Selective Cell Isolation by Transferrin Functionalized Silane–Carbon Soot Mediated Superhydrophobic Micropatterns

Govind P. Chate, Narendra R. Kale, Vrushali Khobragade, Chinmay Rahane, Marcelo Calderón, Shashwat S. Banerjee,* and Jayant J. Khandare*

Surfaces that facilitate selective cell adhesion using specific targeting moieties have great implications in diagnostics, tissue engineering, and high throughput screenings. However, designing robust and spatially confined micropatterns for selective cell isolation on portable platform is highly challenging. Here, wettable, silane (Si) micropatterns holding covalently attached transferrin (Tf) for targeting Tf overexpressing cancer cells are reported. These micropatterns are separated by carbon soot based superhydrophobic regions that turn these targeting sites into surface tension confined “microwells.” These microwells facilitate capture of human colorectal carcinoma cells (HCT 116) and human cervical adenocarcinoma cells (HeLa) by confining their attachment to wettable region, thereby making isolation and spotting of the targeted cells more efficient. In addition, owing to its transparent trait, the Tf conjugated wettability based patterned chip offer real time optical monitoring of cell adhesion, cell growth, and cell behavior. The specific cell isolation using such surface has applications in devising cancer recurrence monitoring tests.

Designing patterned superhydrophobic (SH) surfaces is highly challenging, since the fabrication of wettable and biologically imperative surfaces with functionalized patterns necessitates control for fluid handling in confined space.1–7 However, imparting chemical functionality to wettable microframes surrounded by SH regions is highly challenging. This is because, few techniques have been proposed to obtain wettable patterned surfaces by using surface modification techniques namely “ink” printing. Furthermore, functional groups have been generated using UV irradiation.8,9 However, the targeting moieties have not been conjugated covalently on wettable patterns surrounded by SH region. Therefore, a novel chemical approach is envisioned to design functionalized micrometer-sized patterns embedded with targeting moieties for selective cell isolation.

We report design and synthesis of functionalized patterns by covalently conjugating transferrin (Tf) on silanized glass surfaces bordered by CS regions. We hypothesize that the covalently conjugated Tf on wettable silanized glass surfaces will demonstrate specific capture of Tf overexpressing cells. Tf is a serum glycoprotein that is widely used as a targeting moiety in cancer therapeutics. Cancer cells overexpress Tf receptors (TfRs) when compared with normal cells. The reason behind overexpressing TfRs by malignant cells is because they are rapidly dividing and growing. This property of malignant cells proves to be their Achilles’ heel.10 Recently, we reported the use of Tf as cell targeting moiety to isolate and capture cancer cells from cancer patients blood samples though 3D matrix substrates.11–14 Similarly, the targeted drug delivery systems designed by Tf-decorated nanoparticles (NPs) demonstrate its cell specific accumulation in tumor, highlighting the enhanced targeting ability after conjugation with Tf.15–19 It is envisioned that Tf targeting moiety covalently attached on wettable patterns will target TfRs overexpressing cells and shall offer several advantages over surfaces that have no confined space limited by boundary layer. Therefore, the uniqueness of such surfaces is fourfold: (a) Tf-mediated wettable cell adhesion sites, (b) nonwettable Cassie–Baxter state inducing SH regions surrounding adhesion sites for preventing cell attachment, (c) high media holding capacity of patterned wells, and (d) ease of monitoring the cell growth studies due to translucent surface.

To demonstrate the efficiency of Tf silane (Si-Tf) functionalized chip, HCT 116 and HeLa cells were used to evaluate the cell isolation efficiency from cell enriching medium. Earlier, Si-Tf chip was prepared by a multistep process as depicted in Figure 1. Number of wettable patterns per Si-Tf chip can be increased, depending on the size of the glass substrate used and the volume of cell suspension from which cell isolation has to be carried out. The process for preparation of Si-Tf chip with multiple wettable sites (Figure 1) and Si-Tf chip with single wettable pattern (Figure 2a) is similar, except the number of masking beads used. Glass cover slips were silanized to impart –NH₂ functional groups for further chemical conjugation. Each glass slip was estimated to contain ≈19 amine groups per nm².20 Quantitation of the number of amine groups per nm²...
area was performed using orange II dye method. This amine group quantitation method was used to confirm silanization. Consequently, silane-functionalized glass slips were then subjected to Tf conjugation at marked sites where patterns were developed. Tf was chemically immobilized with amine-functionalized silanated glass using $N$-(3-dimethylaminopropyl)-$N$-ethylcarbodiimide HCl (EDC·HCl) coupling reaction. The silanization provides the necessary amine groups to be

Figure 1. Scheme of events leading to formation of spatially controlled Tf-conjugated patterns on a glass substrate. a, b) Glass cover slips were used as substrate and were oxidized using piranha solution and subsequently silanized. c) Tf was covalently conjugated on the silanized surface using EDC·HCl as coupling agent. d) Functionalized spots were masked with glass beads of 2 mm diameter to avoid CS deposition. Rest of the silanized glass was covered with PDMS layer (PDMS:curing agent 10:1). e, f) CS was deposited on PDMS layer as well as on glass beads and non-SH patterns were carved out by removing CS-covered glass beads.

Figure 2. Cell isolation in 3D nanostructured patterns. a) Schematic representation of Si-Tf chip and artificial cell suspension. Si-Tf chip is dropped in the cell suspension where wettable target sites allow target cells to get adhered along with some cell media. The presence of nonwettable CS NPs layer around wettable spots prevents cell suspension attachment to the surface except target site. b) Fluorescence microscope images of HeLa cells isolated in wettable patterns 24 h after their isolation were acquired using fluorescence microscopy. Brightfield, Eosin, DAPI, and multichannel overlay images demonstrate the wettability assisted cell isolation from cell suspension. Scale bar is 10 µm.
introduced on the acidified glass substrate. EDC coupling facilitates covalent conjugation of Tf in wettable silanized patterns without formation of any intermediate byproducts. This results in excellent coupling of Tf to wettable patterns as evident from its quantitation. The amount of Tf conjugation was quantitated to be \( \approx 12 \mu g \) per pattern using Bradford assay. On the other hand, nonwettable regions were fabricated by depositing CS over semicured polydimethylsiloxane (PDMS) mixed with curing agent and mapped around conjugation site. The semicured PDMS allows more firm attachment of CS as compared to completely cured PDMS as evident from the CA measured for 10 \( \mu L \) water droplet on surfaces prepared by semicured and completely cured PDMS. CA for 10 \( \mu L \) water droplet on semicured PDMS turned SHS was 162\(^\circ\) while SHS resulting from completely cured PDMS was 138\(^\circ\). Glass beads were used as covers to mask the conjugation site and to prevent CS deposition on conjugated Tf. Finally, glass beads covered on Si surface were removed to form the wettability tuned Si-Tf chip.

Scanning electron microscopy (SEM) images of CS NPs deposited on PDMS surface are shown in Figure 3a. The image indicates, CS NPs of \( \approx 40 \) nm size were formed at uppermost layer. Interestingly, the static contact angle (\( \Theta \)) for a 5 \( \mu L \) sessile water droplet on nonwettable region was observed to be 162\(^\circ\) (Figure 3d) showing the SH nature of the nonwettable region. This is expected to assist the cell media droplet to assume Cassie–Baxter state by trapping air pockets beneath the contact line of cell media drops. The robustness of nonwettable region of Si-Tf chip was evaluated by examining change in CA for 10 \( \mu L \) water droplet on nonwettable region after washing. The washing process involved passing 50 mL distilled water over 30 s as water current and repeating the process three times. Superhydrophobicity of the nonwettable section of the Si-Tf chip was maintained even after repeated washings as evident from the <2\(^\circ\) change in CA for a 10 \( \mu L \) water droplet measured before and after washings.

Figure 3. Characterization of Si-Tf chip. a) SEM micrographs of CS particles deposited on PDMS layer by combustion of carnauba wax candle flame. b) AFM micrograph indicating surface geometry of nonwettable region depicting arrangement of CS particles. c) Schematic representation of concentric patterns of Tf-functionalized Si-Tf chip. d) Static contact angle of 5 \( \mu L \) water drop on nonwettable region. e) SEM microscopy images of interface of functionalized wettable pattern and surrounding SH region.

Notably, cell adhesion molecules (CAMs) do not find adhesion sites when the cell suspension drop comes in contact with nonwettable region, thereby preventing the cell attachment.[21] Furthermore, the surface tension induced by CS layer with very low surface energy allows the water droplet to roll off from the surface even at 2\(^\circ\) inclination (Figure S2, Supporting Information). As evident from atomic force microscopy (AFM) images, the height of CS aggregates in top layer was below 500 nm formed by individual CS particles of 40–50 nm size that were attached to the PDMS during the curing process (Figure 3a). While, AFM studies showed nanoscale roughness on PDMS template resulting from CS deposition (Figure 3b). CS NPs were deposited as uneven patterns resulting into nanoscale roughness. This arrangement of CS aggregates allowed the formation of pillars of varying heights that prevented the water droplets and polar solvents from wetting the surface. Further, the arrangement of CS aggregates at the interface of wettable and nonwettable pattern resulted in cell media droplet confinement only to wettable region (Figure 3e).[22] Cell media drops adhered to the wettable patterns could be easily transferred from Si-Tf chip to 6-well plate by using micropipette for cell incubation and microscopy studies. Interestingly, cell media drops remain in these patterns even when the Si-Tf chip was rotated at 180\(^\circ\) owing to its adhesive force enabled attachment to the wettable pattern (Figure S1, Supporting Information).

Cell targeting and cell isolation efficiency was studied by seeding HCT 116 and HeLa cells using wettable pattern on Si-Tf surface that was initially immersed in cell suspension for 30 s and later repeatedly washed with excess amount of cell media. The droplets were then observed under fluorescence microscope and total number of cells attached per pattern was accounted. The surface area (SA) of each pattern was calculated to correlate the number of cells attached in each wettable pattern (Table S1, Supporting Information). The number of cells captured for Si-Tf was found to be \( \approx 7 \) times higher than...
nonfunctionalized surface indicating the role of Tf moiety. In order to compare the efficiency of Tf functionalized Si-Tf versus nonfunctionalized pattern, the SA required to isolate cells was considered as a prudent parameter. Area required to isolate a single cell in Si-Tf patterns was found to be 177.25 µm². On the other hand, the Tf negative patterns required 1256.16 µm² area to isolate a single cell. This significantly lower area required for cell isolation by functionalized patterns can be attributed to the presence of Tf and its cell specificity. TfRs overexpressing HCT 116 colorectal cancer cell isolation enhancement is in line with the presence of Tf and its cell specificity. TfRs overexpressing HCT 116 colorectal cancer cell isolation enhancement is in line with our hypothesis and supported by earlier reports.[23,24]

To further investigate the influence of Tf on cell capture efficiency, we employed wettable patterns of Tf conjugated (SHS-Tf), silanized glass (SHS-Sil), and plain oxidized glass (SHS-glass) for capturing HeLa and HCT 116 (Figure 4). Silanized glass surface isolated more number of cells (1256.16 µm² surface area required for single isolated cell) over untreated glass patterns (2235.15 µm² surface area for single cell) indicating the significance of surface functionalization induced by silanization. The linear chains created on the surface after silanization have amide groups at the terminal that enables Tf attachment to this surface through EDC coupling reaction. Rough silanized surface isolated more cells as compared to its untreated glass counterpart highlighting the importance of surface roughness for CAM attachment. Tf conjugated to silanized surface further enhances the capturing ability of Si-Tf for Tf overexpressing tumor cells. Our results further showed that SHS-Tf surface isolated highest number of cells (~7 times) per unit area indicating the enhanced efficiency of surface after conjugation with Tf. Enhanced cell isolation after covalent conjugation of Tf to silanized wettable surface proves the need for chemically attaching targeting moieties on wettable spots as hypothesized by us. It was noted that no fraction of cell suspension remained attached to the nonwettable region highlighting the Cassie–Baxter state attained by media droplet, resulting from the air entrapped in upper layer of surface beneath the droplet. Further, no fraction of CS was observed in the wettable pattern proving the robust attachment of CS to PDMS, thereby maintaining nonwettability throughout the experiment.

Tf conjugated in patterns of Si-Tf chip act as favored as well as enhanced interacting sites for cancer cells and thereby a high number of cancer cells are attached on these patterns. Thus, the surface tension confined system enables cell attachment in wettable patterns, whereas nonwettability induced by superhydrophobicity assisted in directing the cell suspension droplets to wettable patterns. Importantly, cell adhesion and cell growth is facilitated in Si-Tf patterns that provide nanorough wettable surface for enhanced cell interactions with CAMs. Si-Tf pattern (Figure S3b, Supporting Information) accounts ~1 mm² surface area that could hold 330 µL cell media inside individual pattern (Figure S3a, Supporting Information). Cell growth in the Si-Tf pattern is uniform, as evident from cell growth at boundary region of the pattern indicating uniformity of Tf conjugation in Si-Tf chip functionalized wettable spots (Figure S4, Supporting Information).

In order to assess the feasibility of Si-Tf to be used for monitoring cell growth, study of its cell media holding capability was essential. Moreover, the cell media held in wettable patterns should be sufficient enough to provide the necessary nutrients to cells during cell growth phase. In addition, we studied the rate of evaporation of cell media, i.e., ~50 µL McCoy’s 5A (modified) medium droplet was used at room temperature. After 24 h, 45% reduction in droplet volume was observed. Likewise ~48% droplet size reduction was observed for Dulbecco’s Modified Eagle Medium (DMEM) after 48 h. When compared with the amount of media required for cell growth observation in case of 96-well plate (300 µL), there was a sixfold decrease in cell media requirement. This makes the cell isolation and growth studies fairly economical as compared to conventional techniques. Toward this, cell growth studies were carried out on Si-Tf patterns for 72 h with periodic replenishment of cell media. Cell growth images were taken after regular time intervals of 4, 12, and 24 h. The cell growth images taken after 24 h are as shown in Figure S4 (Supporting Information). These results underline the potential of Si-Tf patterns to monitor cell growth parameters in real time.

Figure 4. HCT 116 cell isolation using Si-Tf chip. a) Images of schematic representation depicting cell attachment to conjugated Tf in wettable pattern of Si-Tf chip. The Si-Tf chip is shown containing Tf covalently attached to wettable pattern, thereby making it a substrate that acts as a trap for targeted cells. The cancer cell attachment to Tf is shown in these schematics with marked reference to actual cells isolated in Si-Tf chip. b) HCT 116 cell growth over a period of 48 h in patterns functionalized with Tf, without Tf conjugation, and without silanization. Scale bar is 20 µm.
In another set of experiments, we evaluated the cell media evaporation rate in Si-Tf patterns to be used as prospective platform for cell aggregation studies (Figure S5, Supporting Information). Cell suspension droplet adhered to Si-Tf pattern has fixed contact line spanning across the diameter of wettable pattern. 10–50 µL cell suspension droplets when placed on the wettable patterns assume a convex shape with their boundaries lying completely within the wettable region. But as the volume of droplets is increased, although the contact line remains fixed, the droplet starts spreading over to the nonwettable region. The nonwettable region has low surface energy, which in combination with surface tension of water makes the droplet assume semispherical shape (Figure S3, Supporting Information). When cell suspension droplet is placed in wettable pattern, the cells start settling at the bottom under the influence of gravitational pull. Meanwhile, the droplet starts shrinking as the evaporation starts, bringing the cells settled on the concave portion of the droplet toward the fixed contact line. This ultimately resulted into aggregation of cells specifically limited to area of contact line. The shape of the sediment changed with the number of cells present in the droplet. In this study, we observed the shrinking pattern of the droplet as well as the reduction in its area. 100 µL cell suspension droplet shrinks 70% in volume after 80 min. The contact line of the droplet remains fixed during evaporation (Figure S5, Supporting Information). In order to carry out cell growth of the aggregated cells, it is necessary that cell media volume is maintained which changes during evaporation. Si-Tf chip facilitates compensation of loss of media through evaporation by manually adding fresh cell media during cell growth studies. These results indicate the feasibility of using Si-Tf as a platform for cell aggregation studies for prolonged duration. Cell media droplet can be held in inverted state as shown in Figure S1a (Supporting Information). This makes the Si-Tf chip, a prospective platform for studying 3D tumor spheroid formation. Cell aggregate formation studied by us yielded clustered cells in wettable patterns (Figure S1b, Supporting Information), but further studies are needed to be carried out using the Si-Tf chip in inverted state holding cells suspended in anchored droplet.

In summary, we have prepared a novel Si-Tf cell targeting and isolation chip where wettable patterns are formed by functionalization of glass substrates to covalently attach a targeting moiety, Tf. This covalent attachment binds the Tf in targeting spots, allows selective capturing of Tf overexpressing cancer cells when Si-Tf is exposed to cell suspension. We demonstrated that Tf can be covalently conjugated to functionalized wettable patterns through EDC coupling reaction carried out on silanized glass pattern surrounded by CS NPs induced superhydrophobicity. CS-based SH layer surrounding the wettable pattern facilitates surface tension induced confinement of cell suspension drop. We effectively isolated 55 HCT 116 cells in Si-Tf patterns spanning cumulative area of 9749 μm² spread over four wettable spots. In comparison, number of cells isolated in Si-Tf wettable patterns without Tf, was 9 in 4 spots with cumulative area of 11305 μm². Ease of cell media transfer to and from the wettable spot allows cell growth studies to be conducted for 48 h. The cell suspension droplet evaporation studies revealed that the droplet shrinking takes place along the fixed contact line, thereby facilitating the cell aggregation in wettable spot.

We conclude that transferrin functionalized silane–carbon soot mediated superhydrophobic micropattern is a potential platform for cell targeting which can be developed into a diagnostic tool to isolate cells of interest in confined space. Various other biologically implicated targeting moieties can be conjugated to the functionalized glass substrates by altering the functionality imparted to wettable spots, which provide confined sites for cell growth parameter studies. In addition, results presented here also highlight that designing wettability controlled patterns can prove to be a choice of surface patterning for containment and confinement of fluids and their motion, which will act as guiding template for bio-functionalized materials. Overall, this study present the feasibility of chemically attaching targeting moieties with spatial conformities coupled with prospect of selecting targeting moiety for specific cell isolation.

**Experimental Section**

*Preparation of Functionalized Glass Substrates:* Glass cover slips were procured from a local supplier and treated with Piranha solution (conc. H₂SO₄ and H₂O₂ in 3:1 proportion) at 80 °C for 2 h with constant stirring. Treated cover slips were washed thoroughly by double distilled water and dried in oven at 40 °C. The oxidized cover slips were further functionalized with 3% [3-aminopropyl] trimethoxysilane in toluene. After overnight stirring, silanized glass substrates were washed with double distilled water and dried at room temperature.

*Attaching Tf to Silanized Glass:* EDC.HCl and Tf (30 µg mL⁻¹) were mixed at pH 6.0 for 30 min. 500 µL of Tf and EDC.HCl solution were placed on the sites of pattern formation and incubated at room temperature for 3 h. The glass substrate was washed with DI water to remove any unattached Tf from the sites and subsequently dried at room temperature. These Tf functionalized wettable patterned cover slips were UV sterilized for 15 min and stored in sterile conditions to be used for cell capture studies.

*PDMS-Glass Bead Mask Formation:* Sylgard 184 elastomer base and curing agent were mixed in a proportion of 10:1. Silanized glass slips were covered with thin layer PDMS except for the sites of Tf conjugation. Tf conjugated sites were masked with glass beads surrounded by PDMS layer. Uniformly spread PDMS layer is allowed to cure at 80 °C for 30 min and cooled to room temperature.

*Wettability Controlled Confined Pattern Formation on Silanized Glass:* Masked silanized glass was deposited with nonwettable CS layer using carnauba wax candle flame. CS started emitting from candle flame during wax combustion in oxygen-deprived state resulting from glass slip capping the flame tip. After CS depostion, the glass slip was exposed to water current to remove excess and unbound CS. Glass beads were removed leaving behind Tf spots surrounded by CS layer.

*Evaluation of Contact Angle of Silanized Glass and Patterned Surface:* Contact angle Goniometer was used to evaluate the static contact angle of silanized glass as well as functionalized glass substrate. A 5 µL water drop was placed on the nonwettable region and the angle at the three-phase contact line was measured by the software interface. Captured images were further processed with image processing freeware ImageJ using Low Bond Axisymmetrical Drop Shape Analysis (LB-ADSA) plugin of ImageJ.

*Cell Suspension Preparation:* HCT 116 colorectal cancer cell line and HeLa cervical cancer cell line were used to evaluate Si-Tf chip. HCT 116 cell line was maintained in 90% McCoy's 5A medium in addition to 9% fetal bovine serum and 1% antibiotic in cell culture flask. HeLa cell line was maintained in DMEM supplemented with 9% fetal bovine serum and 1% antibiotic; incubated at 37 °C and 5% CO₂ concentration. These cell lines were periodically inspected for contamination. Cells in
To the PDMS layer on the silanized glass were evaluated for their superhydrophobicity through their arrangement. The morphology of CS particles attached to PDMS that form the nanoaggregates to accommodate the air pockets were also observed. The morphology of CS particles forming the nonwettable part were observed under fluorescence microscope (Carl Zeiss Axio Observer A1) as shown in Figure 4b.

**Comparison of Cell Adhesion on Silanized Glass versus Tf Functionalized Glass:** HCT 116 and HeLa cells adhered on Si-Tf chip patterns were observed under fluorescence microscope. Number of cells adhered in patterns with conjugated Tf and non-Tf conjugated patterns were compared. Area of each pattern was measured using Zeiss Core software to calculate the number of cells adhered per pattern on Si-Tf chip and control surface. Efficiency of Si-Tf chip patterns was calculated by measuring the area of each pattern and comparing the number of cells per pattern.

**Observation of Cell Growth in Wettable, Functionalized Patterns:** Si-Tf chip and control surface with adhered cell suspension drops were transferred to a 6-well plate. Completed Cellular and control medium was added to HCT 116 cell suspension drops and 50 µL Dulbecco’s Modified Eagle’s medium was added to HeLa cell suspension drops. The 6-well plate containing the pattern was transferred to a bioincubator maintained at 37 °C and 5% CO2 concentration. Cell growth was observed at every 3 h interval.

**Attachment of Cells on Nonwettable Region:** In a control experiment, cell attachment to nonwettable CS layer was observed by placing 10 µL of HCT 116 cell suspension with 2 × 106 cells mL−1 concentration on CS layer for 2 h. 1 µL nuclear staining dye DAPI (0.5 mg mL−1) and 1 µL cytoplasm staining dye Eosin (5 mg mL−1) were added to the drop and incubated for 30 min and subsequently washed with phosphate buffer. Cell suspension drop was removed from the surface to observe the number of cells attached to CS layer. CS pattern was observed under fluorescence microscope for detection of attached cells.

**SEM Characterization of Nonwettable and Boundary Regions on Si-Tf Chip:** The morphology of CS particles forming the nonwettable part of the chip was characterized by SEM (FEI, Quanta 200-USA). The functionalized patterned wells and CS particles present at the interface were also observed. The morphology of the CS particles attached to PDMS that form the nanoaggregates to accommodate the air pockets imparting nonwettability was observed.

**AFM Characterization of CS Layer:** Patterned Tf nonwettable surfaces were characterized using the Park XE 150 Atomic Force Microscope attached to a Labram high-resolution spectrometer. CS NPs attached to the PDMS layer on the silanized glass were evaluated for their attachment to assess the formation of air pockets which imparts superhydrophobicity through their arrangement.

**Supporting Information**

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

**Acknowledgements**

The authors acknowledge the financial support of the Department of Science and Technology, Government of India for the Fund for Improvement of Science and Technology infrastructure (FIST-DST) and the Department of Biotechnology (DBT) grant. The authors also appreciate Freie Universität, Berlin, Alumni research grant and Prof. Marcelo Calderón Group’s support for SEM and AFM studies.

**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

cell isolation, conjugation, superhydrophobic, transferrin, wettability