

# GRAPHENE OXIDE COATED FABRIC LAYERS FOR THE EFFICIENT ISOLATION OF CIRCULATING TUMOR CELLS

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## ABSTRACT

We present the fabric sheet layers, functionalized with graphene oxide (GO) for the sensitive isolation of circulating tumor cells (CTCs). Compared to the previous microfluidic-based CTC detection devices, which require complicated and long fabrication processes, the fabric sheet layers are simply fabricated by conventional textile manufacturing; thus the device is mass producible at extremely low-cost. Furthermore, since the present layers are composed of polyester yarns, GO can be directly modified on the surface after only a half hour of surface modification preprocess with protein, bovine serum albumin (BSA); thus, it has boundless potential for bioapplications. Here we demonstrated the fabric sheet layers and their simple modification methods to achieve high sensitive CTC isolation.

## INTRODUCTION

Liquid biopsy based on the circulating tumor cells (CTCs) is an emerging field of cancer diagnosis that enables multiple testing and continuous monitoring of the disease under clinical treatments, such as drug and surgical dissection of a tumor in non-invasive manners. Due to its advantages, a number of microfluidic-based devices have been introduced. Recently, various nanomaterials have been applied on the microfluidic devices, in purpose of enhancing the sensitivity during CTC isolation. Nanomaterials including graphene oxide (GO)<sup>1</sup>, nanowires<sup>2</sup>, and carbon nanotubes<sup>3</sup> have been used for increasing the immobilized antibodies on the surface of microfluidic devices. However, the conventional nanomaterial-based CTC isolation devices are accompanied by complicated fabrication processes of expensive materials; thus suffer from low productivity and usability, which is undesirable for the serial monitoring of cancer in clinics.

Here, we present anti-epithelial cell adhesion molecule antibody (anti-EpCAM antibody)-immobilized CTC isolation platform which is not only mass producible but also capable of sensitive CTC isolations with high purity at the same time, by functionalizing GO on the surface of the fabric sheets composed of polyester monofilament yarns. GO is one of the most extensively used nanomaterials for enhancing the sensitivity during the detection of various biomolecules. GO can be easily functionalized on the surface of polyester fabric sheets by electrostatic self-assembly<sup>4</sup>, only after a simple surface modification process involving bovine serum albumin (BSA) treatment. We have detected the concentration of oxygen ratio compared to the carbon (O/C ratio) after dipping BSA-adsorbed fabric (BmF) sheets on GO

solution for different time length, to determine the optimal time for GO functionalization. GO-modified fabrics (GmF) were then immobilized with anti-EpCAM antibodies (Ab-GmF) and the concentration of nitrogen over carbon (N/C ratio) was compared with non-modified fabrics (NmF) and antibody immobilized NmF (Ab-NmF), to confirm that GO modification improves the density of antibody. To verify the platform to be used for rare cancer cell isolation, we measure the cell capture efficiency and specificity using breast cancer cell lines. The present device provides high efficient CTC isolation without using any complicated fabrication processes, thus has potential to be used in CTC-based cancer diagnosis.

## MATERIAL AND METHODS

### Design of Fabric Sheet Layers

Polyester fabric sheet layers were made based on the conventional textile manufacturing process which involves weaving, sizing, washing, and calendaring. Polyester mono yarns having 20 denier (50 – 60  $\mu\text{m}$  thickness) were used for the sheet production. As shown in Figure 1, the sheet is composed of 3 by 1 twill with 592 ends and 200 picks per inch. As a result, fabric sheets had pores with  $\sim$ 12  $\mu\text{m}$  and  $\sim$ 148  $\mu\text{m}$ , in between neighboring warps and wefts, respectively. Fabric sheet layers were cut into circle having diameter of 20 mm. Three of fabric sheet layers were inserted into jigs which can be connected to the commercial syringe.

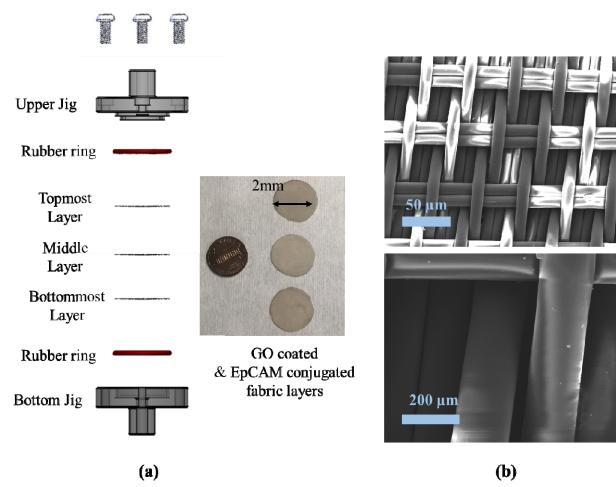


Figure 1: The GO coated & EpCAM conjugated fabric sheet layers: (a) schematic diagram and images of fabric sheet layers and jigs; (b) scanning electron microscope image of fabric sheet layers

## Graphene Oxide Functionalization

In order to modify the fabric sheets with GO, the surface was repeatedly washed to remove the impurities. Then, bovine serum albumin (BSA) was used as a glue-like material for the adsorption of GO layer through electrostatic adsorption. After that, BSA-adsorbed sheets were incubated with 1 mg/ml of GO solution at RT for 1 hour and the incubation time was varied from 30 mins to 12 hours for the optimization of the GO functionalization.

## Antibody Immobilization

For the immobilization of the antibodies on the GO-modified fabric sheets, the surface was modified through silanization followed by a bonding with crosslinkers. Then, antibody immobilization was achieved by taking a conjugation route which consist biotinylated BSA solution, avidin, and biotinylated anti-EpCAM antibody. Each step was determined to be 1-hour process at RT with repetitive washing by PBS buffer solution. Lastly, the antibody-immobilized fabric sheets were treated with 1% BSA solution for preventing nonspecific adsorption.

## Contact Angle (CA) Analysis

The fabric sheets were characterized by contact angle analyzer using EasyDrop Contact Angle Measuring Instrument (KRUSS GmbH, Germany). To confirm the change of the hydrophilicity according to the modification step, a drop of the water (approximately 5  $\mu\text{l}$ ) is placed on the surfaces using micro syringe. The samples were enclosed inside an environmental chamber for controlling humidity and the measurements were performed with capturing photographic image simultaneously. Each sample was analyzed five times; the mean and the standard deviation of a set of data were acquired, respectively.

## Field Emission Scanning Electron Microscope (FE-SEM) Measurement

The results of the GO modification were confirmed using FE-SEM. The equipment was operated at an acceleration voltage of 5 kV and with a working distance of 2.5 mm. All images were magnified by a factor of 1.00 K. The prepared fabric sheets were cut into small pieces, attached to double-sided adhesive tape mounted on SEM stub, and coated with osmium of 1.0 nm thickness in order to avoid degradation or charging effects of biomolecules.

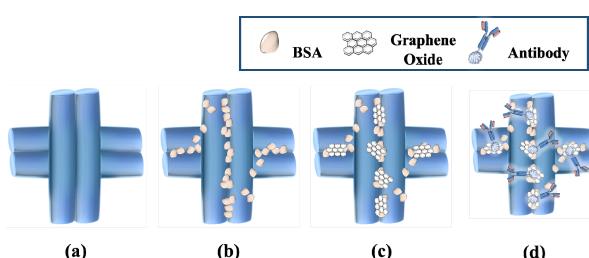


Figure 2: The schematic diagram of polyester fabric modification: (a) non-modified polyester fabric; (b) BSA-adsorbed polyester fabric; (c) GO-modified polyester fabric (GmF); (d) antibody-immobilized GmF (Ab-GmF).

## X-ray Photoelectron Spectroscopy (XPS) Analysis

The changes induced by surface modification and immobilization process were also verified by XPS analysis. Using by a standard monochromatic Al  $\text{K}\alpha$  excitation source ( $\text{h}\nu = 1486.6 \text{ eV}$ ), the surface was analyzed at 35° of the standard beam and detector input angle. Survey spectra were recorded over a range of 0-1,200 eV with pass energy of 100 eV and a step of 1.0 eV. High-resolution spectra of C1s, O1s, and N1s were also collected for the calculation of atomic concentration: Oxygen-to-Carbon (O/C) and Nitrogen-to-Carbon (N/C) ratio.

## Sample Preparation

The sensitivity of the present device was evaluated using breast cancer cell line, MCF-7. Cells were incubated under general cell culture conditions, Roswell Park Memorial Institute (RPMI) media containing 1% penicillin and 10% FBS for 3 days at 37°C, supplemented with 5% CO<sub>2</sub>. The cultured cells were stained with CellTracker Green (CTG) to measure the capture efficiency. Cancer cells, were trypsinized and detached cells were spiked into 1x PBS solution or human whole blood, for the capture efficiency verification. In case of validating the purity, human whole blood samples were obtained from healthy donors with KAIST institutional review board (IRB) approval.

## Sample Processing

Three GO-modified fabric sheet layers were stacked and inserted to the syringe filter-like jigs for simple use. Cell mixtures (~50 cells spiked into 1mL of PBS solution or whole blood) were processed at the flow rate of 5 mL/h after initial washing with 1mL of PBS solution. Then, 2 mL of PBS solution was processed at the identical flow rate for the additional washing. Cells captured in each of three layers were measured and results are compared with in case of using Ab-NmF and NmF sheets instead of Ab-GmF.

For the purification process, jigs were unloaded and each of three layers were re-inserted in three different jigs. 5mL of PBS solution was reversely flown through the jig at the flow rate of 100 mL/h to eliminate the untargeted leukocytes. Cancer cell loss during the purification process and the number of leukocytes after trypsin treatment were validated to confirm the purity. The sample processing steps are summarized in Figure 3.

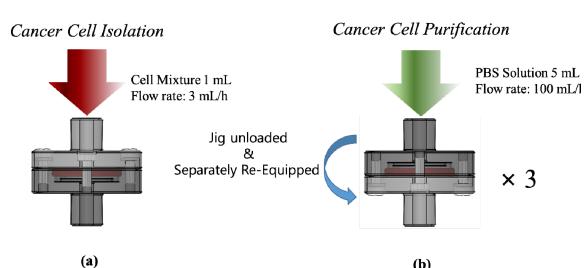


Figure 3: Sample processing: (a) CTC isolation; (b) CTC purification

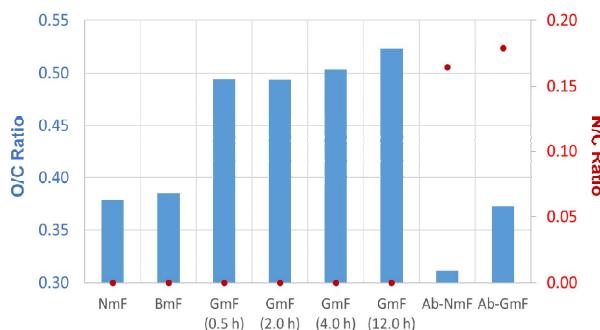
## RESULTS AND DISCUSSION

### Confirmation of the GO modification Based on Wetting Property

In order to confirm the results of GO modification, we measured surface wettability of the samples by using contact angle analyzer. The contact angle of the NmF and BmF sheet was  $81.7 \pm 1.4^\circ$  and  $53.8 \pm 2.1^\circ$ . Since pristine polyester have hydrophobic nature, the change of the hydrophilicity indicates the presence of GO, an oxygen-containing material. However, the wettability of the surface was unmeasurable due to extremely high concentration of hydroxyl group in the GO layer because a drop of water momentarily spread on the surfaces. Considering that extremely high concentration of hydroxyl group in the GO layer, it may be an explainable result; therefore, we concluded that GO layer was properly formed on the BmF sheet. According to our experience, the angle increased again after immobilization step onto GO-modified fabric (GmF) due to the nature of the immobilized proteins (data not shown).

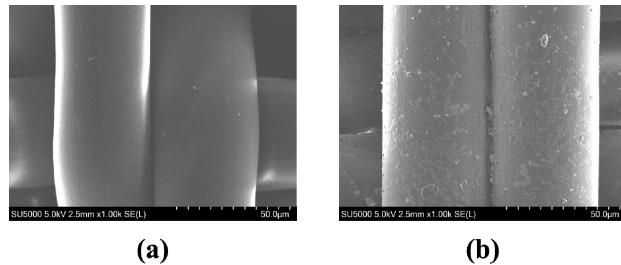
### GO Functionalization and Density of Antibody Enhancement

The changes induced by surface modification and immobilization process were verified by XPS analysis (Figure 4). First, the increases in O/C ratio supports the existence of GO layers. It was 0.38 and 0.39 on NmF and BmF, but dramatically changed after GO modification (0.49~0.52) following to the incubation time ( $0.5 \sim 12.0$  hours). Based on the results, we determined the optimal incubation time for the GO functionalization: as it can be seen in Figure 2b, atomic concentration of oxygen species or O/C ratio of GO modified fabric (GmF) sheet was gradually saturated after 30 minutes and seldom changed by 12 hours. Therefore, we concluded 30 minutes of incubation with 1mg/ml of GO solution was enough to modify the fabric sheets. Second, the changes in N/C ratio indicates the results of antibody immobilization. Whereas



**Figure 4:** O/C ratio (blue, column) and N/C ratio (red dot, scatter) of the polyester fabrics verified by XPS analysis. The sample name is abbreviated as follow: NmF= Non-modified fabric; BmF= BSA-adsorbed fabric; GmF= GO-modified fabric; Ab-NmF= Antibody-immobilized NmF; Ab-GmF= Antibody-immobilized GmF. The numbers in parentheses refer to the incubation time of GO modification process.

proteins are nitrogen-rich molecules, neither pristine

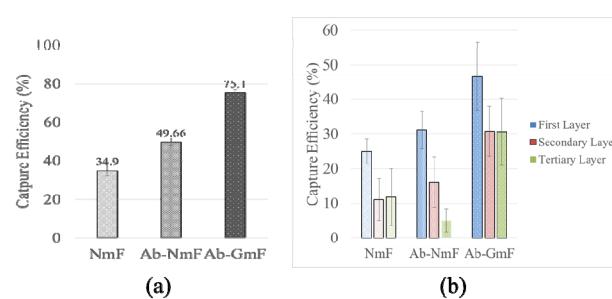


**Figure 5:** The confirmation of appearance of the polyester fabrics using FE-SEM: (a) GO-modified polyester fabric (GmF); (b) Antibody-immobilized GmF after experiment. All images were magnified by a factor of 1.00 K and taken at acceleration voltage of 5.0 kV.

polyester nor GO does not contain nitrogen atom. It was 0.18 and 0.16 on Ab-NmF and Ab-GmF. From the difference between two immobilization pathways, we hypothesized that antibody immobilization can be enhanced in relation to quantity and quality through GO modification process. Because GO has ultrahigh specific surface area with the plentiful oxygen-containing groups, there might be more possibility of antibody immobilization with better distribution and less hindrance.

### Stability of GO-modified Fabric Sheets

Figure 5 shows the FE-SEM images of the fabric sheets fibers at an intersect point. We found that GO modification and antibody immobilization process did not affect the original structure of fibers. Nothing unusual was found on GmF except little impurities since the changes induced by BSA or GO might not be confirmed in micrometer-scale. We also confirmed the surface after using biological samples; even though the fiber was partly covered by the aggregated proteins, enzymes, and small molecules, the surface of the fibers did not damage during experiment. These results show that the feasibility of the fabric sheets as an alternative substrate for processing biosamples because these sheets can be simply applied to the previously established bioapplication.



**Figure 6:** The capture efficiency of GO modified fabric layers: (a) the total capture efficiency compared to the control samples; (b) the capture efficiency in each layer.

## Cancer Cell Isolation

The total cancer cell capture efficiency after the immobilization of anti-EpCAM antibody on (Ab-GmF) showed 75.2% when spiked into PBS solution, which was significantly higher than that obtained from NmF (34.9%) or Ab-NmF sheet layers (49.7%). Furthermore, since high isolation sensitivities were maintained for the middle and the bottommost layers in case of GO-fabric layers (over 30%), higher sensitivity can be easily achieved by stacking up more layers. In case of NmF and Ab-NmF, most of the cells were captured in the topmost layers due to the size effect, while additional layers show very low capture efficiencies (5 – 20%). The strong binding between the cancer cells and high-density antibodies, attached in Ab-GmF interface was beneficial for improving the sensitivity during cancer cell isolation process. Even when cancer cells were spiked into human whole blood instead of PBS solution, the capture efficiency still maintained over 73 % (data not shown). The effect of high viscosity and large-sized leukocytes, which degrade the cancer cell isolation, are negligible since the difference were in the margin of error.

## Cancer Cell Purification

GO-based antibody immobilization was also advantageous for enhancing the specificity; only ~5% of cells were detached from the layer with the reverse flow of PBS solution during the purification process, while over 30 % of cancer cells were lost in NmF layers. The total number of leukocytes after the purification process were in between 70 to 280 per 1 mL of human whole blood. The leukocytes remained on the fabric sheets were gathered after applying trypsin through reverse direction and identified by staining with CD45 and DAPI. We verified that since our device is capable for separating the layers and purifying each of layers through high flow rates, it is possible to easily improve the purity during CTC isolation.

## CONCLUSION

Here, we proposed the mass producible fabric sheet layers functionalized with GO for the effective isolation of CTCs. With the simple modification process with the help of BSA-GO combination, GO-modified fabric sheets can be simply prepared; thus, we expanded this as an anti-EpCAM-immobilized substrate for CTC application. The present device showed enhanced antibody attachment and result in capture efficiency of 75-80 % for cells with high EpCAM expressions. The GO-modified fabrics were also beneficial for maintaining high specificity by only capturing 71-270 leukocytes per 1mL of human whole blood. The ideal combination of fabric sheets and GO enables us to achieve a simple, mass-producible, cost-effective, hygienic, disposable material for the bioapplication. We expect that high productivity of the GO-modified fabric sheet layers are beneficial for our device to be used in diagnosis of cancer or examination of metastatic potential in clinics.

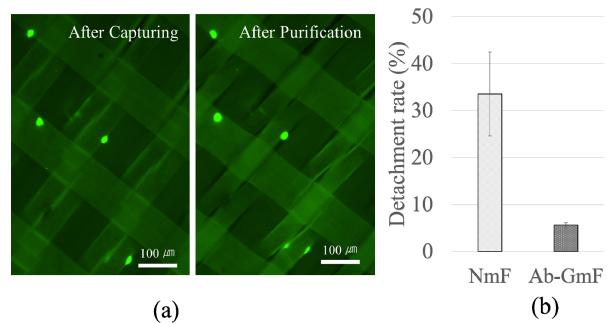


Figure 7: The image of isolated cancer cells and cancer cell detachment rate: (a) the captured cancer cells after the capturing and the purification process; (b) cell detachment rate during the purification process.

## ACKNOWLEDGEMENTS

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