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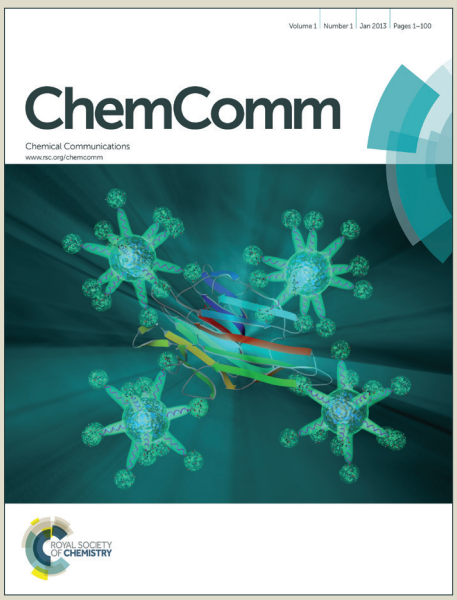

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

A Size Amplified Immune Magnetic Microbeads Strategy in Circulating Tumor Cells Rapid Detection

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

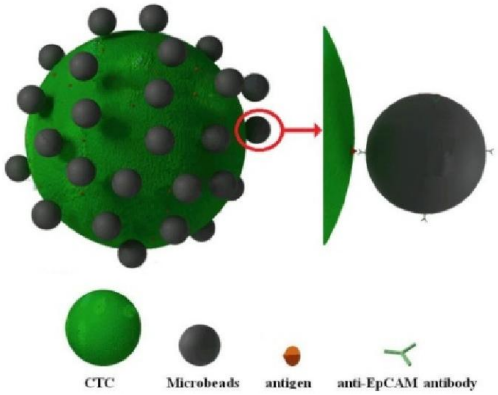
DOI: 10.1039/b000000x

Using anti-EpCAM antibody modified magnetic microbeads allowed us to simultaneously apply size-amplification and magnetic labelling of CTCs to the capture and purification of CTCs by membrane filtration and immune-magnetic separation. High purity capture (>98%), rapid (<2 hours) and simple detection of CTCs were realized.

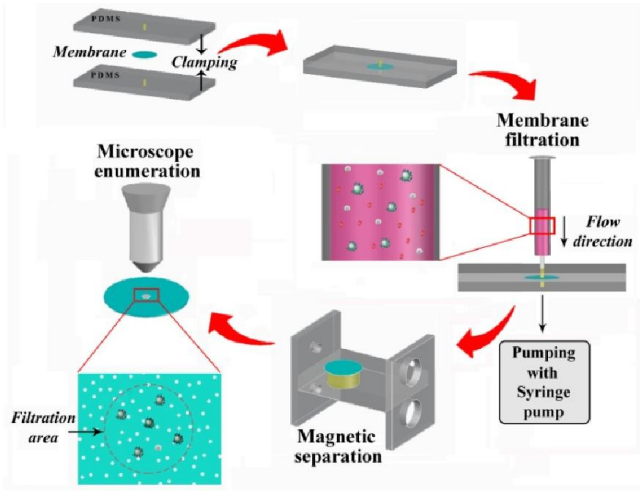
It has been recognized for decades that cancers metastasize because primary tumors shed cells into the blood, which carries them to other organs where they seed new tumors.[1, 2] Only in the past 10 years, however, have researchers figured out how to efficiently capture Circulating Tumor Cells (CTCs) from a blood sample.[3] Now, it is believed that the capture and count of CTCs can be used as a new "liquid biopsy" for tracking the spread of cancer or predicting the survival benefit from treatments.[4-8] To efficiently capture and enrich the minuscule number of tumor cells that circulate in patient's blood, immune-magnetic separation based on magnetic microbeads[9] or nanospheres,[10, 11] size-based membrane or chip filtration,[12-15] density gradient sedimentation,[16] and microfluidic chip based methods,[3, 17-19] were frequently reported. As described by Kim *et al*.,[20] in developing CTC capture technology, the critical criteria are high capture efficiency and high purity. Current isolation methods often suffer from an inherent trade-off between these two goals. The Cell Search System based on the immune-magnetic separation has been cleared by the U.S. Food and Drug Administration for routine clinical use in metastatic breast cancer.[21, 22] However, this kind of immune-magnetic separation often had unsatisfactory capture efficiency.[23, 24] In order to attain the goal of early accurate diagnosis, improvement in capture efficiency and purity has become a primary focus. In the last few years, to improve the capture efficiency, many size-based methods using membrane or chip filtration were reported.[12-15, 19] Although the high capture efficiency could be obtained, the capture purity was often poor due to the existence of large scale leukocytes, the likelihood to deform and the highly variable sizes of CTCs.[12, 14, 15] To improve the capture purity, Lee *et al*.[25] devised an isolation strategy using microbeads-based size amplification and a high-pore-density filter. Kim *et al*.[20] developed a CTC isolation method using selective size amplification and a multi-obstacle architecture filter. Zhao *et al*.[17] designed a polymer nanofiber-embedded microchip for high-purity prostate CTC isolation. These reports showed that the selective size amplification or the modification of the microchip could contribute to improve the efficiency and purity, but the results of capture purity had not been detailedly and definitely described. Judging by the staining images after CTC isolation from blood, many leukocytes were still retained.[9, 17, 20] Thus, development of a CTC capture method with high capture efficiency and high capture purity still remained elusive.

Therefore, we put forward a Size Amplified Immune Magnetic Microbeads (SAIMM) strategy which combines the size amplification-membrane filtration with immune-magnetic separation to achieve both the high capture efficiency and high purity. The capture efficiency and purity of membrane filtration generally depended on the CTCs' size differentiation with leukocytes and the degree of CTCs' deformation. The immune-magnetic microbeads could selectively amplify the CTCs, thus bypassing the size overlap issue with leukocytes. In addition, conjugation with magnetic microbeads could help to avoid the deformation of CTCs. Following membrane filtration, the residual leukocytes were further removed by a magnetic separation step. Together, these techniques would simultaneously contribute to the improvement of CTCs capture efficiency and purity.

The overall scheme was that the magnetic microbeads were modified with anti-epithelial cell adhesion molecule (EpCAM) antibodies. The immune-magnetic microbeads would specifically recognize the EpCAM antigens on the surface of CTCs, as shown in Scheme 1. Following incubation, membrane filtration was used to remove the majority of leukocytes and free magnetic microbeads, and then residual leukocytes were removed by a



Scheme 1 Schematic view of the CTC recognized by the immune-magnetic complex.



Scheme 2. Procedure of the membrane filtration and magnetic separation.

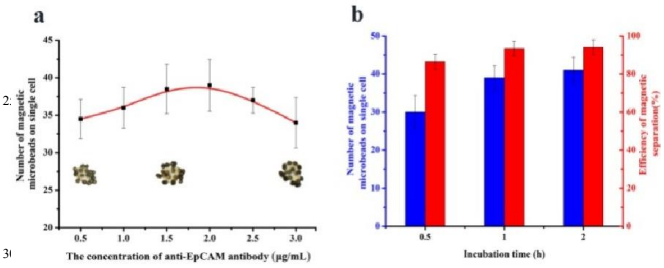


Fig. 1 Optimization results of the microbeads recognizing the cells. (a) Influence of the concentration of anti-EpCAM antibody. (b) Influence of the cell incubation time.

magnetic separation step. To simplify the procedure and reduce the analysis time, the membrane-retained CTCs were directly observed and counted under an optical microscope. Scheme 2 shows the procedures of the membrane filtration and magnetic separation.

This SAIMM strategy based portable and rapid detection method could be applied to large sample screening, and positive samples would be confirmed by confocal microscopy. It was not necessary for most negative samples to identify CTCs by staining with at least three fluorescent markers and observing by confocal microscopy which was not afforded by every laboratory.[3, 8, 14, 15, 26] Applying our SAIMM strategy to CTCs detection, we managed to simultaneously achieve high efficiency and purity capture, simple and rapid large sample screening.

The magnetic microbeads were modified with anti-EpCAM antibodies. The concentration of anti-EpCAM antibodies was optimized in the range of 0.5-3 $\mu g \cdot mL^{-1}$. Low impact of the antibodies' concentration showed on the number of magnetic microbeads on single cell and 1.5 $\mu g \cdot mL^{-1}$ was chosen (Figure 1a).

To gain the rapid detection, the incubation time of cells by magnetic microbeads should be as shorter as possible, so the incubation time was optimized as detailed below. For 0.5, 1 and 2 hours' incubation, the number of magnetic microbeads on single cell and the efficiency of magnetic separation all reached platform maximums after 1 hour's incubation (Figure 1b).

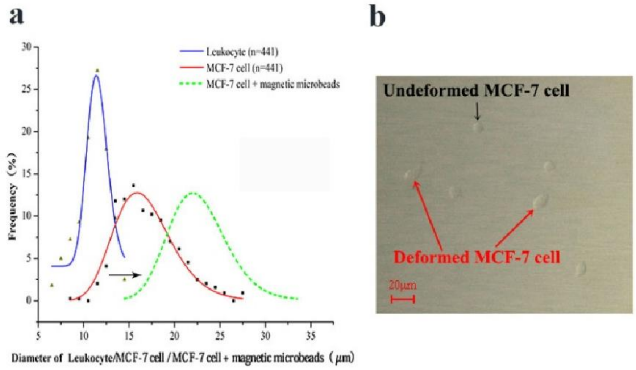


Fig. 2 Factors influencing the membrane filtration. (a) Size distribution curves of MCF-7 cells and leukocytes. (b) The deformation of MCF-7 cells filtered through membrane.

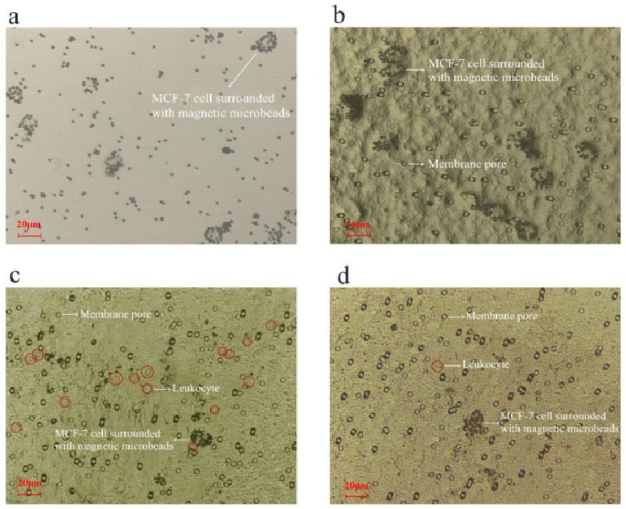


Fig. 3 Microscopy images of MCF-7 cells incubation (a) and capture (b-d). (a) MCF-7 cells surrounded with immune-magnetic microbeads on slide. (b) MCF-7 cells surrounded with immune-magnetic microbeads on membrane after membrane filtration and immune-magnetic separation. (c, d) Capture purity comparison of the membrane filtration (c) with the membrane filtration combined with magnetic separation (d).

To investigate the CTCs' deformation and the size differentiation with leukocytes, a breast carcinoma cell line, MCF-7 cells were used. MCF-7 cells with a size larger than the pore diameter (8 μm) could be filtered through membrane and severely deformed compared with those less than 8 μm (Figure 2b). The round cells were undeformed (marked with black arrow) and the oval cells were deformed (marked with red arrow). This would seriously affect the capture efficiency of membrane filtration.

To establish the size overlap between the MCF-7 cells and the leukocytes, and illustrate the selective size amplification of MCF-7 cells by magnetic microbeads, the size distribution curves of MCF-7 cells, leukocytes and size-amplified MCF-7 cells were constructed (Figure 2a). The green curve modeled the size

distribution of size-amplified MCF-7 cells, got by shifting the red curve of 6 μm (length of two microbeads' diameters, Scheme 1). The arrow pointed to the translating direction. The immune-magnetic microbeads could selectively size-amplify the MCF-7 cells, so as to definitively differentiate them from leukocytes, thus negating the size overlap of the cells in their native form. At the same time, the conjugation with magnetic microbeads helped to avoid the deformation of MCF-7 cells. These would simultaneously contribute to the improvement of the capture efficiency and purity. According to the results of Kim's report[20] , 3 μm was determined to be the optimal microbead diameter. So we directly selected the 3 μm magnetic microbeads for subsequent experiments.

Different from the only membrane filtration based method reported by Lee *et al*.[25] and Kim's *et al*.[20], in our SAIMM strategy, by means of the immune-magnetic microbeads, not only the membrane filtration, but also the immune-magnetic separation could be improved. The MCF-7 cells could be successfully recognized by the anti-EpCAM antibodies on the magnetic microbeads (Figure 3a). The cells coated with magnetic microbeads on the membrane after membrane filtration and magnetic separation were shown in Figure 3b. Due to its transparent nature, the Nuclepore Track-Etch filter membrane was selected, allowing for visualization of CTCs coated with microbeads using an optical microscope.

Following membrane filtration and immune-magnetic separation, the number of MCF-7 cells spiked in human blood was counted under an optical microscope. The results of spiked experiments are shown in Table 1. For cell number of 50-200，the recovery ratios were more than 90%. Mean recovery ratios of 78.64% and 81.52% for 5 and 10 cells spiked samples were obtained (Table 1). That could satisfy the requirement of rapid screening method. In the case of cell number less than 2, the influence of self aggregation of the magnetic microbeads would be dominant, so that the recoveries of samples were more than 120%.

Table 1. Detection results of MCF-7 cells spiked in human blood

Methods	Spiked cell' number	Capture efficiency (%)
Membrane filtration combined with magnetic separation	200	93.21 ± 8.26
	150	93.54 ± 9.05
	50	91.62 ± 8.44
	10	81.52 ±11.61
	5	78.64 ± 15.45
	2	133.34 ± 43.18
Only magnetic separation	150	83.28 ± 14.80
	50	80.29 ± 12.36
	10	61.31 ± 11.02

Furthermore, the method of membrane filtration combined with magnetic separation was compared with only membrane filtration or magnetic separation, alone. The purity means the number of leukocytes near each MCF-7 cell captured on the membrane, and the more of the leukocytes, the low of the purity. Figure 3c, d showed that if only membrane filtration was adopted[20, 25] without the step of magnetic separation, there were far more leukocytes near each MCF-7 cell than the combined method. So the SAIMM strategy could markedly improve the capture purity by the further step of magnetic separation. There was almost no residual leukocyte for most experiments (the capture purity >98%), which was far better than the results reported by Hosokawa *et al.*[27] and Kamande *et al.*[28] (>86%). Capture efficiencies for the combined method were also higher than only magnetic separation without the step of membrane filtration (Table 1).

Conclusions

A novel capture and detection method of CTCs was designed and constructed. The high capture efficiency and capture purity were achieved simultaneously by combined use of size amplification-membrane filtration and immune-magnetic separation. At the same time, this rapid detection method was also applied to human blood sample, the minimum detection limit could reach 5 cells in 1 mL blood, and the overall operation could be finished in 2 hours. We believe that it has the potential to be applied to early clinical diagnosis and can benefit the analysis of other types of cells or bacteria as well.

This work was supported by the 973 Program (2013CB933800), National Natural Science Foundation of China (21105058, 21227005, 21390411, 91313302, 21035003), and Program for Changjiang Scholars and Innovative Research.

Notes and references

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† Electronic Supplementary Information (ESI) available: [Ethics statement, detailed experiments of culture and preparation of MCF-7 cells, preparation of the immune-magnetic microbeads, capture device and procedure, capture and detection of MCF-7 cells]. See DOI: 10.1039/b000000x/

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