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We report a photo-cleavable material for tight trapping of nonadherent cells to substrate surfaces. Model immunocytes were selectively trapped in a non-irradiated area as single cells after the projection of a light pattern and withstood high-speed laminar flow, achieving light-guided cell release from the substrates.

Single-cell analysis is a rapidly growing research field in biomedical science[1] and biotechnological fields[2]. Image-based methods for single-cell analysis have been actively studied because they can provide accurate and diverse information on individual cells through, for example, real-time monitoring of cellular behaviors and phenotypes[2,3], observation of the dynamics of marker molecules[4] and detection of secreted factors[5]. In such methods, one of the key steps is to separate individual cells on substrates at a high-density for successive high-throughput analysis. In conventional systems, single cells are trapped with determined separation using a microwell array[2,3,5], microfluidic cell capture array[6,7], dielectrophoretic[8] or magnetic devices[9]. Photo-responsive materials have also been reported for single-cell trapping[10]. Compared with methods that require a multiple-step microfabrication process, such material-based approaches are very simple: the cell patterning surface can be produced by only single irradiation with a pattern of light on material-coated substrates. We also reported a photo-responsive cell trapping material[11] and applied it to single-cell analyses[4,12]. This material consists of a long poly(ethylene glycol) (PEG) chain, an oleyl group and a photo-cleavable linker (PL) moiety. On the material-coated surface, the oleyl groups are displayed on the PEG layer for trapping living cells via interaction with the cell membrane, whereas, on the photo-irradiated areas, cells are not trapped because the oleyl group is released via photo-induced cleavage[11]. Because there is no need for biological cell adhesion, cell trapping on this material is extremely rapid and applicable to non- or weakly-adherent cells. On the trapped immunocytes, the normal cell behaviours such as ligand-induced activation and internalization of membrane receptors were observed at a single-cell level[4,12]. However, the cell-trapping ability of this photo-cleavable PEG-lipid is very low, although that of a normal PEG-lipid is extremely strong[13]. Therefore, there is a need to treat the cell pattern on a material with utmost care, such as when the surface is rinsed for exchanging medium, staining the cells, or washing to remove non-specifically adsorbed cells. Therefore, increasing

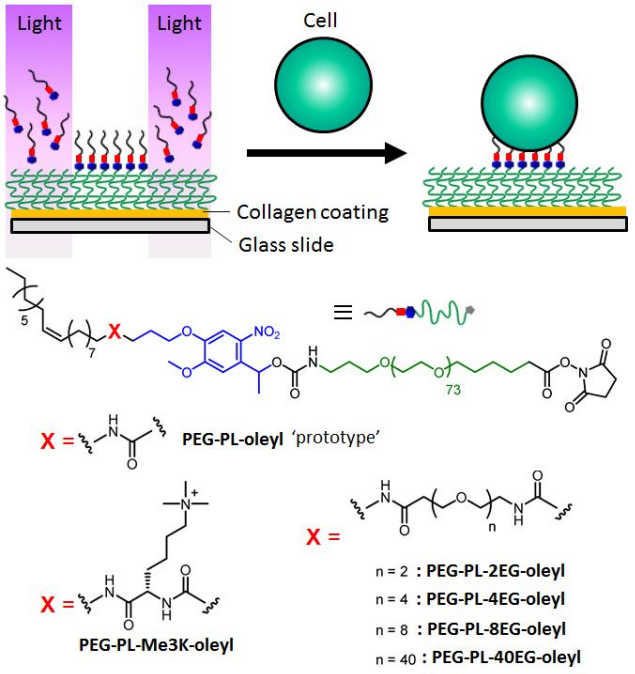

Fig. 1 Schematic illustration of light-guided cell patterning on a glass substrate coated with photo-cleavable PEG-lipids and the chemical structures of both the candidate materials and previously reported 'prototype'.

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the cell-trapping ability of the photo-cleavable material is required for a reliable and reproducible cell manipulation system.

Here, we report the development of photo-responsive materials with strong cell-trapping ability. Five candidate materials were designed and synthesized based on the previously reported photo-cleavable PEG-lipid. In this study, we focused on the chemical structure between the lipid and PL moieties, and incorporated a cationic linker and oligo-(ethylene glycol) linkers. The cell-trapping abilities of surfaces coated with the candidate materials were examined by counting the trapped cells under microfluidic laminar flow. On the optimized material, light-guided patterning of living cells were achieved at a single-cell level in microfluidic devices.

In Regarding the molecular design, an additional linker moiety was incorporated between the oleyl and PL moieties of the 'prototype' of photo-cleavable PEG-lipid (**PEG-PL-oleyl**)(Fig. 1, position '**X**')[11] as it is located at the cell surface when the lipid moiety of the PEG-lipid is inserted into cell membranes. First, a trimethyl lysine moiety was incorporated as a cationic linker to strengthen the binding ability by electrostatic interaction with the anionic components on cell surfaces such as sialic acid (Fig. 1, **PEG-PL-Me3K-oleyl**). The designed material was synthesized and identified by ^{1}H-NMR (see Supporting Information). Collagen-coated glass substrates were then modified with **PEG-PL-oleyl** and **PEG-PL-Me3K-oleyl**. In addition, a substrate modified by PEG-oleyl without PL (**PEG-oleyl**) was prepared as a positive control. To examine the cell-trapping ability of these materials, the number of cells that withstood the sheer stress of microfluidic flow on the surface of a micro-flow path were counted (Fig. 2a, see Supporting Information). As a model nonadherent immunocyte, Ba/F3 cells from the murine pro-B cell line were loaded into the flow path. After incubation for 30 min, phosphate buffered saline (PBS) was pumped into the flow path at 0.1 mL/min for 1 min to remove non-trapped cells. Then, to examine the cell-trapping ability against a microfluidic stress, PBS was poured at 5 mL/min for 1 min, and the remaining cells on the substrate were counted before and after the microfluidic stress. In Fig. 2b, the cell remaining ratio on the **PEG-PL-oleyl**-modified surface was less than 40%, compared with that of **PEG-oleyl**. From this result, it was quantitatively confirmed that the cell-trapping ability drastically decreased by incorporating the PL moiety into the **PEG-oleyl**. Furthermore, conversely to our design, the cell-trapping ability of **PEG-PL-Me3K-oleyl** was less than that of **PEG-PL-oleyl**. Therefore, we hypothesized that the incorporation of bulky chemical structures, such as a nitrobenzene derivative and a branched alkyle chain, might impact the the binding ability of the lipid moiety to cell membranes.

According to the results of the first trial, we designed a photo-cleavable PEG-lipid in which a tetra(ethylene glycol) spacer was incorporated at position **X** (Fig. 1, **PEG-PL-4EG-oleyl**) with the intention the chemical structure neighboring the oleyl group was the same as that of **PEG-oleyl**. As a result, the cell remaining ratio on the **PEG-PL-4EG-oleyl**–modified surface was significantly improved to above 80% (Fig. 2b). This

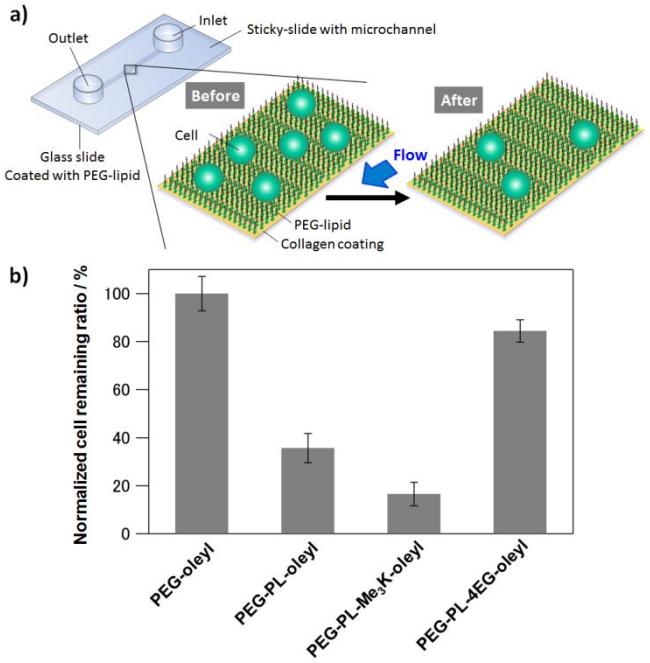

Fig. 2. Evaluation of the cell-trapping ability of photo-cleavable PEG-lipids on the substrate. (a) Schematic illustration of the evaluation system in which the trapped cells were exposed to the sheer stress of laminar flow using the microchannel with the bottom surface modified with PEG-lipid derivatives. (b) The normalized cell remaining ratios after exposure to the laminar flow at 5 mL/min. The cell remaining ratio was normalized with that on the **PEG-oleyl** surface. Values are means ± standard error (n = 3).

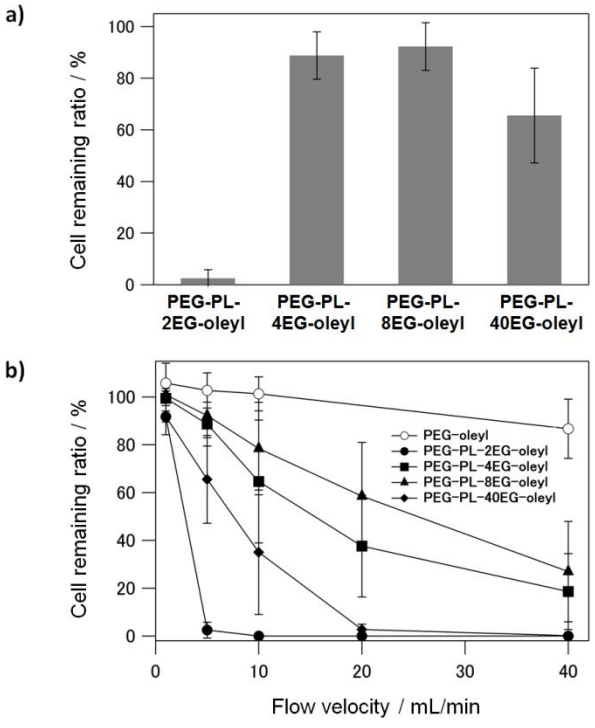

Fig. 3 Cell-trapping ability of oligo-(ethylene glycol) spacer-incorporated photo-cleavable PEG-lipids. (a) The cell remaining ratios after exposure to the laminar flow at 5 mL/min and (b) at various flow velocities. The cell remaining ratios of **PEG-oleyl** (open circle), **PEG-PL-2EG-oleyl** (closed circle), **PEG-PL-4EG-oleyl** (closed squire), **PEG-PL-8EG-oleyl** (closed triangle) and **PEG-PL-40EG-oleyl** (closed diamond) were plotted. Values are means ± standard error (n = 4).

result suggests that the oligo (ethylene glycol) structure neighboring the oleyl group may be required for tightly binding to cell membranes. To investigate the influence of the length of this oligo (ethylene glycol) spacer, three PEG-lipids including the spacers with various lengths (n = 2, 8 and 40 in Fig. 1) were also synthesized, and subject to the same microfluidic experiment (see Supporting Information, Fig. S3). In Fig. 3a, the cell remaining ratio of **PEG-PL-4EG-oleyl** and **PEG-PL-8EG-oleyl** was greater than those of the other candidates. Moreover, when the flow velocity was increased to 10, 20 and 40 mL/min, the cell-trapping ability of **PEG-PL-8EG-oleyl** was clearly greater than **PEG-PL-4EG-oleyl** (Fig. 3b). Accordingly, **PEG-PL-8EG-oleyl** was employed in further experiments as the optimized material for light-guided cell trapping.

Light-guided cell micropatterning was performed on the optimized material surface. In this experiment, the bottom of the flow path was irradiated with ultraviolet (UV) light (360 nm) through a photomask printed with array patterns of circular spots (with the spot diameters: 14–22 μm). After irradiation, BaF3 cells were trapped on the bottom as described above. Figure 4a shows a microscopic image of the cell patterns. By image analysis, the micropatterns were evaluated by their ratios of the numbers of single cell spots to the total spots. The optimal spot diameter was 18 μm (Fig. 4b and 4c). At larger diameters (20 or 22 μm), the number of multiple-cell spots containing more than two cells was increased, and at smaller diameters (14 or 16 μm), the empty spots that did not contain a cell increased because the area of the oleyl-coated surface was not adequate for immobilizing each cell against the shear stress during the wash process (Fig. 4c). This optimized diameter of the photomask was larger than that of the BaF3 cell (approximately 10–12 μm). In this experiment, the photomask was below the glass bottom during light irradiation, and therefore, the light diffracted at the mask was broadened by the thickness of the glass substrate, leading to substantial narrowing of the non-irradiated spots on the upper surface of the glass bottom. Thus, a fine single-cell array was prepared without careful control of the flow rate due to the strong cell-trapping ability of the material.

Finally, we examined light-guided cell release from the material-coated surface (Fig. 4d). BaF3 cells were trapped on the bottom of the flow path, and the bottom was irradiated with various doses of UVA light (360 nm), followed by washing at a flow rate of 1 mL/min to remove the detached cells. Figure 4e and 4f show the microscopic images of the remaining cells and their remaining ratio after light irradiation, respectively. The number of the remaining cells decreased according to the light dose, and nearly all cells were released from the surface after light irradiation at 3 J/cm^2 (Fig. 4e, bottom and 4f). In our previous reports[11], this light dose was confirmed to not decrease the cell viability. Accordingly, this result suggests that the cells of interest on the material are potentially recovered alive in a light-guided selective manner, leading to facile image-based cell sorting after single-cell analysis.

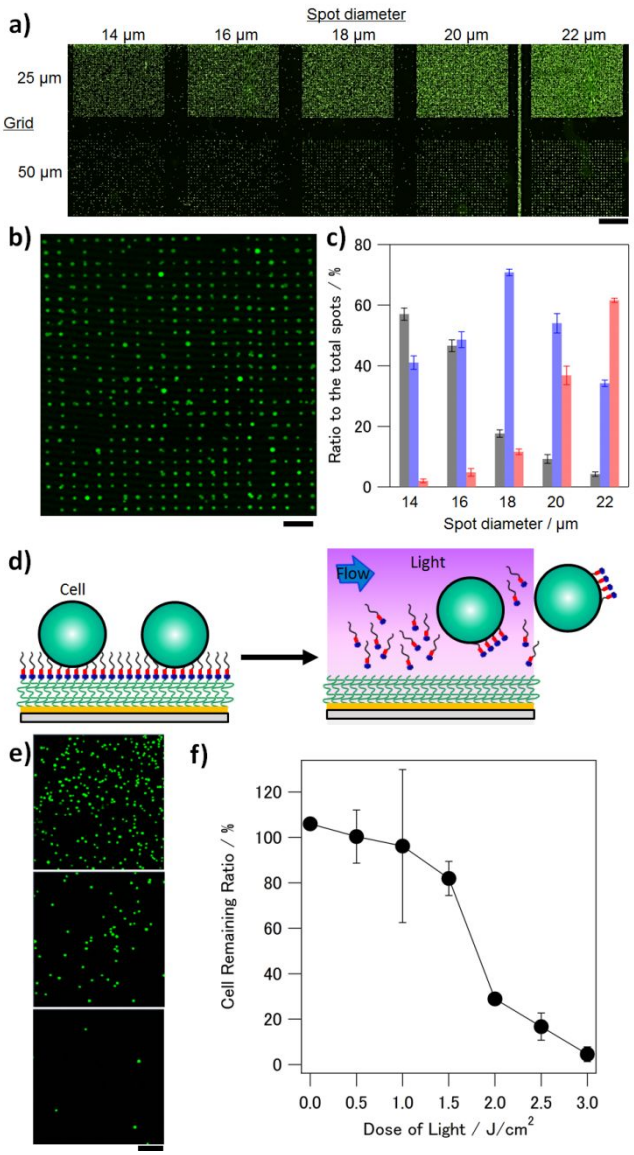

Fig. 4 Light-guided cell manipulation on the photo-cleavable PEG-lipid-modified substrates. (a) The fluorescent microscopic image of the light-guided cell patterns of EGFP-expressing cells (spot diameters of the photomask: 14–22 μm; array grid: 25 μm × 25 μm or 50 μm × 50 μm). Scale bar: 500 μm. (b) The image of the cell array (the spot diameter: 18 μm; the array grid: 50 μm × 50 μm). Scale bar: 100 μm. (c) Ratio of the empty spot (black bars), single-cell spot (blue bars) and multiple-cell spot (red bars) to the total spots at various spot diameters. Values are means ± standard error (n = 5). (d) Schematic illustration of light-guided cell release from the material-modified surface. (e) The fluorescent microscopic images of the cells remaining on the substrate after light irradiation at 0 (top), 2 (middle) and 3 J/cm^2 (bottom). Scale bar: 200 μm. (f) The cell remaining ratio in response to the dose of light. Values are means ± standard error (n = 3).

In this study, the photo-cleavable PEG-lipids with strong cell-trapping ability were obtained through structural optimization, and the optimized composition was demonstrated on the microfluidic device. As a single-cell analysis system, microfluidic systems are widely employed due to the following advantages; (1) the extremely small space of the devices requires a small amount of analyte cells and expensive reagents; (2) the small volume achieves highly sensitive detection; (3) microfluidics facilitate changing the medium

conditions, leading to simple automation and verification. In such microfluidic system, more accurate and rapid manipulation of analyte cells creates more reliable information at higher throughput. From the experimental results, more than 80% of the trapped cells withstood the laminar flow at high flow velocities adequate for removing non-specifically adsorbed cells under the optimal condition (Fig. 4a). Therefore, the present material can serve as a useful tool for enabling reliable and high-throughput analysis. In particular, the PEG-lipid-based materials could rapidly trap cells within 30 min, while most other materials require more than several hours to tightly attach analyte cells because they use biological adhesion for cell attachment. This advantage in the trapping speed can lead to shorter analysis times. Furthermore, since cell adhesion is not an obligatory feature, the present material is applicable to non- or weakly-adherent cells, which include immunocytes and some cancer cells such as circular tumor cells (CTCs) that have attracted growing attention in cancer immunotherapy[14] and diagnostics[15]. In addition, the trapped cells were released from the substrate by irradiating with a low dose of light (Fig. 4e and 4f). From these results, the present material is promising for light-guided isolation of the cells of interest in a rapid and high-throughput manner, although both a high-precision light irradiation system and flow system for cell recovery are further needed.

Conclusions

Photo-responsive materials with strong cell-trapping ability were developed for light-guided preparation of single-cell arrays. On the material-coated substrate, the cells were trapped only in non-irradiated areas after the projection of a light pattern and remained even after treatment with high-speed laminar flow, leading to accurate and reliable preparation of a single-cell array of nonadherent cells. Furthermore, light-responsive cell release from the substrate was demonstrated at a non-cytotoxic dose of light. This material is a potentially facile tool for image-based single-cell analysis and light-guided single-cell sorting for immunotherapies, stem cell researches and drug development.

Conflicts of interest

There are no conflicts to declare.

Acknowledgement

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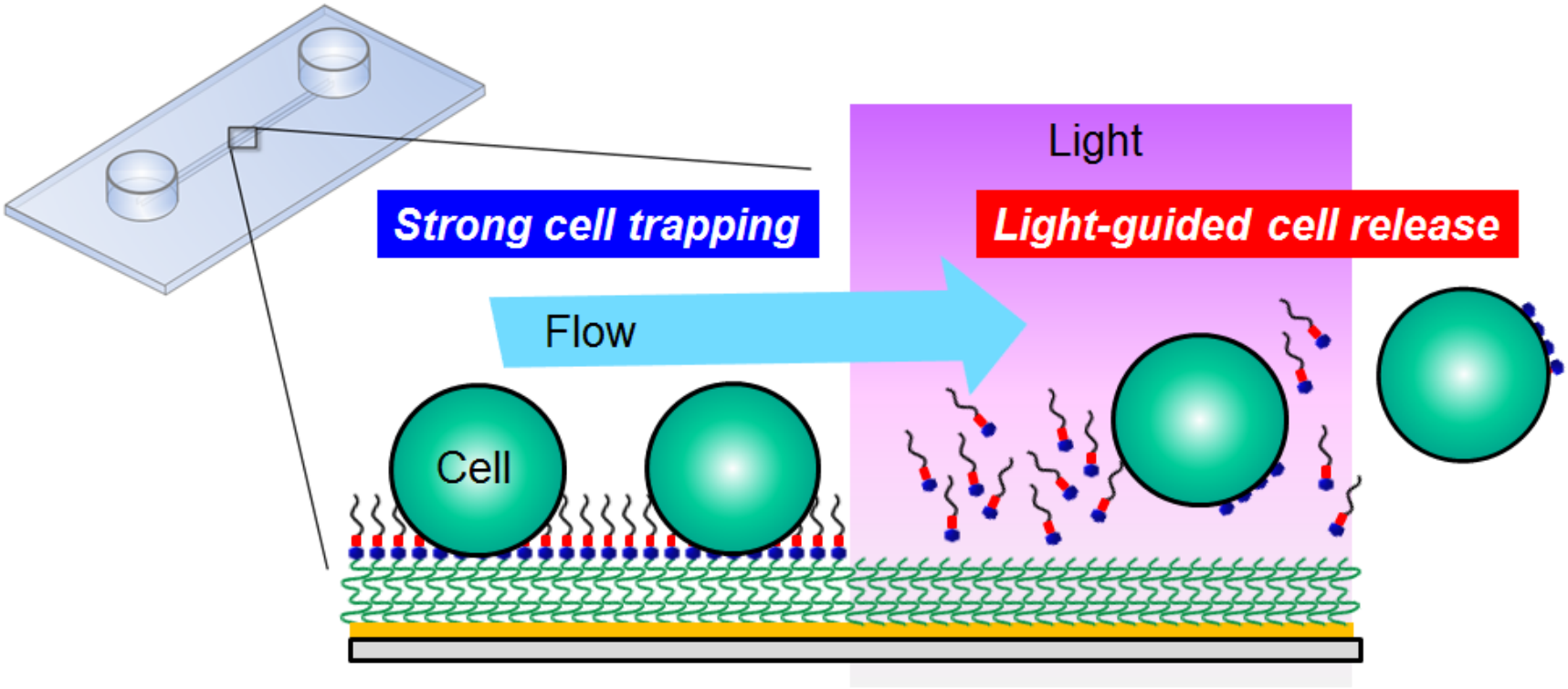

Optimized photocleavable PEG-lipids tightly trapped cells on the substrateunder high-speed flow conditionand release cells in a light-guided manner.