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Rapid agglutination of whole blood by antiserum and its integration in a blood pretreatment module with dual function

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ABSTRACT

Blood pretreatment for many clinical applications in most situations involves removing blood cells, and today mainly depends on centrifugation. Centrifugation, however, needs machinery which consumes power. In the point-of-care-testing area in particular, harvesting plasma or serum from undiluted whole blood has been both an important hurdle and target at the same time. We describe herein a rapid and integrated method for the separation of plasma from undiluted blood in dozens of seconds without any power supply. We generated polyclonal antibodies from mice which captures human blood cells to make cellular aggregates, resulting in agglutination in a few seconds. The generated anti-human blood antiserum accelerated the sedimentation of blood cells, which was four times faster than the natural sedimentation, even from undiluted whole blood. Additionally, a plasma albumin reduction module was combined to make a pretreatment module having dual functions. The volume of recovered plasma increased with the decrease in albumin. The pretreatment of undiluted whole blood was finished in 30 s without any detectable blood cells using this dual function pretreatment module.

1. Introduction

Gathering plasma from whole blood is one of the most important and frequently requested pretreatments and is also an inevitable bottleneck to the application of field-applied diagnostic chips. Centrifugation may be the most efficient and fast method for this purpose, but an additional power supply must accompany the centrifuge itself. There have been a variety of reports concerning plasma preparation for point-of-care testing applications (reviewed in reference [1]). Those previous methods have largely relied on complicated principles or devices to realize a single aim, the removal of blood cells. We introduce a cell removal method based on a simple principle, and which also performs an additional function.

Plasma can be obtained after the simple sedimentation of blood cells using only gravitational force, but this method takes too long a time to harvest enough plasma from whole blood, and is therefore not adequate for field applications, which usually require prompt processes. In order to speed up the sedimentation of blood cells without an exogenous power supply, we devised a novel method in which blood cells are aggregated by antibodies which can recognize and bind human blood cells.

The main concept of blood aggregation using the anti-human blood polyclonal antibody is depicted in Fig. 1. Human blood cells including red blood cells and white blood cells will interact with antibodies raised against the human blood cells themselves. Because of the nature of polyclonal antibody – which bind to a variety of antigenic determinants – the blood cells will make complex aggregates through the diverse epitopes of cells. The aggregation of blood cells will make compact lumps leaving cell-free plasma. Consequently, this makes it possible to use a smaller number of filters with larger pore sizes to remove blood cells from whole blood. This will reduce the time spent filtering blood cells and increase the amount of harvested plasma at the same time.

Additionally, the removal of albumin, which is more than 50% of total plasma protein [2], is also an invaluable but not previously attempted pretreatment in microsystems. Reduction of albumin can be helpful in detecting other tiny amounts of proteins during general diagnosis. Moreover, because albumin is the material

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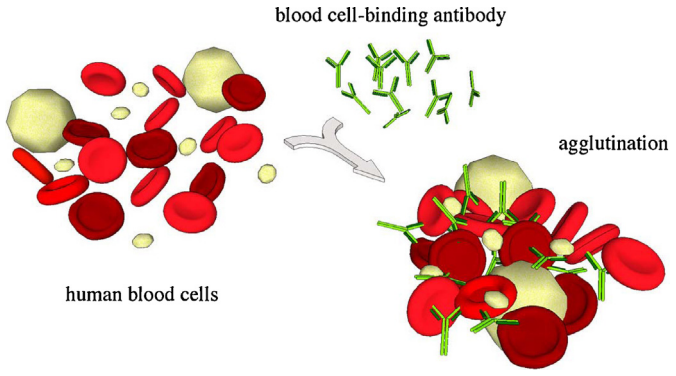

Fig. 1. The principle of blood cell aggregation by anti-human blood antibody. The polyclonal antibody binds to human blood cells and produces three dimensional aggregates with human blood cells resulting in agglutination. Note that only red blood cells, the major blood cells, have been depicted for clear demonstration.

which causes blood viscosity, plasma with reduced albumin will show improved fluidity especially in microstructures, owing to the increase of Reynolds number [3].

Cibacron Blue F3GA is known to bind albumin through hydrophobic fatty acid-anion-binding sites and bilirubin-binding site of albumin [4]. Although this dye is also known to bind some other proteins, such as catalase, glucose oxidase and lysozyme [5], Cibacron Blue F3GA has been a promising choice to remove albumin and is widely applied by immobilization on several kinds of structures, including microspheres [6], microchannels [7], and fibers [8,9]. Currently, there are many kinds of commercial products using this dye but only for macro-scaled column systems. This report is the first application of Cibacron Blue F3GA-conjugated beads in an integrated module with another functional unit.

2. Materials and methods

2.1. Generation of anti-human blood polyclonal antibody

Human whole blood cells were used as antigen in order to generate polyclonal antibody which binds to human blood cells. Type O human whole blood was drawn from a volunteer's fingertips. The drawn blood was immediately washed with PBS (phosphate-buffered saline, pH 7.4) 3 times. The washed blood cells were fixed by 2% (v/v) of formaldehyde and 0.2% (v/v) of glutaraldehyde to prolong the integrity of the cellular membrane after inoculation. The fixed cells were washed again with PBS 3 times to remove the fixing solution. Female C57/BL6 mice, 5 weeks old, were intraperitoneally immunized with the prepared human blood cells [10]. Each inoculum contained human blood cells from 10 μL of original whole blood, which corresponds to about 5×10^7 cells. Immunizations were performed 5 times with 3 week intervals. Blood was collected from each mouse two weeks after the final immunization. The drawn whole blood was left at room temperature for sufficient clogging. Serum which contained the anti-human blood polyclonal antibody was harvested after centrifugation at 15,000 × *g* for 20 min at 4 °C. Aliquots of the harvested anti-human blood antiserum were frozen at −20 °C until each usage.

2.2. Fabrication of pretreatment module

We previously introduced a blood filter module which could be integrated into any kind of pre-made biochips. This module was adopted with modifications in order to verify the integrated dual function reported here. Schematics and pictures of fabricated modules are depicted in Fig. 5(a)–(c), and the detailed fabrication of a magnetically actuated filter module was previously described [11]. Briefly, circular membranes of 4 mm in diameter were prepared by puncturing commercially available membranes made for retarding the movement of blood cells (Advanced Microdevices P. Ltd., FR1 of 0.6 mm thickness). Cylindrical plastic containers were made of acrylic plates, of which the inner diameter was 4.2 mm. A cylindrical Nd-Fe-B permanent magnet (4 mm in diameter and 3 mm long, LG magnet) was inserted into the plastic container, which would be used for squeezing membrane stack in cooperation with an external magnet. After stacking a few membranes on this magnet, 5 μL of anti-human blood antiserum was absorbed into this membrane. And then, an albumin adsorption beads block was stacked. Blue Sepharose 6 Fast Flow (GE Healthcare) was adopted as the Cibacron Blue F3GA-linked albumin adsorption beads. 30 μL of original bead slurry was washed 3 times with distilled water, and the final volume was made to 60 μL, which corresponds to 15 μL of beads for each pretreatment module. This bead slurry was shaped into a cylindrical block using another empty cylindrical plastic container, and any excess moisture was removed through absorption by filter paper (Whatman no. 1, GE Healthcare) prior to applications. Finally, a circular double-sided tape (3 M, model #9495LE) with a central hole of 1 mm was attached on the crest of the container. The finished module was turned up to position the magnet over the membranes.

The magnet within the module was pulled downward by an additional Nd-Fe-B magnet (10-mm diameter and 10-mm length with 5030 Gauss, LG magnet) which was positioned below the module. The downward movement of the module, that is the distance between the module and the exterior magnet, was controlled by a digital elevator. Otherwise, the magnet within the module was manually pushed down to squeeze out the plasma in the module.

2.3. Analysis of pretreatment performance

Whole blood was treated with a minimal amount of anticoagulant just after being gathered from a volunteer's finger tips. One fiftieth volume of EDTA (ethylenediaminetetraacetic acid) solution was added to the whole blood to make 10 mM. The treated whole blood was used to validate performance, and the harvested samples were examined through a light microscope (Eclipse TE2000-U, Nikon) to count the number of red blood cells. 0.5 μL of each recovered sample was also analyzed through conventional SDS-PAGE using 12% gel [12] along with 0.5 μL of untreated plasma in order to compare the amount of albumin among samples. ImageJ (version 1.45s) was obtained from the NIH webpage, and SDS-PAGE bands were analyzed according to the supplied instructions [13].

2.4. Quantitative evaluation of pretreatments

Purified amyloid beta 1-40 peptide (Covance Research Products) was spiked into human whole blood to be 1 ng/mL. Plasma was obtained by a pretreatment module or by centrifugation at 15,000 × *g* for 2 min at room temperature. The quantity of amyloid beta 40 was measured using an ELISA kit (Colorimetric BetaMark x-40, Covance Research Product) according to the manufacturer's instruction. Otherwise, 2 μL of IgG-conjugated horseradish peroxidase (anti-rabbit IgG-peroxidase, Sigma–Aldrich), 8–22 μg according to the manufacturer's data sheet, was spiked into 200 μL of human whole blood, and plasma was obtained by two methods as mentioned above. The peroxidase activity existing in plasma was measured by mixing 1 μL of 10-fold diluted obtained plasma with 100 μL of TMB (3,3′,5,5′-tetramethylbenzidine, Sigma–Aldrich). The colorimetric reaction was stopped by adding 100 μL of 1 N H_2SO_4 before being read at 450 nm by a microplate reader (VersaMax, Molecular Devices).