries employed. For this particular microarray choice, the order of postfunctionalization required the conjugation of **8** selectively to the NHS cross-linker **2** spots followed by hydrazone formation between **11** and the aldehyde cross-linker **5** spots. When the order of postfunctionalization was reversed, namely the addition of **11** before **8**, some non-specific conjugation between **11** and NHS cross-linker **2** was observed.

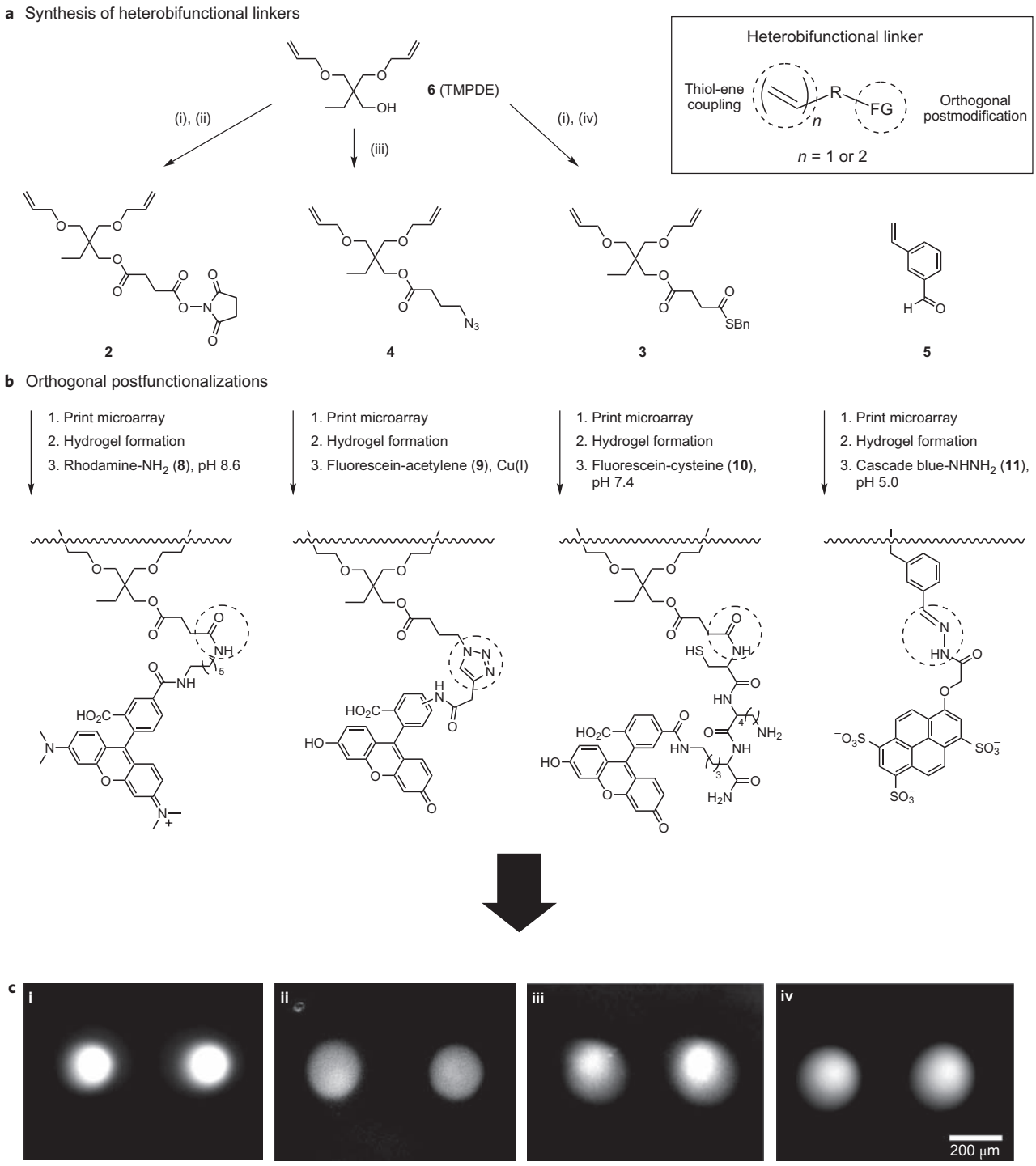

Figure 4 | Synthesis of orthogonal heterobifunctional crosslinkers and their postfunctionalization in printed microarrays. a, Synthesis of heterobifunctional crosslinkers, **2**–**5** (**5** is commercially available): (i) succinic anhydride, DMAP, pyridine (ii) *N*-hydroxysuccinimide, DCC, DMAP (iii) 4-azidobutanoic anhydride, DMAP, pyridine (iv) benzyl mercaptan, DCC, DMAP. **b**, Schematic of the orthogonal post-functionalization of rhodamine-NH_2 (**8**) to *N*-hydroxysuccinimide ester microarrays, fluorescein-alkyne (**9**) to azide microarrays, *N*-terminal cysteine functional fluorescein peptide (**10**) to thioester microarrays, and Cascade blue-hydrazide (**11**) to aldehyde microarrays. **c**, Fluorescence images of the dyes in **b** specifically attached to the spots on the hydrogel microarrays. DCC = *N*,*N*′-dicyclohexylcarbodiimide; DMAP = 4-dimethylaminopyridine; TMPDE = trimethylolpropane diallyl ether.

To further emphasize the orthogonal nature of the hydrogel microarrays, a hybrid strategy was then developed through a combination of the direct and postfunctionalization techniques to prepare multifunctional arrays. Thiol-functional peptides (CRGDS and CRGES) and the heterobifunctional NHS cross-linker **2** were printed from a single solution, which led to arrays that contained both the peptide and reactive NHS groups (Fig. 6). The activated esters were then coupled with an amino-functionalized dye (**8**), and these composite arrays subsequently seeded with fibroblast cells. As expected, the cells were found to attach and spread only in the CRGDS printed spots, with no attachment for the CRGES control peptide. An overlay of the brightfield image with the fluorescence image showed that the cells remained in the same area in which the rhodamine dye had been coupled, which demonstrates that

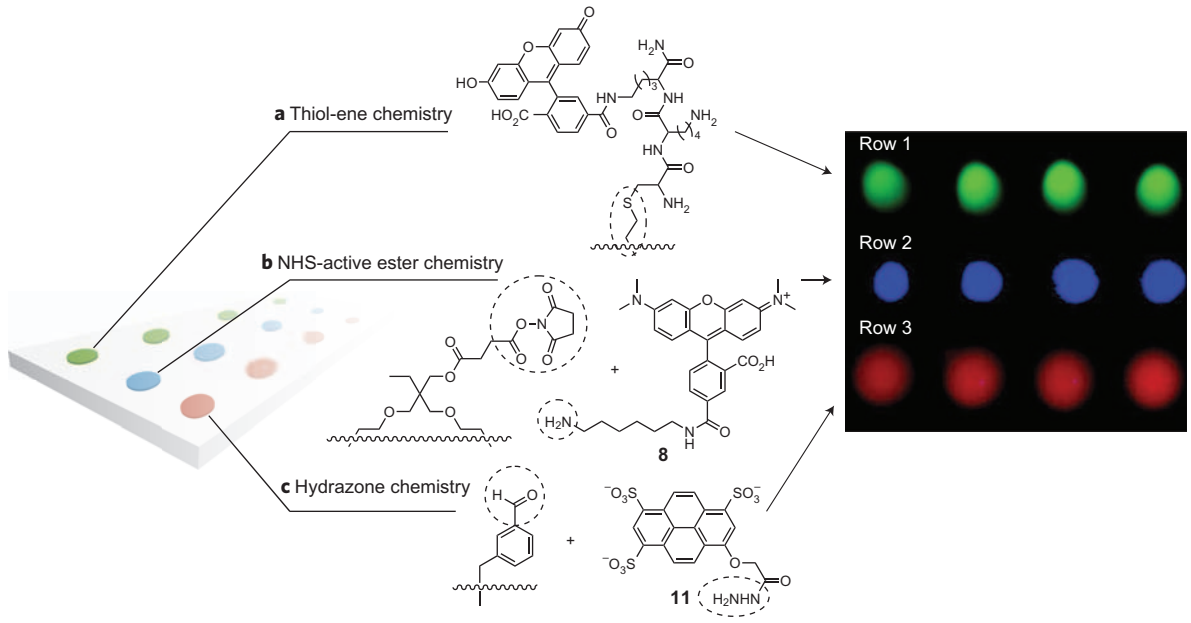

Figure 5 | Orthogonal ligation reaction on a single microarray using thiol-ene, hydrazone and NHS-active ester chemistries. Sequential ligation of the three dyes was performed by initial printing of **9**, **5** and **2** in rows 1, 2 and 3, respectively, using thiol-ene chemistry. Subsequent postfunctionalizations with **8** and **11** using NHS-active ester and hydrazone orthogonal ligation strategies yielded the functionalized microarray.

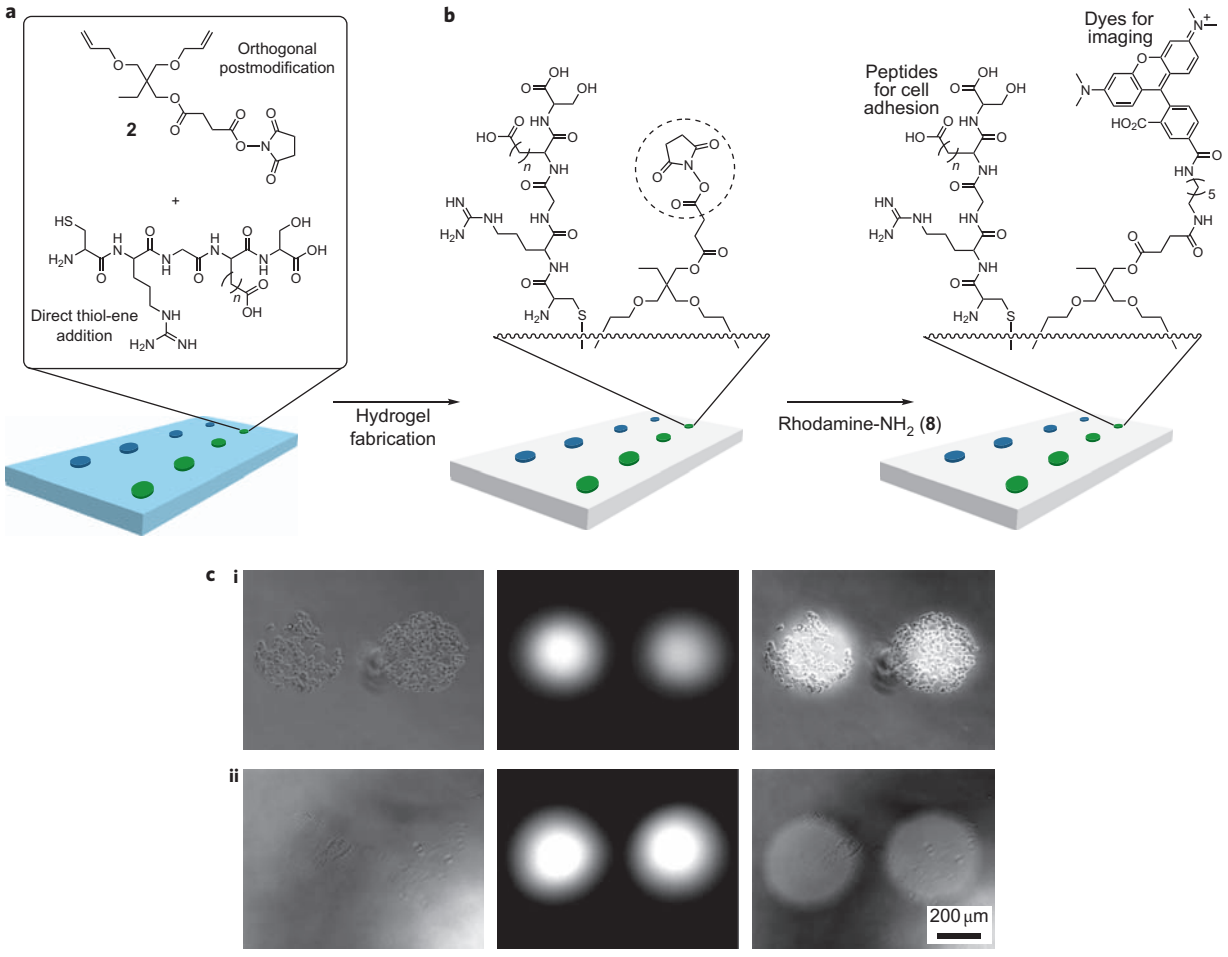

Figure 6 | Combination of direct printing of peptides with orthogonal postfunctionalization on individual microarray spots. a, Microarray fabrication. Cysteine-containing peptides were printed in combination with **2** and transferred to the hydrogel as a microarray. **b**, Postfunctionalization. Microarrays were further conjugated with **8**. $n = 1$, CRGDS; $n = 2$, CRGES. **c**, Microscopy images shown are of CRGDS (**i**) and CRGES (**ii**) in combination with **8** through coupling with the co-printed **2**. Bright field images (left column), fluorescence images (centre column) and overlay of brightfield and fluorescence images (right column).

multiple functionalities can be printed in a single array (Fig. 6c). The advantage of dye labelling in a separate step for the arrays is not only that it greatly simplifies the identification of the array, but also that it points towards the potential advantages of multilabelled systems, and so exploits multiple peptide, dye–peptide and peptide–saccharide combinations, among many others.

Conclusion

Through the combination of microarray printing and the orthogonality of thiol-ene chemistry, different strategies to fabricate functionalized hydrogel microarrays have been developed. The robust nature of the chemistry used to fabricate the arrays allows three-dimensional localization for combinations of functional molecules in defined areas at the surface of cross-linked PEG substrates. This use of a high-yield, user-friendly chemistry and mild reaction conditions for the curing and array-transfer steps also presents a simple method to produce high-throughput, polymeric microarrays in which only minimal amounts of active agents are required. The flexibility of the reported approaches (direct functionalization, orthogonal postfunctionalization and hybrid strategies) provides a variety of platforms that permit the display of assorted chemical signals on non-fouling substrates with tunable mechanical properties. Potential applications range from the analysis of cell–matrix interactions using libraries of biomolecules to the display of mixtures of catalytic moieties.

Methods

Microarray printing. Printing was performed with an Affymetrix 417 arrayer and a setup with a 96-well plate. The printing solutions used for the direct printing of peptides contained a 50:50 ratio of 100 mg ml^{-1} peptide to 100 mg ml^{-1} four-arm PEG–thiol. The NHS cross-linker **2** thioester cross-linker **3** and azide cross-linker **4** were printed as a 25% vol/vol mixture with precursor solution (four-arm PEG–thiol/polyethylene glycol diacrylate 700 (0.115 g/0.1 g)). The vinylbenzaldehye cross-linker (aldehyde cross-linker) **5** was printed as a 10% vol/vol mixture with precursor solution. For microarrays with varying concentrations of NHS cross-linker, the different ratios (vol/vol) were printed from separate wells with different concentrations of NHS cross-linker on a single array. Printing was performed on pre-cleaned 3 × 1 inch glass microscope slides, ~1 mm thick.

Microarray transfer. After the array had been printed, it was transferred to a PEG-based backing. The solution for the polymer backing consisted of a precursor solution and less than 0.1 wt% dimethylaminopyridine (**1**). A droplet of the solution was sandwiched between a clean glass slide and the slide with the microarray printed on it using Teflon spacers (0.4 mm), and cured under ultraviolet light (365 nm) for two minutes. After curing, the array was peeled from the glass slide.

Rhodamine-NH_2 (8), fluorescein–alkyne (9), fluorescein–cysteine (10) and Cascade Blue–$NHNH_2$ (11) conjugations. Microarrays of NHS **2**, thioester **3**, azide **4** and aldehyde **5** cross-linkers were formed as described above. The NHS cross-linker microarrays were incubated (in the dark) at room temperature with light mixing in a pH 8.6 solution of **8** at a concentration of 0.2 μg ml^{-1} for two hours. The azide cross-linker microarrays were incubated (dark) at room temperature with light mixing in a solution of the fluorescein–alkyne dye **9** (0.2 μg ml^{-1}) and a 1:1:1 solution of $CuSO_4$ (10 mg ml^{-1}), sodium ascorbate (10 mg ml^{-1}) and MeOH for two hours. The aldehyde cross-linker microarrays were incubated (dark) at room temperature with light mixing in a pH 5.4 solution of **11** at a concentration of 0.2 μg ml^{-1} for two hours. The thioester cross-linker microarrays were incubated (dark) at 37 °C with light mixing in a pH 7.4 solution of **10** at a concentration of 0.2 μg ml^{-1} for eight hours. The gels were removed from the dye solutions and washed in water for two days, and imaged by fluorescence microscopy using dye-specific filters.

Cell studies. Eight replicates of each hydrogel sample were cut and placed in 96-well plates. Gels were sterilized by incubating with 250 μl of Normocin (5×) overnight at 4 °C. The samples were thoroughly rinsed with phosphate buffer saline (1×) and then incubated in phosphate buffer saline for 24 hours before cell seeding. NIH 3T3 fibroblast cells were seeded at 30K cells cm^{-2} in serum-free Dulbecco's Modified Eagle Medium media with 1% penicillin–streptomycin. After three hours the cells were examined for attachment in Dulbecco's Modified Eagle Medium. Then 10% vol/vol of GIBCO fetal bovine serum was added to four replicates for long-term proliferation studies. Cells were imaged at 8, 24 and 72 hours with media change just before imaging at 72 hours.

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Author contributions

C.J.H, L.M.C. and N.G. contributed to the conception and experimental design. N.G. performed the experiments. B.F.L. contributed to the cell studies. M.D. contributed to the X-ray photoelectron spectroscopy experiments. N.D.T. contributed to the profilometry experiments. M.V.T., E.J.K., D.O.C. and S.T.H. contributed to experimental analysis. All authors contributed to discussion of the results and commented on the manuscript.

Additional information

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