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PAPER

Peptide-based isolation of circulating tumor cells by magnetic nanoparticles

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The detection of rare circulating tumor cells (CTCs) in the peripheral blood of metastatic cancer patients has shown promise for improved diagnosis, staging and prognosis of cancers. Epithelial cell adhesion molecule (EpCAM) has been revealed to be over-expressed in CTCs while absent in normal blood cells, and has been used as an efficient diagnosis and therapeutic target on CTCs, especially in CTC isolation and detection. Most of the CTC isolation technologies are based on nanomaterials or nanostructured surfaces functionalized with EpCAM antibody. Herein, instead of anti-EpCAM, we report a new CTC isolation method with high efficiency by using the EpCAM recognition peptide functioned iron oxide magnetic nanoparticles (MNPs) (Pep@MNPs). The *de novo* designed peptide, Pep10, with comparable binding affinity K_D (1.98×10^{-9} mol · L^{-1}) to that of the anti-EpCAM (2.69×10^{-10} mol · L^{-1}) is attached onto MNPs *via* biotin-avidin interaction. We demonstrate that Pep10@MNPs (200 nm) have the comparable capture efficiency (reaching above 90%) and purity (reaching above 93%) to anti-EpCAM@MNPs for breast, prostate and liver cancers from spiked human blood. Furthermore, the captured cells still maintain viability for further molecular biological analysis with this method. The peptide-based CTC isolation method could be beneficial for the cancer prognosis and metastasis prevention by increasing the stability and reproducibility.

1.Introduction

Despite recent advances of new diagnostic and treatment strategies, cancer accounts for about 10 million deaths per year worldwide.[1] It is generally recognized that most cancer-related mortalities result from metastatic disease.[2-3] Circulating tumor cells (CTCs) are the cells that detach from the primary tumor and circulate in the bloodstream. CTCs have a malignant potential and are able to form overt metastases in distant organs. Moreover, reports have also noted that CTCs can be present in peripheral blood prior to detection of the primary tumor *via* conventional screening methods.[4,5] Therefore, elucidating the presence and number of CTCs in peripheral blood can serve as an indicator for the judging of prognosis, evaluation of therapeutic efficiency and disease stage forecasting as well.[6] However, CTCs are an extremely rare component within cancer human blood with the majority cellular components, that is several cells/mL of CTCs in the background of $\sim 10^9$ cells/mL of erythrocytes and $\sim 10^6$ cells/mL of leukocytes.[7] To isolate and identify CTCs from the abundant background blood cells (BCs), researchers have developed many techniques based on morphological or chemical differences between the target CTCs and normal hematopoietic species.[8] These methods can be divided into two categories: physical and immune separation. Because of the better reliability and reproducibility, CTC enrichment based on antibody-modified immunomagnetic beads is the most widely used strategy for CTC isolation. CellSearch® system based on this strategy is the first and the only system approved by the U.S. Food and Drug Administration (FDA) for evaluating the diagnosis and prognostic effect of metastatic breast and colorectal cancer patients.[6]

The epithelial cell adhesion molecule (EpCAM) is a widely used biomarker for CTC detection which is highly expressed in breast carcinoma and other metastatic cancers.[9] Recently, many CTC isolation techniques have been reported by using nanomaterials[10,11] and micro-nanostructured surfaces[12-19], functionalized with antibody against EpCAM. For example, Massachusetts General Hospital (MGH) team[7] designed a new microchip which has 78,000 interaction points modified with anti-EpCAM antibody to capture CTCs, which has been applied in clinical trials for liver and lung cancer detection in 2007. Tseng group respectively demonstrated that a three-dimensionally (3D) nanostructured substrate[13], nanostructured silicon substrates[14] and electrospun TiO_2 nanofiber[15] coated with anti-EpCAM exhibited outstanding CTC capture efficiency and sensitivity. Recently, A. Wang group[10] and S. Nagrath[11] reported the antibody-coated nano-iron oxide particles and graphene oxide nanosheets for isolating CTCs, in which the capture efficiency has been greatly improved with the maximum sorting rate reaching to above 80%. S. Wang and L. Jiang groups have successfully constructed different antibody functionalized nanostructured interfaces[16-19] to improve the capture efficiency by the effect on the cell morphology. Although antibody functionalization have highly improved CTC capture efficiency and CellSearch system has also been approved by FDA, widespread clinical application of the antibody-based CTC isolation technologies is still limited by the stability, reproducibility, mass production and expensive price.[20] From the viewpoint of molecular recognition, peptides play key roles in participating in ligand–receptor and protein–protein interactions, since the recognitions are mainly involving in the short peptides at the contact interface. If the binding affinity of peptides to target proteins are comparable with antibodies,

together with the possible higher stability, reproducibility and mass production by chemical synthesis, peptides could be very promising candidates for disease diagnosis, drug therapies and targeting delivery systems.[21,22] For instance, peptides have been applied as tumor targeting molecules[23-25], drugs for cancers[26], and gene carrier vehicles[27-29]. So peptides could also be ligands targeting at the antigens and receptors on the cells[30-33]. In addition, the antibody cross-linked on the surface of magnetic nanoparticles usually exhibits an incomplete contact between the biological macromolecules and ligands as a result of steric hindrance, affecting ligand utilization[34]. So peptide-modified magnetic nanoparticles could be promising technology for CTC isolations.

The widely used techniques for screening novel peptides to recognize cell surface receptors are phage display, mRNA display and ribosome display[22], while the construction of random peptide library and the selection of the high affinity candidates are time-consuming. To date, no bioactive peptides binding to EpCAM have been selected *via* these *in vitro* display techniques. In the current study, we tested a collection of chemically synthesized peptides based on the amyloid proteins, fragments of various proteins including EpCAM and others, and the cell-based selection process is performed for screening of the peptide ligands with high binding affinity. This collection of peptides were examined in our previous studies for unraveling various interaction, hydrogen bond, electrostatic and hydrophobic interactions between side chains, in the peptide assemblies using scanning probe microscopy[35-38]. Only the peptide sequence screened out with reasonable binding affinity to EpCAM is reported in this manuscript, which is also commonly practiced for other recognition probe screening strategies, such as phage display peptides and aptamers[39,40]. In this paper, as shown in Fig.1, we identified a CTC recognition peptide Pep10 (VRRDAPRFSMQGLDACGGNNCNN) with highest binding affinity and selectivity to target CTC cells by cell-based selection from a series of *de novo* designed peptides. Then the iron oxide magnetic nanoparticles (MNPs, about 200 nm) are functionalized by the recognition peptide through biotin-streptavidin interaction for CTC isolation. We demonstrated the successful isolation of CTCs with high capture efficiency by peptide functionalized magnetic nanoparticles from spiked human blood under a magnetic field.

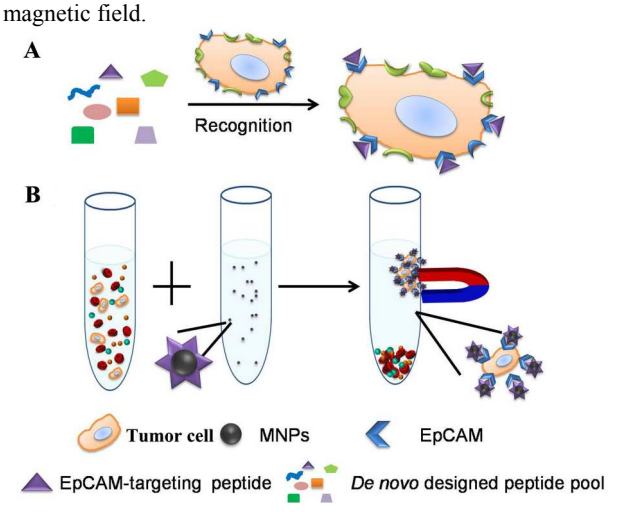

Fig.1. Schematic diagram of CTC isolation with MNPs modified with EpCAM targeting peptide. (A) Screening of EpCAM targeting peptides from *de novo* designed peptide pool. (B) Isolation of CTCs in magnetic field.

2. Materials and methods

2.1 Chemicals

All the peptides used in this experiment were purchased from SPB technology Co. Ltd (Shanghai, China).

RPMI-1640 cell culture media was purchased from HyClone (USA). Hoechst.33342 dye was purchased from Sigma-Aldrich (USA).

Fluorescein isothiocyanate (FITC)-labeled anti-human EpCAM antibody and FITC-labeled mouse IgG2bκ isotype as positive and negative controls were obtained from BioLegend (San Diego, USA). Recombinant human EpCAM/trop-1 *Fc* chimera was purchased from R&D (Minneapolis，USA). Phosphate buffer saline (PBS) was prepared according to standard laboratory procedure.

FITC-labeled anti-human CD45 and allophycocyanin (APC)-labeled anti-human cytokeratin (CK) were respectively obtained from Sino Biological (Beijing, China) and Abnova (Taiwan, China).

2.2 Instrumentations

Peptide screening was performed on a flow cytometry (FCM, FACSCalibur, BD, America). *Zeta* potential and particle size are measured by using NanoZS Zetasizer (Malvern, Worcestershire, UK). OD_{595nm} was measured with plate reader (Tecan Infinite-M200, Männedorf, Switzerland). The binding constant between peptide and EpCAM was obtained by surface plasma resonance (SPR) technology on a PlexArray HT system (Plexera, USA). All the cell images were obtained on an IX71 microscope (Olympus, Japan).

2.3 Cell culture

Human blood samples were supplied by Department of Blood Transfusion, Affiliated Hospital of Academy of Military Medical Sciences (Beijing, China). Human breast cancer MCF-7 and SK-BR-3 cells, prostate cancer PC3 cells, liver cancer Hep G2 cells, human promyelocytic leukemia HL-60 cells, human peripheral blood leukemia Jurkat cells and human leukemia U937 cells were purchased from the Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). The cells were cultured in a flask containing RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% streptomycin/penicillin. The flask was placed in a humidified atmosphere with 5% CO_2 at 37 ℃ in a cell culture incubator. The media was replaced once every three days. The cells were cultured to about 80～90% confluence before harvest. During harvest, the cells were washed twice with PBS buffer followed by trypsinization with 1 mL of trypsin-ethylenediaminetetraacetic (EDTA) solution (0.25 w/v % trypsin, 2.5 g/L EDTA) at 37 ℃ for 5 min to detach the cells from the flask. The trypsin was neutralized by adding 5 mL of fresh supplemented RPMI-1640 medium, and the harvested cells in medium suspension was transferred into a centrifuge tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cells were re-suspended in fresh medium.

2.4 Flow cytometry

Subconfluent cultures were trypsinized into single cell

suspension, counted, washed with PBS buffer, and stained with FITC-labeled anti-EpCAM and FITC-labeled recognition peptides. For each staining reaction, 100,000 cells were incubated with 15 μL of each compound for 30 min at 4 ºC. Unbound compounds were washed off and cells were analyzed on a BD FACS Calibur system. Mouse IgG2bκ-FITC was included as isotype controls.

2.5 SPR

The binding kinetic parameters of the EpCAM with surface immobilized recognition peptides and anti-EpCAM were measured using BIAcoreX SPR system. All SPR measurements were performed at 25 ºC. Deposit a thickness of 1.5-5.0 nm of Cr and 50 nm thickness of Au on to the SPR glass substrate (Plexera, KxV5) *via* thermal evaporation and plasma sputtering for 5 minutes. Then gold chip was modified with a self-assembled layer of the carboxyl group-containing thiol molecules *via* a covalent bond. Then its surface was washed with ethanol twice. Drop 0.2 μL 5mg/mL N-terminal free recognition peptides and 12.5 μg/mL anti-EpCAM. Copious bio-grade water (18 MΩ·cm deionized water filtered by a 2 μm filter and radiated by UV light) was used to rinse the acid-treated chips before it was set on the BIAcoreX instrument for the following real-time biomolecular interaction analysis.

EpCAM was diluted at a series of concentrations (1.56, 3.13, 6.25, 12.50, 25.00 μg/mL) using triple distilled water. 800 μL EpCAM dilutions were injected into the channels at a flow rate of 120 μL/min at 25°C. No regeneration was performed. All sensor grams were recorded from the start of the injection to the end of the dissociation phase. BIAcore analysis was performed using a BIAcore 3000 reader (Applied Biosystems, Uppsala, Sweden). Binding studies were performed in a running buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant. The rate constants and the equilibrium constants were determined from the sensor grams.

2.6 Functionalization of MNPs with recognition peptide

The biotin-conjugated recognition peptide, Pep10, was conjugated to streptavidin-conjugated MNPs [200 nm (Ademtech, France)] *via* biotin-streptavidin interaction. Briefly, MNPs (10 mg Fe/mL) was incubated with peptides (1 mg/mL) at a volume ratio 1:2 for 30～40 min in PBS (pH=7.2) at room temperature. The resulting conjugates Pep10@MNPs was washed with PBS more than 5 times under a high magnetic field (116 mT) and the final concentration of the conjugates are 10 mg Fe/mL. The hydrodynamic radius and the *zeta* potential of the MNPs before/after conjugation with the peptide were determined using dynamic light scattering (DLS) (Zetasizer NanoZS, Malvern, England). As the control of the CTC capture experiment, biotin-labeled anti-EpCAM was modified onto MNPs for obtaining the anti-EpCAM@MNPs by using the same method.

2.7 Isolation of MCF-7 cancer cells from a mixed sample

Pep10@MNPs was used to capture rare tumor cells in synthetic CTC samples. For ease of detection, MCF-7 was incubated with Hoechst.33342 blue fluorescent dye, followed by three PBS washes. Then the mixed samples were prepared by respectively mixing about 50 pre-stained MCF-7 cells with 10^8 (approaching to the number of leukocytes in blood) HL-60, U937 and Jurkat cells in 1 mL PBS. 10 μL obtained Pep10@MNPs was added to the above samples to be incubated with gentle shaking at 37°C for 30 min (the optimal condition). Then they were isolated and washed with PBS at least 3 times under a magnetic field (116 mT). The captured MCF-7 and background cells were all counted using the fluorescence microscope to calculate the capture efficiency and capture purity.

2.8 Isolation of cancer cells spiked in blood

For the cell capture experiment from artificial CTCs-containing clinical samples, the samples were prepared by spiking about 50 Hoechst.33342-stained MCF-7 cells into 1 mL human blood. Add 10 μL obtained Pep10@MNPs or anti-EpCAM@MNPs to the mixed cell suspension in the 1.5 mL centrifuge tube. After incubation of the mixed suspension in shaker at 37°C for 30 min (the optimal condition), the captured cells were gently washed with PBS at least 5 times under a high magnetic field (116 mT). The capture efficiency was measured by imaging and counting the pre-stained MCF-7 cells using the fluorescence microscope. Capture efficiencies from 1 mL human whole blood spiked with 50 cells of three other kinds of tumor cells (SK-BR-3, PC3 and Hep G2 cells) were also investigated to confirm the general applicability of this strategy. Whole blood samples were collected from healthy people and put into EDTA-coated vacutainer tubes for use with in 24 h.

2.9 Confirmation of CTCs with fluorescence immunocytochemistry

In order to confirm the cell type of the captured cells from blood sample, commonly used three-color immunocytochemistry method was applied. The captured cell samples were incubated with FITC-labeled anti-CD45 and APC-labeled anti-CK as well as Hoechst.33342 for 30 min respectively and followed by PBS washing at least 3 times. The cell type confirmation was performed using fluorescence microscope.

2.10 Environmental scanning electron microscopy (ESEM)

In order to observe the primary morphology of the captured tumor cells, we performed the ESEM imaging of the captured cells (FEI Quanta 200) at low vacuum (93 Pa). Before observation, the captured cells were fixed with 2.5% glutaraldehyde for more than 4 h at room temperature. Then the samples were dehydrated by alcohol solution with a series of concentrations (30%, 50%, 75%, 80%, 90% and 100%), followed by drying with supercritical carbon dioxide. Then, the captured cells were investigated by ESEM.

2.11 Cell viability analysis

A commonly used cell live/dead analysis method[43] was utilized to analyze the viability of the isolated cells. Briefly, the cells were stained with 10 μM calcein AM and10 μM propidium iodide (PI) at room temperature for 30 min. Then the cells were observed under a fluorescence microscope. The experiments were repeated three times.

3. Results and Discussion

3.1 Screening of EpCAM recognition peptides

EpCAM protein comprised of a large extracellular domain (265 amino acids), a single transmembrane region (23 amino

acids), and a short (26 amino acids) cytoplasmatic tail, was usually the detection marker of CTCs in clinical.[7] Electrostatic interaction, hydrogen bonding and hydrophobic interactions widely exist between the proteins and peptides as revealed in our previous studies on various amyloidal peptide assemblies by using scanning tunneling microscopy[35-38]. In the current work, we report the test outcome from a collection of peptides interacting with EpCAM with relatively high binding affinity.

The screening process of recognition peptides was monitored using FCM on four EpCAM positive cancer cell lines[33,41,42], MCF-7, SK-BR-3, PC3 and Hep G2 and three EpCAM negative cell lines HL-60， U937 and Jurkat[43-45]. For all the *de novo* designed peptides labeled with FITC, the fluorescence intensity of the cells can reflect the binding capacity of the peptides to the cells. From the binding capacity of the antibody to all the seven cell lines (Fig.2, the FCM graphics were shown in Fig.S1), it can be clearly identified that the low population of EpCAM (Fig.2A) on HL-60, U937 and Jurkat cells comparable to IgG. This result confirmed the EpCAM negative property of the three cell lines, which are consistent with the previous reports[29,44,45]. The high EpCAM population (Fig.2B) on MCF-7, SK-BR-3, PC3 and Hep G2 cells confirmed the EpCAM positive property of the four cell lines. Among all the designed peptides, Pep10 (VRRDAPRFSMQGLDACGGNNCNN) demonstrates the highest binding rates to EpCAM positive cells. As shown in Fig.2, Pep10 demonstrates the high binding rates to MCF-7 (88.5 ± 1.6%), SK-BR-3 (90.7 ± 3.2%), PC3 (96.8 ± 1.4%) and Hep G2 (89.6 ± 1.4%) cells, while very low binding rates to the negative controls, HL-60 (8.6 ± 1.7%), U937 (15.9 ± 1.3%), Jurkat (6.0 ± 1.1%), under the same conditions. That is, the Pep10 can selectively bind to EpCAM positive cells. Moreover, the comparable binding capability and selectivity of Pep10 with the anti-EpCAM indicate that Pep10 could be the possible candidate for CTC isolation targeting surface EpCAM proteins.

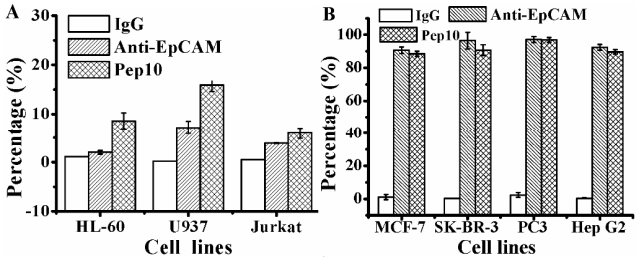

Fig.2. The binding capacity for IgG, anti-EpCAM and Pep10 on HL-60, U937 and Jurkat cell lines (A) and on MCF-7, SK-BR-3, PC3 and Hep G2 cell lines (B) were determined by FCM. Percentage refers to the percentage of the cells fluorescence by FITC binding to the total cells. Experiments were done in triplicates and the reported error is the standard deviations.

3.2 Binding affinity assay

To validate the binding affinity, the equilibrium dissociation constant (K_D) of Pep10 and anti-EpCAM with EpCAM are measured by SPR technique. Usually the surface is very close to saturation after one injection and needs the regeneration prior to a following ligand/analyte binding experiment. While sometime acceptable regeneration condition is hard to find, especially for stable ligand-analyte complexes, where dissociation rate constant (k_d) is relatively small (in the order of 10^{-4} s^{-1})[46]. In this system, no acceptable regeneration protocol was discovered by changing regeneration buffer many times. So the reported non-regeneration protocol[47] was used for kinetic parameter measurements, in which subsequent ligand/analyte interactions can be measured without regeneration.

The kinetic parameters of the Pep10 binding to EpCAM were determined with anti-EpCAM as a positive control. The SPR sensor grams (Supporting Information, Fig.S2) were recorded for the calculation of the quantitative binding kinetic parameters, such as association rate constant (k_a) and k_d as shown in Table 1. K_D were calculated from the measured k_a and k_d ($K_D = k_d/k_a$), where a lower value of K_D corresponds to a stronger binding affinity. As listed in Table 1, Pep10 shows comparable binding affinity (1.98×10^{-9} mol · L^{-1}) to that of anti-EpCAM (2.69×10^{-10} mol · L^{-1}), which is in agreement with the above FCM data. This result indicates that Pep10 can be the possible candidate in CTC isolation as a complementary surface modification agent.

Table 1. Kinetic parameters for the binding of anti-EpCAM and peptides to EpCAM by SPR.

	Kinetic parameters		
	k_a ($L\cdot mol^{-1}\cdot s^{-1}$)	k_d (s^{-1})	K_D ($mol\cdot L^{-1}$)
anti-EpCAM	1.60×10^{4}	4.30×10^{-6}	2.69×10^{-10}
Pep 10	9.64×10^{3}	1.90×10^{-5}	1.98×10^{-9}

3.3 Characterizations of MNPs and its conjugates

Magnetic microbeads have shown great potential in magnetic separation in clinical and biological experiments, while the limitations obstruct their applications, such as fast aggregation and coagulation, low surface to volume ratio, interference on the fluorescence and optical microscopic observation[48,49]. In this work, MNPs with diameters of around 200 nm (Ademtech, France) is selected for magnetic separation of CTCs from the blood samples. The selected recognition peptide, Pep10, is attached onto the MNPs surface (Fig.S3) *via* biotin-streptavidin interaction for improving the binding affinity and selectivity of MNPs to CTCs. The functionalization of the peptide on MNPs is characterized by DLS. As shown in Fig.3, the hydrodynamic size of Pep10@MNPs (305 nm) is slightly increased compared to the pristine MNPs (235 nm), which indicates the successful peptide modification. Since the extended length of the Pep10 is around 7.5 nm while the diameter difference in MNPs because of peptide functionalization is 70 nm, multilayer of peptides should be attached onto the nanoparticle surface. The peptide multilayer formation could be associated with the disulfide bond formation between cystine residues. The *zeta* potentials of the Pep10@MNPs are also changed to a more positive value (from -43.2 mV to -27.4 mV), which could be related to the PI value (isoelectric point, 8.23) of Pep10 originated from the positive Arg residues in the sequences. As a result, Pep10 has been successfully conjugated to the MNPs using the biotin-peptide and the streptavidin-MNPs interactions.

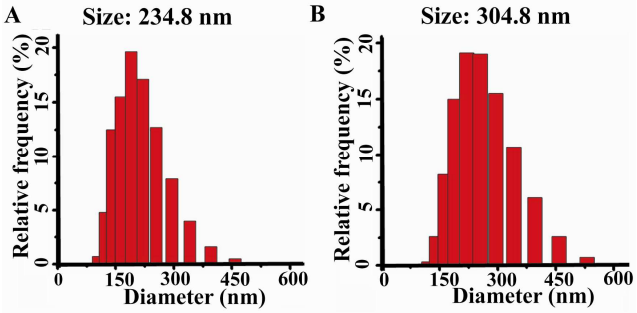

Fig.3. Hydrodynamic diameters of the (A) MNPs and (B) the Pep@MNPs conjugates.

3.4 Isolation of MCF-7 cancer cells from spiked mixed sample

To study the capture efficiency of Pep10@MNPs in isolating CTCs from mimicking CTC samples, 50 Hoechst.33342-stained MCF-7 cells were respectively spiked into 1 mL PBS solution containing 10^8 (corresponding to the number of leukocytes in blood) HL-60, U937 and Jurkat cells[43-45]. As shown in Fig.4, above 45 MCF-7 cells (the total number is about 50) can be isolated with the Pep10@MNPs when the number of the three background cells are as high as 10^8/mL. The capture efficiencies of Pep10@MNPs from all the three background cells are above 90%. These results are coincidence with the high binding affinity of Pep10 to EpCAM protein. Moreover, the capture purity was also calculated by counting the captured background cells and MCF-7 cells (as shown in Fig.4). The capture purity of the MCF-7 cells isolated by Pep10@MNPs from HL-60, U937 and Jurkat cells are 92.5 ± 5.6%, 86.6±5.1%, 92.7±7.3%, respectively. The very low population of captured background cells from initially extremely high number of EpCAM negative cells demonstrated the low binding rate of Pep10 MNPs to the negative cells. In short, the MNPs functionalized by EpCAM recognition peptide, Pep10, can be used to selectively isolate the EpCAM positive cells.

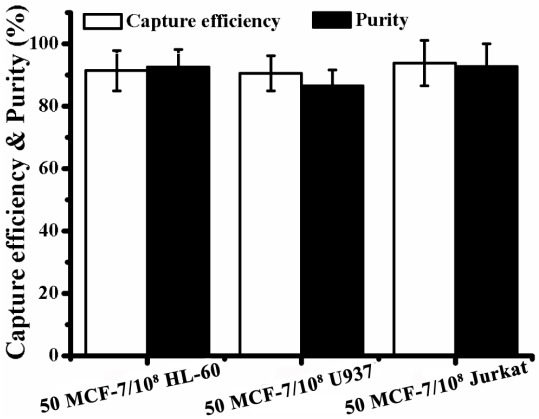

Fig.4. Pep10@MNPs captured MCF-7 cells from mixed samples. Capture efficiency (ratio of captured cancer cells to the spiked cancer cells) and capture purity (ratio of captured cancer cells to total captured cells) in mixture of 50 Hoechst.33342 pre-stained MCF-7 and three different background cells, HL-60, U937 and Jurkat cells with concentration of 10^8 cells·mL^{-1}. The reported error is the standard deviation (n=3).

3.5 Isolation of cancer cells with Pep@MNPs conjugates

To demonstrate the effectiveness of Pep10@MNPs to capture CTCs in whole blood, cancer cells were spiked into healthy human whole blood with a concentration of approximately 50 cells mL^{-1} to prepare closely mimicking clinical samples. The results are shown in Fig.5, 50 Hoechst.33342 pre-stained MCF-7 cells were added to 1 mL whole blood, and the Pep10@MNPs was introduced into the spiked blood sample allowing for the selective binding of the Pep@MNPs to the MCF-7 cells. The typical results for captured cells by Pep10@MNPs are shown in Fig.5A (the other results were shown in Fig.S4). The fluorescence images confirmed that the captured cells are spiked MCF-7 cells with cell nucleus fluorescenced blue by Hoechst.33342 pre-staining. This result demonstrates the successful capture of CTC cells directly from spiked fresh whole blood. The statistical results (Fig.5B) reveal that the average CTC capture efficiency by MNPs@Pep10 reaching up to 90% is comparable to anti-EpCAM@MNPs (91%). Furthermore, the purity of isolated CTC cells by MNPs@Pep10 also can reach up to above 93%, which is also comparable to anti-EpCAM@MNPs (95%). These results demonstrated the considerably high binding affinity and selectivity of Pep10 targeting EpCAM proteins for CTCs isolation and detection.

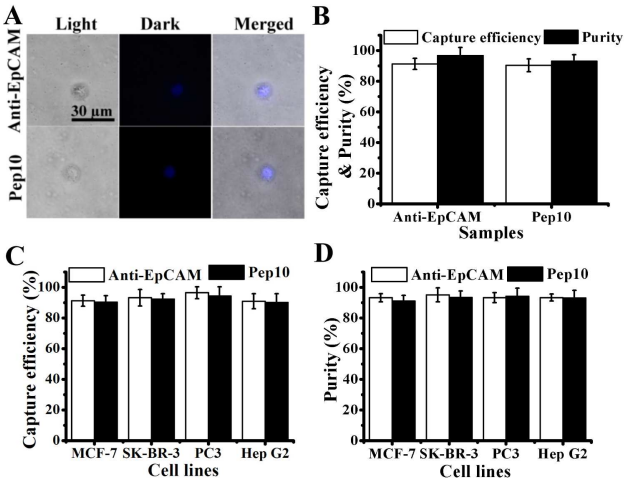

Fig.5. Pep10@MNPs captured tumor cells from spiked human BCs. (A) Fluorescence microscopic images and (B) statistical results of captured cells in mixture of Hoechst.33342 pre-stained MCF-7 and human blood by about 200 nm Pep10@MNPs. (C) Capture efficiency and (D) capture purity from whole blood spiked with four different types of tumor cells: SK-BR-3, PC3, Hep G2, and MCF-7 cells (tumor cell concentration: 50 cells·mL^{-1}). The reported error is the standard deviation (n=3).

In order to confirm the universality of this strategy and the *de novo* designed peptide, we also tested the MNPs@Pep10 system in capturing three other kinds of EpCAM-positive tumor cells (SK-BR-3, PC3 and Hep G2 cells) spiked in whole blood (50 cells·mL^{-1}). The results are shown in Fig.5C and Fig.5D, the capture efficiencies and purity of all these three cell lines also reaching more than 90% and 93%, respectively. This result demonstrates the general applicability of this peptide-based strategy in CTC isolations. From all these experimental results, it could be concluded that 200 nm MNPs functionalized with Pep10 instead of EpCAM antibody is able to capture rare tumor cells from whole blood efficiently.

3.6 Further identification of CTCs

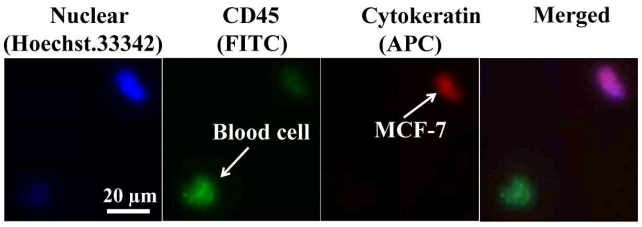

Fig.6. Fluorescent micrographs of MCF-7 captured from blood. Three-color immunocytochemistry method based on APC-labeled anti-CK, FITC-labeled anti-CD45, and Hoechst.33342 nuclear staining was applied to identify CTCs from non-specifically trapped blood cells.

After CTCs were captured from artificial cancer blood samples with the above strategy, a commonly used three-color immunocytochemistry method[50] was applied to identify and enumerate CTCs from non-specifically trapped BCs, including FITC-labeled anti-CD45 (CD45 is a marker for BCs) and APC-labeled anti-CK (CK is a protein marker for epithelial cells) as well as Hoechst.33342 nuclear staining. The fluorescence microscopy was employed to quantify expression levels of CK and CD45 in individual cells. As shown in Fig.6, CTCs exhibit strong CK expression and negligible CD45 signals. In contrast, BCs present low CK and high CD45 expression levels. Hoechst.33342 staining validates that the captured cells retain intact nuclei. The combined information was utilized to identify CTCs (Hoechst+/CK+/CD45-, cell sizes ranging from 10 μm to 40 μm) from BCs (Hoechst+/CK-/CD45+, and cell sizes < 15 μm) and cellular debris.

3.7 Surface structure of cells captured by Pep10@MNPs

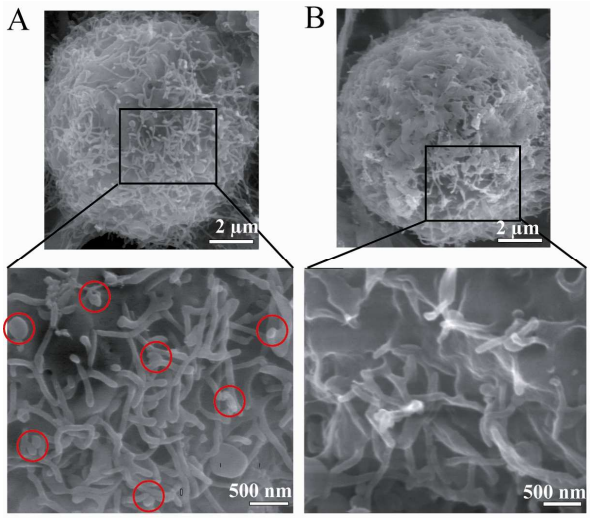

Fig.7. ESEM images (top) and enlarged ESEM images (below) of the MCF-7 cell captured by Pep10@MNPs (A) or without MNPs (B).

To further investigate the interaction and binding ability between cancer cells and Pep10@MNPs, the morphology of MCF-7 cells before and after captured by Pep10@MNPs was analyzed with ESEM. As shown in Fig.7A, although after washed many times, many nanoparticles (marked with red rings) can still be observed to attach on the surface of MCF-7 cells captured by Pep10@MNPs. At the same time, the cells captured by the Pep10@MNPs exhibited more filopodia (Fig.7A, below) than the cells without Pep10@MNPs treatment (Fig.7B). L. Jiang and L. W. Terstappen *et al*. also found that the nanostructures[17-19] and nanoparticals[45] can enhanced the local topographic interactions between the nano-structures and nanoscaled components of the cellular surface (e.g., filopodia and lamellipodia). So we deduce that not only the high binding ability of Pep10 and EpCAM, but also the more filopodia and lamellipodia induced by 200 nm MNPs enhance the interaction between the cells and Pep10@MNPs. These results also verify the high efficiency of Pep10 modified MNPs above.

3.8 Cell viability

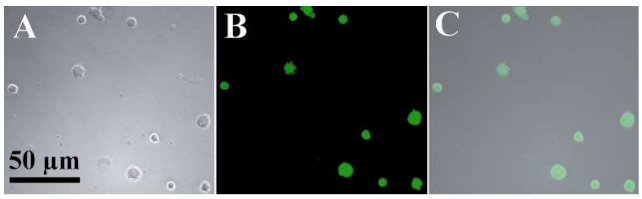

Fig.8. The optical and fluorescence images of the MCF-7 cells captured by Pep10@MNPs with calcein AM and PI dual staining. (A) Optical image, (B) Calcein AM stained living cells showing green fluorescence, (C) merged image of A and B.

The viability of the captured CTC cells is very important for further investigating their malignant nature and assessing the invasive potential of individual CTCs in clinical[43]. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay of the Pep10 on four cancer cells showed negelectable cytotoxicity (Fig.S5). In order to further determine the CTC cell viability, double staining with calcein AM and PI were conducted on the captured MCF-7 cells without removing the Pep10@MNPs. Calcein AM can penetrate the live cell membrane and react with the intracellular esterase to form green fluorescent calcein, while PI is membrane-impermeable nuclear stain that can only stain dead cells resulting in red fluorescence. It can be seen (Fig.8) that nearly all the isolated cells showed green fluorescence, and no red fluorescence can be observed. Thus, we deduce that the captured CTCs with our method can maintain their viability for further molecular biological analysis.

4. Conclusion

In this work, we reported the peptide-based CTC isolation methods, in which MNPs were functionalized with the *de novo* EpCAM recognition peptides for successful magnetic isolation of CTCs. The Pep@MNPs are demonstrated to capture cancer cells in spiked human whole blood without complex pre-treatment process. Results show that Pep10@MNPs (200 nm) have the comparable capture efficiency and purity to anti-EpCAM@MNPs for breast, prostate and liver cancers, reaching above 90% and 93%. Furthermore, the captured cells can still maintain their viability for further molecular biological analysis. These results suggest the promising application potentials of peptide-functionalized magnetic nanoparticles in clinical CTCs detection. The current findings could benefit the improvement of cancer prognosis, evaluation of therapy and the investigations on metastasis mechanisms.

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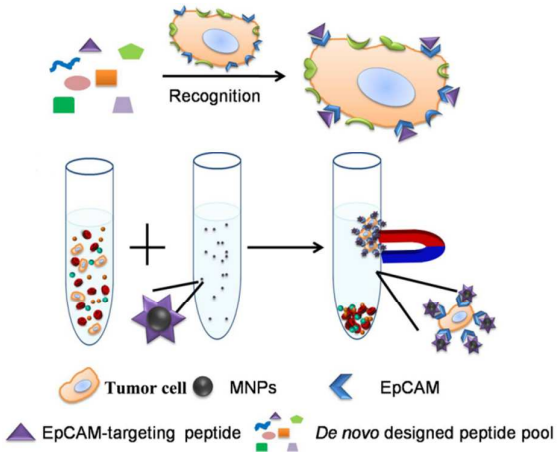

A new CTC isolation method with high efficiency by using the EpCAM recognition peptide functioned magnetic nanoparticles was developed.