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Biotin-Streptavidin Linkage Utilized to Fabricate Dense Packing of Red Blood Cells Immobilized to Protein Monolayer on Solid Support

Taiji FURUNO

ビオチン―ストレプトアビジン結合を利用して赤血球の稠密パッキングを
固体基板表面のタンパク質単分子膜に固定化する方法

古野泰二

Summary——Immobilization of red blood cells (RBCs) onto the surface of solid support is described. Biotinylated carbonic anhydrase (CA) was spread at the air/water interface, unfolded there, transferred onto silicon wafer, and coupled with streptavidin (SAv). Biotinylated RBCs were bound to this SAv layer via biotin-streptavidin linkage forming an immobilized dense packing of RBCs. Upon hemolysis the dense packing of disk-shaped RBCs changed to an in-plane packing of polygon-shaped flattened cells contacting with each other without void space. As an application, patterning of the RBC dense packing was examined.

Key Words: Red blood cell, immobilization, dense packing, streptavidin, biotin, protein monolayer, Langmuir-Schaefer technique

Abbreviations used: RBC, red blood cell; bRBC, biotinylated RBC; SAv, streptavidin; LC-biotin (LC-LC-biotin), sulfo-NHS-LC-biotin (sulfo-NHS-LC-LC-biotin); CA, carbonic anhydrase; Si wafer, silicon wafer; HMDS, hexamethyldisilazane

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1. Introduction

Adhesion or immobilization of biological molecules, organelles and cells on solid supports are essential for both basic studies and industrial applications. Cell adhesion concerns wide interests of cellular biology, biomedicine and bioengineering[1]. The properties of solid surfaces affect interaction with cells. The surface of cell culture vessels, for example, is usually treated hydrophilic by physical means or by coating with polymers depending on cell types[2]. Various strategies have been explored to immobilize cells and enzymes to solid surfaces or into polymer materials[3].

Biotin-streptavidin linkage has been used as a standard biochemical tool to link two biotinylated molecules via streptavidin that has four biotin binding sites. Protein molecules can be biotinylated under mild chemical conditions. Protein antigen was linked to autologous red blood cell surface to study antigen delivery system[4]. Anti-human collagen antibody was linked to the surface of RBC, and the binding of this RBC to collagen-coated solid surface was studied as a drag targeting model[5]. RBCs linked with functional probes on their membrane surface were circulated in bloodstream long days[6].

Densely packed RBCs immobilized on polylysine coated glass slide was used for immunoplaque assay that can be examined by scanning electron microscopy or light microscopy[7]. The adsorption of cells to the polylysine-coated solid support makes use of electrostatic attraction of polylysine to negative ions on the cell surface. On the other hand, biotin-streptavidin method uses the extraordinarily high affinity of streptavidin for biotin with a dissociation constant of $\sim 10^{-15}$ mol/L, so that the bond is unbreakable under ordinary physiological conditions. In the present study, biotinylated carbonic anhydrase (bCA) was spread at the air/water interface forming a monolayer of unfolded bCA and was transferred onto a chip of silicon wafer by Langmuir-Schaefer (L-S) technique[8]. Rat red blood cells (RBCs) were biotinylated and were bound to the SAv arrays coupled with this transferred bCA film (Fig. 1). The binding of RBC to the SAv layer proved to be strong enough to withstand vigorous washing in a buffer solution. Making use of this property, it was shown that the RBC dense packing could be patterned by printing a pattern of biotin binding activity on the SAv layer using deep UV exposure.

2. Materials and Methods

2.1 Materials

Preserved rat blood was purchased from Kohjin Bio Co. (Japan). Biotinylation reagents

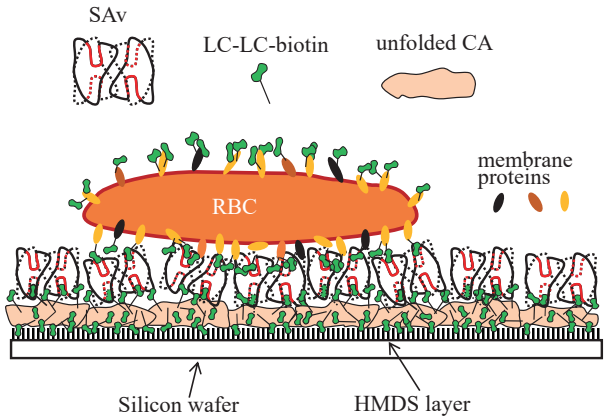

Fig. 1

Schematic for RBC immobilization onto Si chip. The hydrophobic surface of silicon wafer is fully covered with unfolded bCA transferred from the air/water interface by Langmuir-Schaefer method. Streptavidin is bound to the unfolded bCA film. Biotinylated RBCs are immobilized to the SAv layer through biotin-SAv linkage.

Sulfo-NHS-LC (or -LC-LC)-Biotin were obtained from Pierce Chemical Co. Carbonic anhydrase from bovine erythrocytes (CA, 29 kDa, C 3934), streptavidin from Streptomyces avidinii (SAv, 60 kDa, S 4762) and albumin from bovine serum (BSA, A 7030) were purchased from Sigma-Aldrich, Japan. A buffer solution (135 mM NaCl, 10 mM HEPES, 2 mg/mL glucose, 1.0 mg/mL BSA, pH 7.6) abbreviated as Liquid A (LiqA) was used for washing, storing and biotinylating red blood cells.

2.2 Biotinylation of carbonic anhydrase (CA) and red blood cells (RBCs)

CA (1.7 mg/mL) was reacted with Sulfo-NHS-LC (or -LC-LC)-Biotin (1.5 mg/mL) in 100 mM phosphate buffer pH 7.4 (abbreviated as LC-bCA or LCLC-bCA). After incubation for 1 h at room temperature, unreacted reagents were removed by washing with pure water using a molecular filter Amicon Ultra-0.5 Centrifugal Filter Device (molecular weight limit 30 K). Washing was repeated five times with the concentration factor of 10.

Red blood cells (RBCs) were collected by centrifugation. 20 μL of the preserved blood was diluted in 600 μL of LiqA and washed three times. 2 mg of Sulfo-NHS-LC-LC-Biotin was added to the RBC suspension, incubated for 30 min at room temperature. The RBCs were then washed 6 times with 600 uL of LiqA. Optical density at 600 nm (OD_{600}) was taken as a measure for the concentration of biotinylated RBC (bRBC) suspension. A LiqA suspension with OD_{600} of 0.1 ～ 0.7 was used for immobilization experiments. From reported values for RBC count in rat whole blood, we estimated $OD_{600} = 1$ to be about 1×10^7 cells/mL.

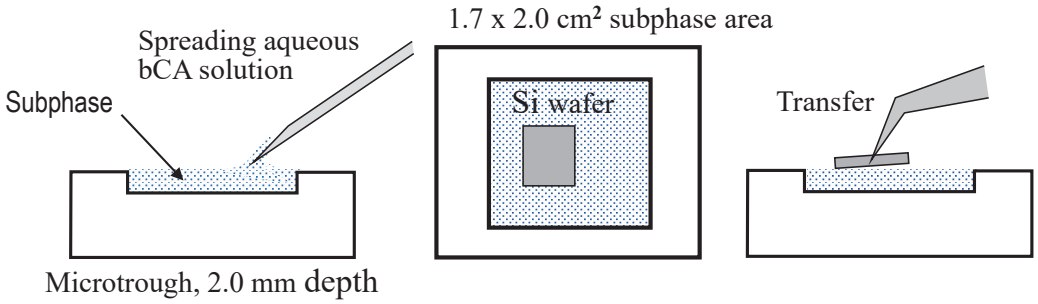

Fig. 2
Schematic for spreading and transfer of unfolded bCA film.

2.3 SAv layer formed on bCA monolayer

In our previous studies, CA molecules dissolved in pure water was spread at the air/water interface and the unfolded CA film was transferred onto hydrophobic silicon surface. This film was biotinylated after the transfer to obtain a dense packing of SAv[8]. In the present study, instead, biotinylated CA (LC-bCA or LCLC-bCA) was spread at the air/water interface. Spreading and transfer conditions for Langmuir monolayers of bCA were optimized by adjusting the amount of spread bCA.

Fig. 2 shows schematically the Langmuir-Schaefer technique. 1.5 uL of 0.7 mg/mL LCLC-bCA solution was spread at the subphase surface (20 mM phosphate buffer, pH 6.4, 3.4 cm^2 surface area). The surface film was transferred onto a chip of hydrophobic silicon wafer after an incubation for 5 min. To achieve SAv binding to the bCA film a drop of SAv solution (100 μg/mL in pure water) was placed on the surface of transferred bCA film and incubated for 40 s with moderate agitation using a micropipette. The chip was rinsed with pure water and finally with 3 % glucose solution. The wet Si chip was placed in an Eppendorf tube and quickly dried by spinning at 6000 rpm. The SAv arrays were thus preserved in glucose and proved to maintain biotin binding activity longer than a month in laboratory air[8].

2.4 Immobilization of biotinylated RBCs onto SAv arrays

Immobilization of biotinylated RBCs onto SAv arrays was achieved quickly by centrifugation (Fig. 3). A wafer chip was placed at the bottom of a 1.5 mL Eppendorf tube containing bRBC suspension. Centrifugation was at 100 ～ 400 × g for 1 min alternating the chip orientation up and down, every time resuspending the precipitated RBCs. The Si chip which emerged fully covered with RBCs was placed in a small plastic dish filled with 0.5 ml of LiqA. The chip was vigorously washed by swinging the dish manually at

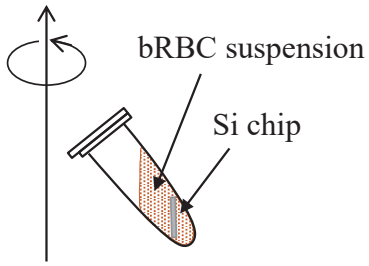

Fig. 3
Schematic for centrifugal deposition of bRBCs on Si chip.

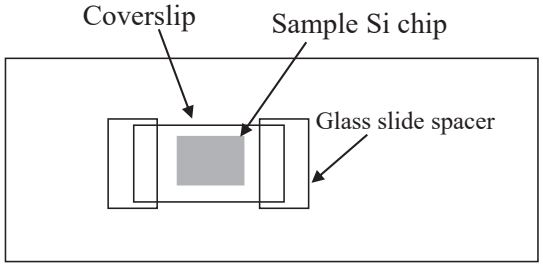

Fig. 4
Schematic of a liquid cell used for Nomarski microscope imaging.

～3 Hz with the oscillation amplitude ～1 cm about 100 times.

Packing of RBCs on the Si surface was observed with a Nomarski microscope using a liquid cell (Fig. 4).

2.5 Patterning of RBC dense packing

Glucose-preserved SAv arrays on Si wafer was made in contact with a mask of quartz plate patterned with 25 μm squares of vacuum deposited chromium (Fig. 6 b). The SAv molecules beneath the unmasked quartz window were exposed to 185 nm deep UV light. The irradiation condition for our apparatus, i.e., the exposure time and location under the UV lamp was optimized by observing detachment of the deposited bRBCs by washing in LiqA. About 8 min of exposure was sufficient to render the SAv surface of the unmasked area inactive for biotin binding.

2.6 Hemolysis of the immobilized RBC

The dynamic structural change in the packing of immobilized bRBCs was observed on introducing pure water using a liquid cell (Fig. 4).

3. Results and Discussion

3.1 Immobilization of RBCs on SAv surface

The centrifugal force acting on the suspended RBCs towards the Si chip was useful to obtain a dense packing of immobilized bRBCs (Fig. 3). A dense packing of immobilized bRBCs was not obtained simply by incubating the SAv-coated Si chip, e.g., in a suspension of OD_{600} ～0.5 for 1 h. The effective centrifugal force acting on bRBC is considered proportional to the density difference between bRBC and solution LiqA. As mentioned

in the experimental section the centrifugal acceleration was 300～400 × g to achieve a dense packing. Fig. 5 a and Fig. 5 c is a comparison between the two samples (Fig. 5 a; OD_{600}～0.35, 100 × g, 1 min, 5 times of centrifugation) and (Fig. 5 c; OD_{600}～0.7, 300 × g, 1 min, 3 times of centrifugation). A stronger centrifugal force with higher RBC concentration resulted obviously in a denser packing. Both Fig. 5 a and Fig. 5 c show apparent anisotropy image for RBC caused by interference microscopy imaging. The shape of bRBC on the SAv surface was convex not the biconcave shape known as discocyte which we usually observe in the rat blood. The RBCs overlapping on the first layer RBCs were removed by washing in LiqA and only the first layer RBCs kept staying on the Si chip, probably due to a strong adhesion force via biotin-SAv linkage. An evidence was that RBCs in Fig. 5 a did not show a Brownian motion.

When the centrifugal acceleration is as high as 300 × g, the force that presses the cells toward SAv surface should be stronger than that at 100 × g. Therefore, at a higher centrifugal force the cells falling to the narrow space between the pre-bound cells gain higher chance to be immobilized despite the repulsive effect from the pre-bound first layer cells. On this occasion, deformation of the cells would readily occur. Comparison between Fig. 5 a (inset) and Fig. 5 c (inset) seems to support this idea. The shape of each cell in Fig. 5 c is difficult to be discerned due to this overlapping of soft deformable cells in the presence of biotin-streptavidin binding that attracts bRBC to the SAv layer. Overlapping between cells might have also occurred in the low-density sample, e.g., as shown in Fig. 5 a (upper left area in the inset). It is evident that Fig. 5 c does not provide unambiguous periphery image of each RBC that is clearly seen in the lower density sample (Fig. 5 a).

Hemolysis of the bound RBCs demonstrated dynamic changes in the packed structure, that completed within 10 min after exchanging LiqA to pure water (Fig. 5 c and Fig. 5 d). RBCs in a medium dense packing changed their shape to isolated flat discs (Fig. 5 b), whereas denser packing as in Fig. 5 c changed to a packing of polygons without space or gap between neighboring cells. Both the periphery of the isolated cell (Fig. 5 b) and the contact lines between hemolyzed polygon cells (Fig. 5 d) appear thicker than central region of each cell. Although it is difficult to interpret the apparent uneven thickness only by light microscopy, it would be concluded at least that the bound RBCs were flattened uniformly without holes, shreds, or wrinkles of micrometer sizes.

Another method to obtain a dense packing of bRBCs was to place the sample Si chip to the bottom of a small dish filled with 0.5 mL RBC suspension agitated by shaking or by pipetting. However, a dense RBC immobilization which changed into a polygon pack-

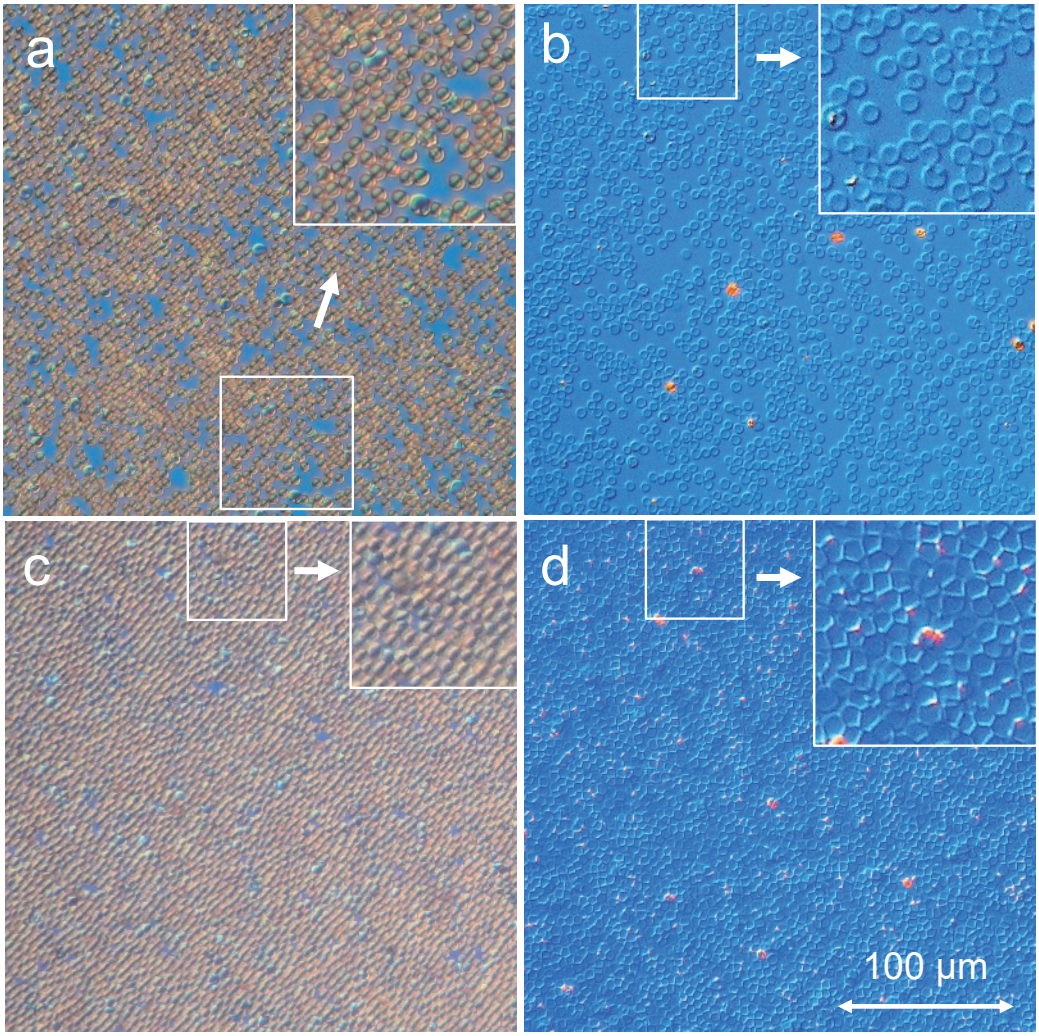

Fig. 5

(a) Nomarski microscope image of the packing of bRBC obtained at lower concentration of RBC and lower centrifugal force.
(b) Dense packing of hemolyzed RBCs corresponding to Fig. 5 a.
(c) Dense packing of bRBC obtained at higher concentration of RBC and higher centrifugal force than in Fig. 5 a.
(d) Dense packing of hemolyzed RBCs corresponding to Fig. 5 c.

ing as shown in Fig. 5 d was not obtained. The centrifugal pressure is thus promising for preparing a full packing of tightly immobilized RBCs.

3.2 Patterning RBC dense packing

The biotinylated RBCs adhere to the SAv surface with a strong biotin-streptavidin linkage. The adsorption of bRBC was examined for other kinds of surface, such as UV irradiated hydrophilic Si surface, HMDS-treated hydrophobic Si surface, monolayers of unfolded bCA or CA transferred onto hydrophobic Si surface, and a cleaned Si surface kept in laboratory air longer than a month. The bRBCs deposited on these surfaces by centrifugation were washed away in LiqA. These results suggest that the surfaces except SAv arrays do not allow tight binding of bRBC. Accordingly, a pattern of biotin binding activity can be fabricated by UV-O_3 cleaning of the SAv film with 185 nm UV light through a mask. Fig. 6 a shows that the bRBCs deposited on the UV-irradiated area of a grid pattern (Fig. 6 b) were washed away by rinsing in LiqA. On the other hand, bRBCs kept adhered to the array of 25 μm squares where the intact SAv layer had been protected by masking. Fig. 6 c shows the packing of hemolyzed RBCs on the squares. If we watch the cells immobilized on the periphery of the 25 μm square, we notice that the outside circumference of each cell is of circular shape, while the cell-to-cell contact inward is of straight line. This indicates that the neighboring cells are pressing each other laterally at an equilibrium internal pressure.

4. Concluding remarks

A tight immobilization of biolinylated red blood cells onto streptavidin layer has been demonstrated.

There are several advantages shown in the present study for RBC immobilization.

1) Preparation of SAv layer on a hydrophobic solid support is simple and the glucose treated SAv layer preserves biotin-binding activity in a dried state at room temperature.
2) Immobilization of bRBCs on SAv surface is so strong that manipulation of each cell in application experiments such as of micro capsules would become easy.
3) Less than 0.5 μL of whole blood is enough to fully cover the substrate surface with 10 mm^2 in area.

The tight packing of RBCs, especially of polygonal packing of hemolyzed cells might attract attention in that the solid surface can be covered with double bilayers of cell plasma membranes almost without vacancies of micrometer size. The system demonstrated in this study thus appears to have fascinating aspects for application studies using RBC.

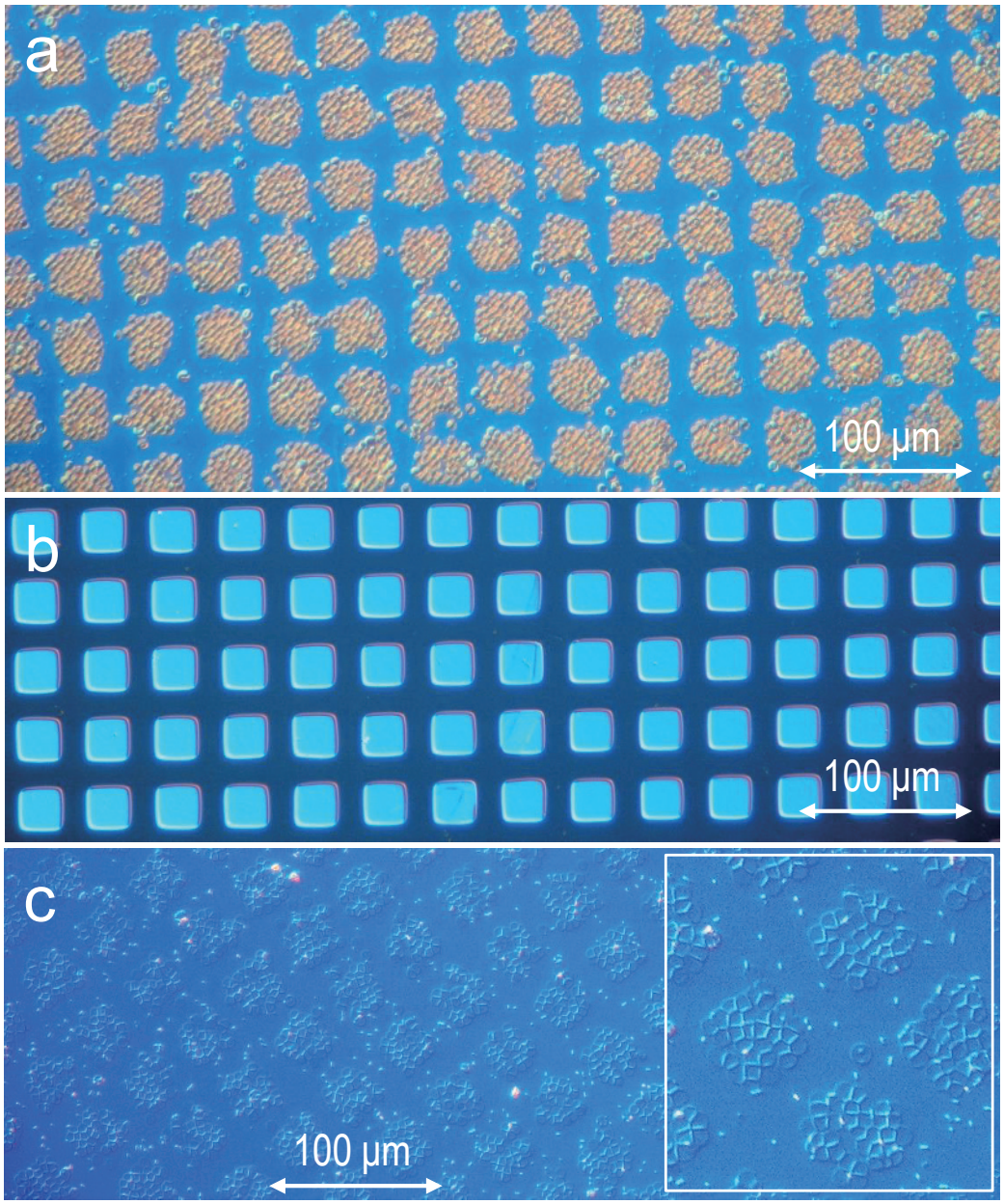

Fig. 6

(a) Dense packing of RBCs immobilized on the array of SAv layers with biotin binding activity.

(b) Mask array of 25 μm squares of chromium deposited on a quartz plate with 0.4 mm thickness.

(c) The RBC packing corresponding to Fig. 6 a was hemolyzed by introducing pure water into the liquid cell under a microscope.

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