

Transparent, biocompatible nanostructured surfaces for cancer cell capture and culture

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Abstract: Circulating tumor cells (CTCs) in the blood which have detached from both the primary tumor and any metastases may be considered as a “liquid biopsy” and are expected to replace tumor biopsies in the monitoring of treatment response and determining patient prognosis. Here, we introduce a facile and efficient CTC detection material made of hydroxyapatite/chitosan (HA/CTS), which is beneficial because of its transparency and excellent biological compatibility. Atomic force microscopy images show that the roughness of the HA/CTS nanofilm (HA/CTS/NF) substrates can be controlled by changing the HA:CTS ratio. Enhanced local topographic interactions between nano-components on cancer cell membranes, and the antibody coated nanostructured substrate lead to improved CTC capture and separation. This remarkable nanostructured substrate has the potential for CTC culture in situ and merits further analysis. CTCs captured from artificial blood samples were observed in culture on HA/CTS/NF substrates over a period of 14 days by using conventional staining methods (hematoxylin eosin and Wright's stain). We conclude that these substrates are multifunctional materials capable of isolating and culturing CTCs for subsequent studies.

Keywords: cell capture, cell culture, nanofilms, hydroxyapatite/chitosan

Introduction

Solid tumors are the most common cause of morbidity and mortality worldwide^{1,2} due to the lack of effective early detection methods and treatments to prevent metastases.^{3,4} Since the first literature report in 1869, circulating tumor cells (CTCs)⁵ (cancer cells that break away from solid tumors)⁶ have been thought to play a crucial role in tumor metastasis and disease progression. In addition, CTCs considered as a “liquid biopsy” of the tumor, are expected to replace surgical tumor biopsies in the monitoring of treatment response and determining patient prognosis. Detection and characterization of CTCs has been technically challenging however, owing to the extremely low abundance of CTCs (a few to hundreds per milliliter) among 10^9 hematologic cells mL^{-1} in the blood.^{7,8} In the past decades, a significant amount of research has been devoted to developing CTC detection technology. Because of this, CTC counts in patients' blood can now serve as prognostic indicators and be used in the monitoring of treatment responses in patients with solid tumors. The immunomagnetic separation-based CellSearchTM Assay (Veridex, Raritan, NJ, USA) is the only US Food and Drug Administration (FDA) cleared CTC technology for the diagnosis of metastatic breast, prostate, and colorectal cancers. Because of its unsatisfactory CTC capture efficiency and high cost, significant efforts have been made to develop new CTC isolation technologies.

A high efficiency CTC detection assay has been reported which uses three-dimensional nanostructured substrates based on silicon.⁹ Enhanced local topographic interactions^{10,11} between nano-components on the cancer cell membranes (eg, microvilli

and filopodia) and antibody-coated nanowires lead to improved CTC capture efficiency. This effect has been further confirmed with other types of nanostructured substrates, including horizontally packed titanium dioxide (TiO₂) nanofibers¹² and polymer-grafted silicon nano-substrates.¹³ In most cases, preparation of these nanostructures requires either specialized equipment or complex process control, so the methods have been limited for high throughput fabrication. Further applications of these platforms are also constrained by their nontransparent nature which makes them incompatible with many optical imaging systems such as those using immunocytochemical techniques.

Herein, we introduce a simple but efficient CTC detection material made of hydroxyapatite/chitosan (HA/CTS), which is beneficial due to its transparency and excellent biological compatibility. Owing to their biocompatibility,^{14–18} HA/CTS-based composites have been widely used in bioengineering applications such as bone repair,^{19,20} tissue engineering,²¹ and controlled drug release.²² However, they have not been used for the development of biomaterials for cancer cell detection. We therefore attempted to reproduce the success with three-dimensional nanostructured substrates previously mentioned by using a layer of horizontally oriented HA/CTS composite nanostructures.

Materials and methods

Preparation and characterization of HA/CTS nanofilm substrates

A description of the HA/CTS nanofilm (HA/CTS/NF) preparation follows. Firstly, the glass was plasma treated: the glass substrate was boiled in piranha solution (3:1 volume/volume [v/v] H₂SO₄/H₂O₂) at 100°C for 1 hour, rinsed in deionized (DI) water several times, treated with oxygen plasma (PDC-32G, Harrick Plasma, Ithaca, NY, USA) for 2 minutes, and then dried with nitrogen gas for further use. Then, a chitosan aqueous solution of 2 weight percentage (wt%) was prepared by dissolving chitosan powder (Haisheng Co, Ltd, Qingdao, People's Republic of China) in distilled water containing 2% acetic acid, then 5 wt% nano-hydroxyapatite (HA) powder (Sigma-Aldrich Co., St Louis, MO, USA) was added slowly. The mixed solution was blended with a magnetic stirrer for 12 hours until thoroughly dissolved and then uniformly coated on the clean glass using a photoresist spinner (6,000 r/h, 60s). In the baking process the coated solution was heated (100°C) on a bake table for 1 hour. Following the baking process the HA/CTS/NF substrate was soaked in 10 wt% sodium hydroxide (NaOH) solution for 10 hours. Finally, the substrate was washed with DI water, dried, and

was now ready for chemical modification. The morphology of the HA/CTS/NF substrate was observed by a field emission scanning electron microscope (SEM, 6700F, JEOL, Tokyo, Japan). The surface roughness of the nanofilm substrate was characterized using an atomic force microscope (AFM) (SPM 9500J3; Shimadzu, Kyoto, Japan). The transmittance of the CTCs capture device was measured by an ultraviolet-visible (UV-Vis) spectrophotometer (UV-2550; Shimadzu).

Surface modification with streptavidin

In order to validate the streptavidin (SA) binding to the HA/CTS/NF substrate, we used Cy5-SA (a red fluorescent labeled SA) (Thermo Fisher Scientific, Waltham, MA, USA) as the detector. First, the substrate was modified with 4% (v/v) 3-mercaptopropyl trimethoxysilane (Sigma-Aldrich Co.) in ethanol at room temperature for 1 hour.²³ Then, the substrate was treated with the coupling agent N-γ-maleimidobutyryloxy succinimide ester (GMBS) 0.25 mM (Sigma-Aldrich Co.) for 1 hour make for GMBS attachment to the substrate. Afterwards, the substrate was treated with 50 μg mL⁻¹ of Cy5-SA at room temperature for 45 minutes, resulting in the immobilization onto GMBS, and subsequently flushed with 1× phosphate buffered saline (PBS) to remove excess SA. The fluorescence images (Figure S1) show that the Cy5-SA is binding to the HA/CTS/NF substrate. According to the statistics, mean fluorescence intensity of the HA/CTS/NF substrate is 5–10 times that of the glass.²⁴ Due to the strong and specific biotin-SA interaction, the HA/CTS/NF substrate could be modified with biotinylated anti-human EpCAM/TROP1 antibody (goat immunoglobulin G) (anti-EpCAM, R&D Systems, Minneapolis, MN, USA) for specific cancer cell capture.

Cells

The colorectal cancer cell line (HCT116), lung cancer cell line (A549), gastric carcinoma cell line (MGC803), cervical cancer cell line (HeLa), and chronic myelogenous leukemia cell line (K562) were harvested from Hubei Key Laboratory of Tumor Biological Behaviors (Hubei, People's Republic of China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (BD Biosciences, San Jose, CA, USA) and supplemented with 10% fetal bovine serum (Sigma-Aldrich Co.) and 1% penicillin/streptomycin solution at 37°C under a humidified 5% CO₂ atmosphere.

Blood samples

Whole blood samples from healthy donors were obtained from the Department of Clinical Laboratory, Zhongnan Hospital of Wuhan University according to Institutional

Review Board (IRB) protocol and cancer patients' blood samples were obtained from the Department of Oncology, Zhongnan Hospital of Wuhan University under a separate IRB-approved protocol. All patients gave their informed consent and the research was approved by the Ethics Committee of Zhongnan hospital of Wuhan University. All blood specimens were collected into anticoagulant tubes (EDTA-K₂, 2 mL, violet cap) (Wuhan Zhiyuan Medical Technology Co, Ltd, Wuhan, People's Republic of China).

Cell capture and culture

Prior to cell capture, a 1×1 cm substrate was placed into a size-matched chamber after which 15 µL of biotinylated anti-EpCAM (10 µg mL⁻¹ in PBS with 1% [w/v] bovine serum albumin) was dropped onto the substrate to incubate for 2 hours before washing with PBS. Then, 1 mL of cell suspensions (10⁵ cells mL⁻¹) was loaded. After placing in a cell incubator for 60 minutes under the conditions of 37°C and 5% CO₂, the substrate was gently washed with PBS at least eight times. The captured cells on the substrate were fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes. Next, the substrate was loaded with 0.5 mL of 0.2% Triton X-100 (Sigma-Aldrich) in PBS and incubated for 10 minutes in order to improve the cell permeability and to allow for intracellular staining, followed by staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.33 µg mL⁻¹ in DI water) on the target cells for 10 minutes. Finally, we imaged and detected targeted cells using a fluorescence microscope (IX81; Olympus, Tokyo, Japan). CTCs captured on substrates were photographed using IPP software (Media Cybernetics Inc., Silver Spring, MD, USA).

For the cell culture experiment, artificial blood samples were prepared by spiking 1,000 HCT116 cells into 1 mL healthy donor blood (1,000 cells mL⁻¹), 1 mL suspension was introduced onto anti-EpCAM-coated HA/CTS NF substrate, after incubation for 1 hour, rinsed three times with PBS to remove nonspecific interaction cells (white blood cells [WBCs]), then cultivated in DMEM over a period of 14 days. Normal plate culture was also examined as a control. The medium was replaced once a day.

Scanning electron microscopy morphology observation

The cell captured sample was prepared following the procedure described in our previous paper, for SEM observation.¹² Briefly, the cells were fixed with 2% glutaraldehyde for 2 hours and then dehydrated sequentially with a series of different concentrations of ethanol (50%, 70%, 90%, 100%,

and 100%) for 15 minutes followed by further dehydration. After completely dried, the samples were sputter coated with gold before being observed with the field emission SEM (6700F; JEOL) at an acceleration voltage of 20 keV.

Cell capture from the artificial CTC blood samples

As summarized in Table S1, the cell capture efficiency of the optimal capture condition was validated using EpCAM-coated HA/CTS NF substrates to capture target cells (HCT116) from the two kinds of artificial blood sample: 1) 1 mL DMEM containing HCT116 cells, and 2) 1 mL human blood (healthy donor) containing HCT116 cells. After rinsing, followed by staining of anti-CK, anti-CD45, and DAPI, the specifically captured HCT116 cells were identified and counted on the substrates.

Results and discussion

Preparation of the HA/CTS NF substrate is controlled by reaction conditions, including reagent concentrations, temperature, and pH, all of which can affect the morphology of the resultant nanofilms. The structure of the substrate is illustrated in Figure 1. In order to obtain an optimal nanostructured substrate, we tested various reaction conditions for the fabrication. Ultimately, five HA/CTS NF-deposited substrates (HA/CTS NF1 to HA/CTS NF5) were prepared using different HA/CTS compositions, as shown in Figure 2A–E. AFM images show that the square average roughness of the substrates can be controlled by changing the HA:CTS ratio (Figure S2 and Table S2). The HA/CTS NF substrates were formed as described in Materials and methods section. The transmittance of the HA/CTS nanofilm on glass – approximately 60% – is shown in Figure 3A, Figure 3B shows HCT116 cells captured by our HA/CTS NF substrate after 14 days of culture, in which the cell structures can be distinguished via hematoxylin eosin stain and Wright's stain. These remarkable properties allow us to observe the process of detecting cancer cells by microscopy, and potentially obtaining biologically functional cancer cells that can be used for ex vivo culture. In addition, they facilitate downstream analyses of circulating tumor cells from cancer patients by using techniques such as immunocytochemistry, fluorescence in situ hybridization, and genomic analysis. During optimization of the HA/CTS NF substrate for cell capture, two critical parameters were found to be closely related to cell capture efficiency:

- 1) dimension and roughness of the nanofilms, with capture depending on enhanced local topographic interactions between nano-components on cancer cell membranes

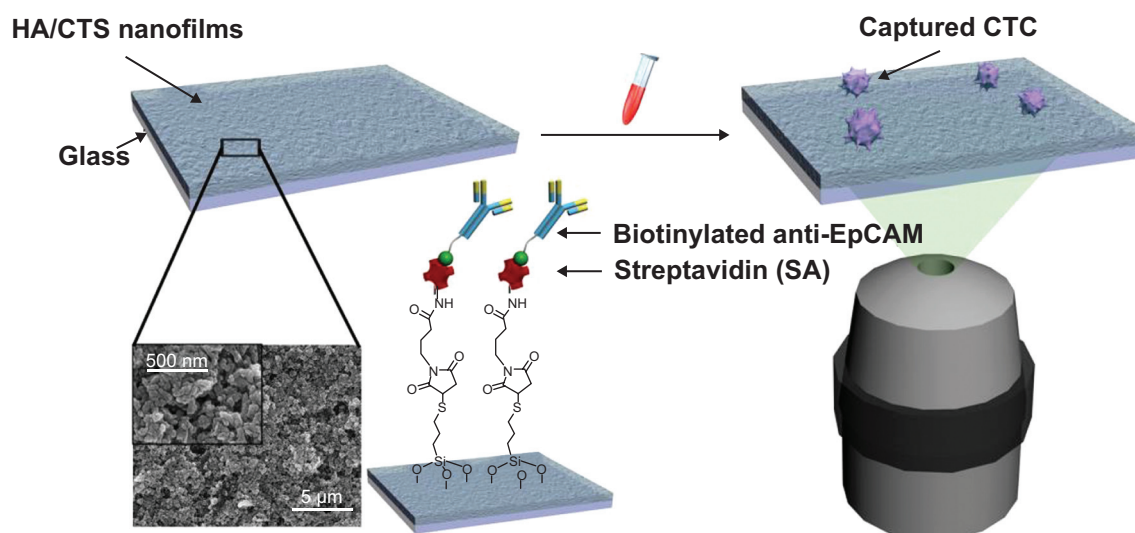


Figure 1 Horizontally packed HA/CTS nanofilm for improved CTC capture by combining cell capture agent and cancer cell preferred nanoscale topography.
Abbreviations: CTC, circulating tumor cell; HA/CTS, hydroxyapatite/chitosan nanofilm.

(eg, microvilli and filopodia) and the antibody-coated nanostructured surface. The optimal surface structure allows increased contact frequency and interaction between the cells and the substrate, thus improving capture efficiency.

- 2) The incubation time, because it takes time, both for the viable cells to approach the substrate and, once contact has been made, for mobility of the cell membrane and extracellular matrix to permit more of the nanostructure to become embedded in the cellular surface components.

To demonstrate how the dimensions and roughness of HA/CTS nanofilm impact the efficiency of cell capture, we prepared an EpCAM-positive cancer cell line (HCT116) in DMEM at a concentration of 10^5 mL^{-1} and captured these spiked cells with anti-EpCAM-grafted substrates prepared with composites having different HA:CTS ratios (1.25:2, 2.5:2, 5:2, 10:2, and 15:2 wt%). As the HA:CTS ratio increased, initially so did the cell capture efficiency, before reaching a maximum, and then declining (Figure 2G and Table S2). When the roughness of the substrate was approximately 150–200 nm, maximum cell capture numbers were achieved for the HCT116 cell line (approximately $55,000 \text{ cells cm}^{-2}$). Presumably, these results indicate that the cellular surface components and the HA/CTS nanofilm are well-matched, allowing sufficient contact and efficient adhesion, resulting in improved cell/substrate affinity. High CTC-capture efficiency was achieved by combining a cell capture agent (anti-EpCAM) with cancer cell-compatible nanoscale topography of the HA/CTS nanofilm substrate. Local topographic interactions correlate with the characteristics of the nano-HA/CTS compos-

ite that include substrate roughness, and cell capture yield reaches a maximum at the optimal roughness level. In our test system, this occurred at a roughness of 150–200 nm (HA/CTS nanofilm3, HA/CTS = 5:2 wt%), which was therefore selected for subsequent studies. Next, to explore how film-formation temperature impacts on the efficiency of cell capture, we carried out experiments with the optimal substrates (HA/CTS nanofilm3, HA/CTS = 5:2 wt%) according to the previously described procedure, but used a different baking temperature (40°C, 70°C, 100°C, 130°C, and 160°C). There were no observable differences in the numbers of HCT116 cancer cells captured when the baking temperature was increased from 70°C to 160°C, nor was any change observed in the HA/CTS nanofilm surface morphology (Figure S3). It was yet another sign that nanostructured materials could affect the efficiency of the cell capture.

We performed experiments to determine the incubation time required for the most efficient cell capture, using the optimal substrate coated with anti-EpCAM, as previously described. HCT116 cancer cells were spiked into DMEM at a concentration of $10^5 \text{ cells mL}^{-1}$ and captured using our platform. Figure 2H shows the cell capture yields for different incubation times. For the EpCAM-positive cell line (HCT116), the recovery yields of cell capture increased significantly when increasing the incubation time. The maximal recovery yields were several-fold greater than the minimal yields in our test, with the maximal (saturation) recovery yields being achieved at incubation times of 60 minutes or longer, leading us to adopt 60 minutes as the minimum incubation time for optimal capture.

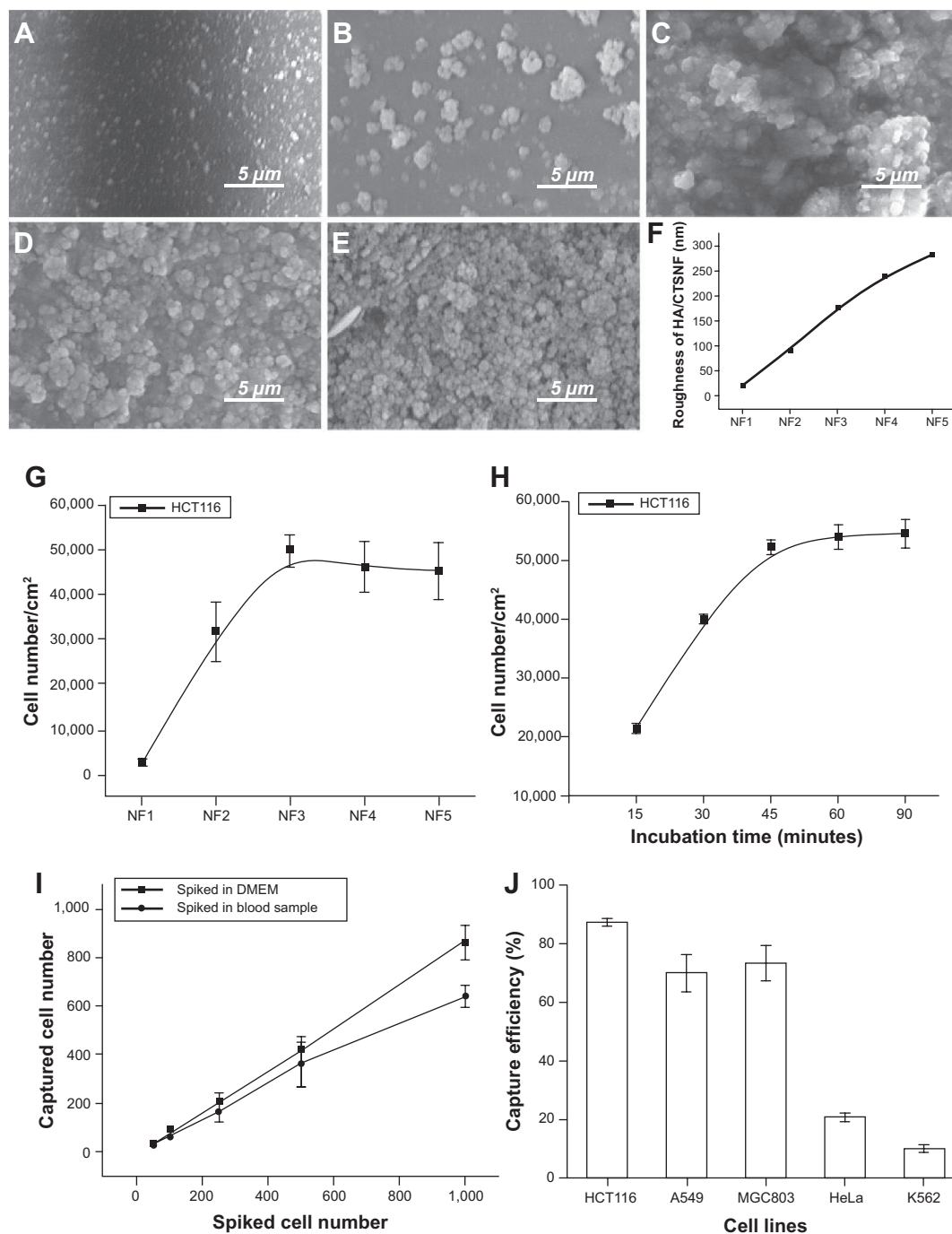


Figure 2 SEM micrograph of HA/CTS nanofilm (HA/CTS/NF) substrates with different HA/CTS ratios.

Notes: (A) 1.25:2 wt% (NF1), (B) 2.5:2 wt% (NF2), (C) 5:2 wt% (NF3), (D) 10:2 wt% (NF4), and (E) 15:2 wt% (NF5). (F) Plotted square average roughness of different HA/CTS/NF samples. The number of cells captured on HA/CTS/NF substrate with (G) different samples of NF1, NF2, NF3, NF4 and NF5, and (H) at different incubation times (with incubation time of 15, 30, 45, 60, and 90 minutes). (I) The captured cell number of CTCs was validated from two kinds of artificial blood samples under optimal conditions (HA/CTS/NF substrate with HA/CTS, 5:2 wt% composite and 60 minutes cell incubation time). (J) Cell capture efficiencies on HA/CTS/NF substrates were validated using suspensions of EpCAM⁺ colon (HCT116), lung (A549), gastric (MGC803), EpCAM⁺ cervical (HeLa), and myelogenous leukemia (K562) cell lines. Error bars show standard deviations (n=3).

Abbreviations: CTCs, circulating tumor cells; DMEM, Dulbecco's Modified Eagle's Medium; SEM, scanning electron microscope; wt%, weight percentage; HA/CTS, hydroxyapatite/chitosan.

We validated the cell capture efficiency of the EpCAM-coated HA/CTS/NF substrates by using a series of artificial blood samples prepared by spiking HCT116 cells into DMEM and healthy donor's blood at concentrations

of approximately 50, 100, 250, 500, 1,000 cells mL⁻¹. The results (Figure 2I and Table S1) indicate that the spiked HCT116 cells were captured from healthy donor's blood with a yield of 64%. These results represent a vast improvement

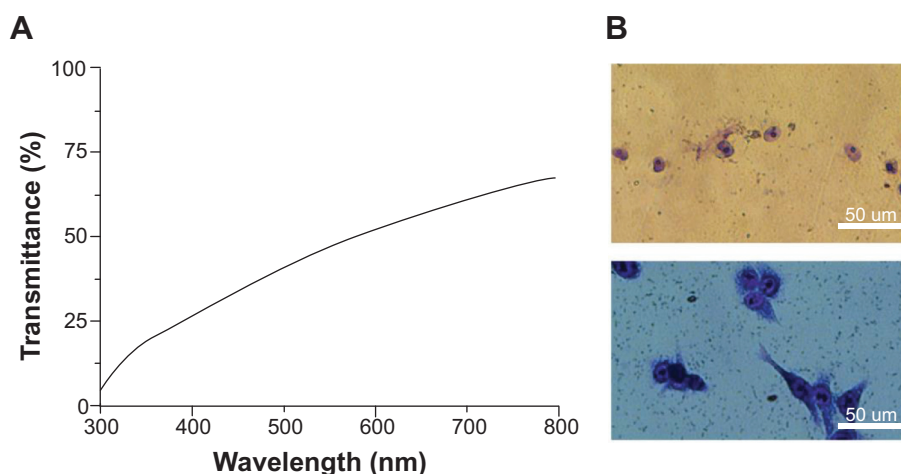


Figure 3 (A) The transmittance of the HA/CTS NF substrate, (B) HE stain (top) and Wright's stain (bottom) of captured HCT116 cells from whole blood samples. **Abbreviations:** HA/CTS NF, hydroxyapatite/chitosan nanofilm; HE, hematoxylin eosin.

in capture efficiency compared with those obtained with commercialized technology.²⁵

Finally, to illustrate the cell capture specificity and optimized cell capture performance of HA/CTS NF, we used three EpCAM-positive cancer cell lines (colorectal cancer cell line HCT116, lung cancer cell line A549, and gastric carcinoma cell line MGC803), and two EpCAM-negative cancer cell lines (chronic myelogenous leukemia cell line K562 and cervical cancer cell line HeLa) for specific cell capture comparison experiments using the optimized cell capture conditions previously described. For the EpCAM-positive cells, the maximal (ie, saturation) cell capture numbers were achieved, whereas only relatively low numbers of EpCAM-negative cells were captured. The results summarized in Figure 2J show that the EpCAM-coated HA/CTS NF substrates were capable of efficiently capturing HCT116, A549, and MGC803 cells, but not HeLa and K562 cells.

To compare the cell capture performance of the HA/CTS NF substrates with an alternative nanoscale substrate, we prepared an HCT116 cell suspension (10^5 cells mL^{-1}) as previously described. One mL HCT116 cell suspensions pre-stained with fluorescein diacetate ($50 \mu\text{g mL}^{-1}$ in acetone) was introduced onto anti-EpCAM-coated HA/CTS NF substrate (SA-anti-EpCAM-HA/CTS NF) and TiO_2 nanofibers substrate (SA-anti-EpCAM-TiNF). A flat glass substrate modified with anti-EpCAM (SA-anti-EpCAM flat glass) acted as a control. After capture, rinsing, and counting, the number of cells captured on HA/CTS NF substrate was equal to that on the horizontally packed TiO_2 nanofibers, and much more than that on the flat glass substrate. The results are summarized in Figure 4A. Importantly, no nanoscale components of the cellular surface (microvilli and filopodia) were observed on the cells on the glass substrate, whereas the cells attached to

the HA/CTS NF substrate exhibited extended pseudopodia. This finding provides further evidence that the mechanism of our nanostructure-based cell-capture system is a specific surface-to-surface interaction. In addition to high capture efficiency equal to that of our previously described TiO_2 nanofibers, cancer cells captured on the HA/CTS NF substrate can be monitored by simultaneous fluorescence and bright-field imaging because of the material's transparent nature. HA/CTS NF is therefore unique in its ability to permit direct monitoring of captured cancer cells. Fluorescein diacetate staining (Figure 4B) shows that the HA/CTS NF-coated area captured considerably more HCT116 cancer cells than the flat glass. To exclude the possibility that HCT116 cells were captured mainly as a result of nonspecific interactions with the HA/CTS NF substrates, we compared the optimized conditions side by side with two control conditions: 1) HA/CTS NF substrate without any surface modification (bare-HA/CTS NF); 2) HA/CTS NF substrate coated only with SA (SA-HA/CTS NF), as showed in Figure 4A. The cell capture efficiency of cells captured on both bare-HA/CTS NF and SA-HA/CTS NF were around 5%, but exceeded 84% when the substrate was modified with both SA and the anti-EpCAM capture agent. These data indicate that non-specific cell capture by pure HA/CTS NF and chemically modified HA/CTS NF is negligible. Anti-EpCAM modified HA/CTS NF substrate can capture target cancer cells with high efficiency and specificity.

Next, we assessed the ability of captured cells to be cultured on HA/CTS NF. We prepared artificial blood samples containing $1,000 \text{ CTC mL}^{-1}$ and used 1 mL for cell capture with anti-EpCAM-coated HA/CTS NF substrate, and cultivated the captured cells in DMEM for 14 days. A normal plate culture was performed in parallel as a control. Morphological observations show (Figure 5A) that

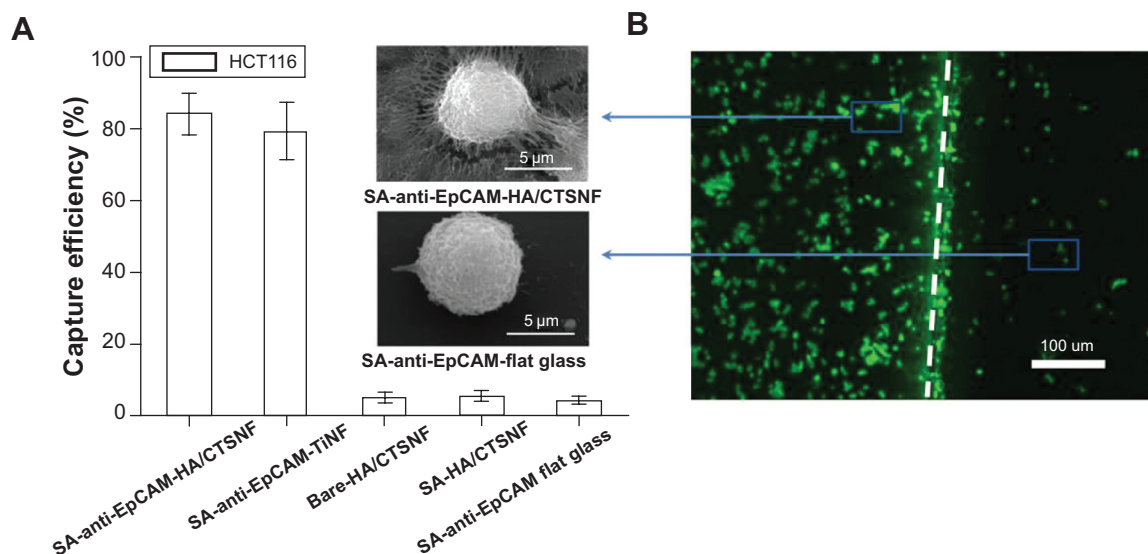


Figure 4 Cell capture performance under five different conditions.

Notes: (A) 1) HA/CTS NF substrate modified with anti-EpCAM (SA-anti-EpCAM-HA/CTS NF), 2) TiO₂ substrate modified with anti-EpCAM (SA-anti-EpCAM-TiNF), 3) HA/CTS NF substrate without any surface modification (bare-HA/CTS NF), 4) HA/CTS NF substrate with only SA coating (SA-HA/CTS NF), and 5) flat glass substrate modified with anti-EpCAM (SA-anti-EpCAM flat glass). An EpCAM-positive cell line (HCT116) was used. SEM images comparing HCT116 cell line captured by HA/CTS NF substrate (top) and flat glass substrate (bottom). (B) Fluorescein diacetate fluorescence micrograph of HCT116 cells captured on HA/CTS NF (left) substrate and glass (right).

Abbreviations: HA/CTS NF, hydroxyapatite/chitosan nanofilm; TiO₂, titanium dioxide; SA, streptavidin.

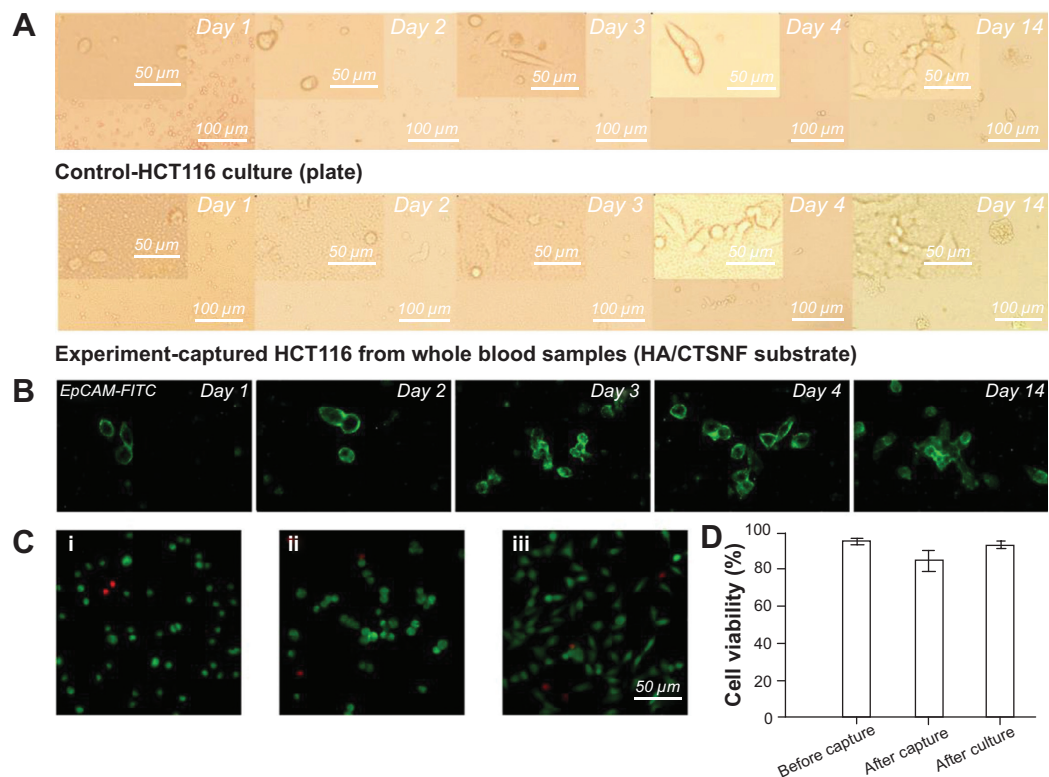


Figure 5 Comparison of cell culture and proliferation between cells.

Notes: (A) Comparison of cell culture and proliferation between cells captured from whole blood samples (1,000 cells mL⁻¹) on anti-EpCAM coated HA/CTS NF substrate and normal plate culture over 14 days. No differences were observed in cell proliferation rates for the HCT116 cancer cells. (B) Fluorescent images of captured HCT116 cancer cells from whole blood samples (1,000 cells mL⁻¹, cultured after capture on anti-EpCAM coated HA/CTS NF substrate). FITC-labeled anti-EpCAM was used. (C) Fluorescent micrographs of fluorescein diacetate-PI stain HCT-116 cancer cells before capture i); after capture ii); and after culture iii). Viable (green) and non-viable (red) cancer cells are distinguishable. (D) Plotted viability of HCT-116 cancer cells at different stages. Error bars show standard deviations (n=3).

Abbreviations: HA/CTS NF, hydroxyapatite/chitosan nanofilm; FITC, fluorescein isothiocyanate; PI, propidium iodide.

the captured HCT116 cells adhered to the substrate, but did not proliferate significantly over the first 3 days, whereas the normal lymphocytes gradually disappeared during the same period. However, after 4 days the cancer cells began to proliferate and migrate on the film, the HA/CTS NF substrate seemed to be more effective for cell adhesion in the initial period of cell culture (up to day 4) because of the good biocompatibility of the nanofilms and their uniform local surface topography. After 14 days, the cell population increased significantly. Figure 5B shows the fluorescein isothiocyanate (FITC) stained HCT116 cancer cells ($1,000 \text{ cells mL}^{-1}$) captured from whole blood samples using anti-EpCAM coated HA/CTS NF substrate and cultured over 14 days. Further, cell viability was tested by fluorescein diacetate-propidium iodide, assay at three stages: before capture, after capture, and after culture. The results indicate that cell viability reached 84% after capture, and 93% after culture, making these cells suitable for subsequent studies (Figures 5C and 5D). The HA/CTS NF substrate clearly appears to have no negative effects on cell morphology, viability or proliferation, exhibits excellent biocompatibility compared with the regular culture plate, and is suitable for cell culture and real time observation, offering an alternate way to obtain more highly purified CTCs. In addition, because the captured cancer cells are alive, they could be isolated from cancer patients and potentially expanded *ex vivo* for further molecular interrogation. The transparency of HA/CTS NF allows direct observation and accurate positioning of the cancer cells it captures, which means they could also potentially be physically isolated for proteomic and genomic analyses.

To validate the clinical utility of the EpCAM-coated HA/CTS NF substrate, we applied the optimized cell capture conditions to study peripheral blood samples from gastrointestinal and lung cancer patients. One mL peripheral blood sample (preserved in EDTA- K_2 tubes) were introduced onto an EpCAM-coated HA/CTS NF substrate and placed into a size-matched chamber ($1 \times 1 \text{ cm}$). After incubation, the captured cells were fixed with 4% PFA and permeabilized with 0.02% Triton-X100. Captured cells were then stained with PE-labeled anti-CK, FITC-labeled anti-CD45 and DAPI.^{26–29} Fluorescence microscopy was used to distinguish specifically captured CTCs (CK+/CD45–/DAPI+) from non-specifically immobilized WBCs (CK–/CD45+/DAPI+) by their fluorescence and larger size (CTCs: 10–30 μm ; 1 WBCs, <15 μm) (Figure 6A). CTC enumeration results are summarized in Figure 6B and Table 1. CTCs were captured from the peripheral blood of the cancer patients with

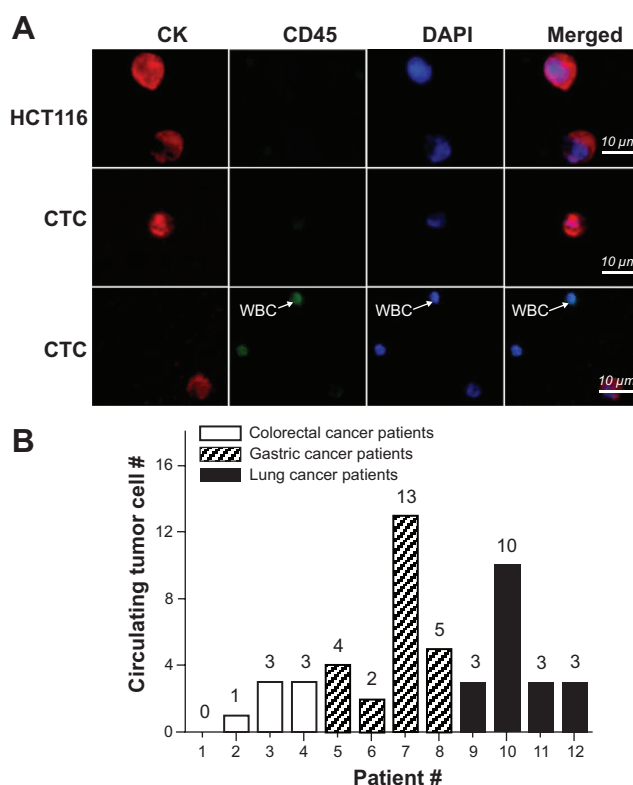


Figure 6 (A) Fluorescent micrographs of CTCs captured from whole blood samples collected from cancer patients. The three-color immunocytochemistry method was used to identify and enumerate CTCs on anti-EpCAM-grafted HA/CTS NF substrate from non-specifically captured WBCs, including FITC-labeled anti-CD45 and PE-labeled anti-CK as well as DAPI nuclear staining. The combined information was used to identify CTCs from WBCs. **(B)** CTC enumeration results obtained from cancer patients (colorectal cancer, gastric cancer and lung cancer).

Abbreviations: CTCs, circulating tumor cells; HA/CTS NF, hydroxyapatite/chitosan nanofilm; FITC, fluorescein isothiocyanate; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; WBCs, white blood cells.

counts ranging from 0–13 per mL. The anti-EpCAM modified HA/CTS NF substrate therefore exhibited considerable CTC capture efficiency.

Conclusion

We have achieved precise control of surface roughness in an HA/CTS NF deposited onto a glass slide to achieve enhanced topographic interactions with CTC surface components. The epithelial-specific capture agent, anti-EpCAM,³⁰ was conjugated onto the surface of the nanosubstrate to enable specific capture of CTCs. HA/CTS NF thus constructed exhibits CTC capture performance equal to that of horizontally packed TiO_2 nanofibers, as well as excellent biocompatibility. In addition, the transparent nature of the HA/CTS NF makes direct observation of the cells possible. These advantageous characteristics enable CTC culture *in situ* and traditional dyeing, as well as accurate positioning of the immobilized cells for subsequent isolation and purification.

Table I Cancer patient CTC counts observed on HA/CTS NF substrates, standard TNM cancer staging; metastatic versus localized

Cancer species	Patient	CTC count (sample =1 mL)	TNM	Metastatic
Colorectal cancer	1	0	T3N2M0	–
	2	1	T3N0M0	–
	3	3	T4N0M0	–
	4	3	T2N0M0	–
Gastric cancer	5	4	T4N3M0	–
	6	2	T1N0M0	–
	7	13	T4N2M1	+
	8	5	T4NXM1	+
Lung cancer	9	3	T1N0M0	–
	10	10	T1N0M0	–
	11	3	T1N1M0	–
	12	3	T2bNXM0	–

Abbreviations: CTC, circulating tumor cell; HA/CTS NF, hydroxyapatite/chitosan nanofilm; TNM, TNM Classification of Malignant Tumours.

CTCs captured from artificial blood samples could be cultured and expanded on HA/CTS NF substrates that represent a multifunctional material capable of CTC isolation and culture, and permitting their immunocytochemical and molecular analysis for clinical applications (monitoring treatment responses and indicating patient prognosis). We anticipate this platform will also be applied to the capture and in situ culture of CTCs for studying drug resistance mechanisms, tumor heterogeneity, and tumor evolution.

Acknowledgment

The work was supported by the National High Technology Research and Development Program of China (grant number 2012AA02A502, 2012AA02A506).

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

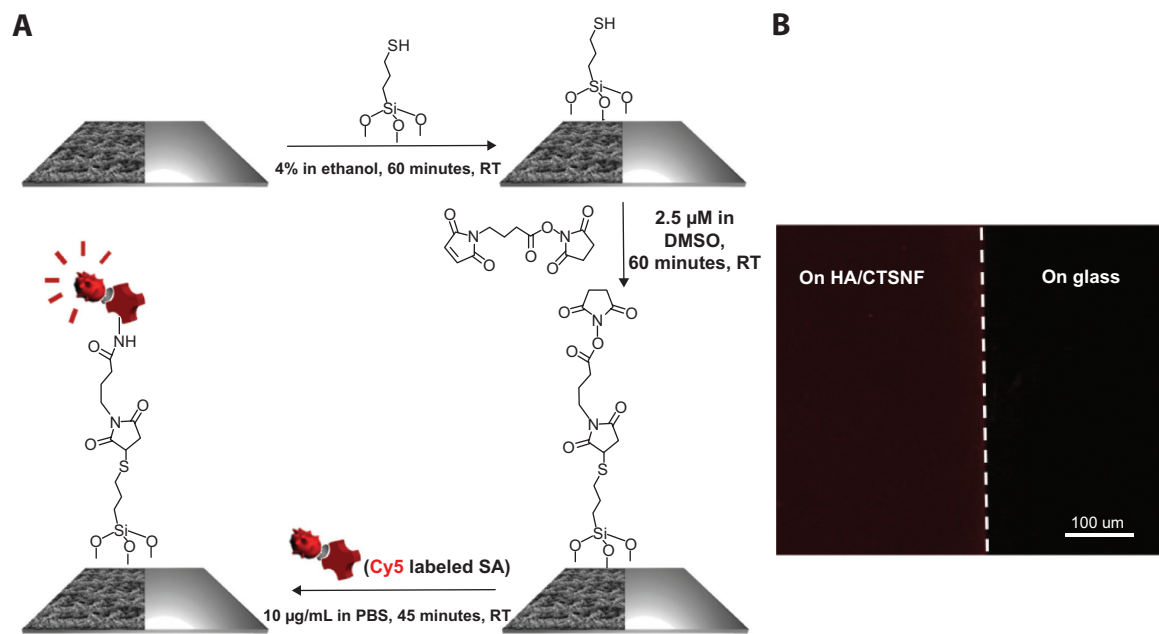


Figure S1 Surface modification with streptavidin (SA). **(A)** Surface modification procedure of the hydroxyapatite/chitosan nanofilm (HA/CTS NF) substrate with SA. **(B)** Fluorescence micrograph of HA/CTS NF substrate (left) and glass (right) coated with Cy5-SA (red).
Abbreviations: DMSO, dimethyl sulfoxide; RT, room temperature; PBS, phosphate buffered saline.

Capture efficiencies of the EpCAM-coated HA/CTS NF substrates

Table S1 Cell capture efficiencies of the optimal capture conditions (HA/CTS NF substrate with HA/CTS 5:2 weight % composite, baking temperature of 100°C, and 60 minutes cell incubation time) were validated using the HA/CTS NF substrates

Cell number/mL	Capture yield		Successful detections out of total trials
	1 mL DMEM containing HCT116 cells	1 mL human blood containing HCT116 cells	
50	85±12%	67±21%	3/3
100	88±20%	66±11%	3/3
250	84±13%	66±15%	3/3
500	85±10%	72±18%	3/3
1,000	86±7%	64±4%	3/3

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; HA/CTS NF, hydroxyapatite/chitosan nanofilm.

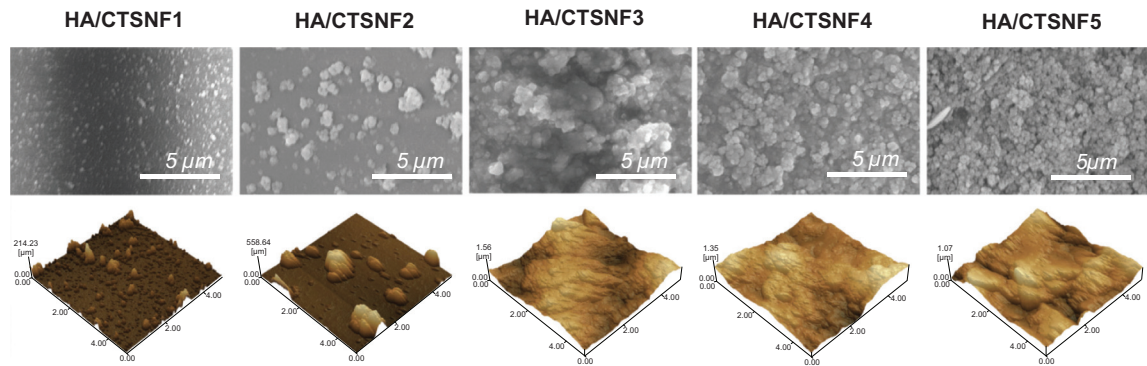


Figure S2 SEM and AFM images of HA/CTS NF substrates with different HA/CTS ratios.
Abbreviations: SEM, scanning electron microscopy; AFM, atomic force microscopy; HA/CTS NF, hydroxyapatite/chitosan nanofilm.

Preparation and characterization of hydroxyapatite/chitosan (HA/CTS) nanofilm substrates

Table S2 Concentrations, roughness, and captured cell numbers of HA/CTS nanofilms (HA/CTS NF1 to HA/CTS NF5)

Sample	Composites (weight %)	Square average roughness (nm)	Captured cell numbers/cm ²
HA/CTS NF1	HA/CTS 1.25:2	20.136	2,833
HA/CTS NF2	HA/CTS 2.5:2	90.863	31,905
HA/CTS NF3	HA/CTS 5:2	176.826	49,960
HA/CTS NF4	HA/CTS 10:2	238.883	46,200
HA/CTS NF5	HA/CTS 15:2	281.266	45,366

Abbreviation: HA/CTS, hydroxyapatite/chitosan.

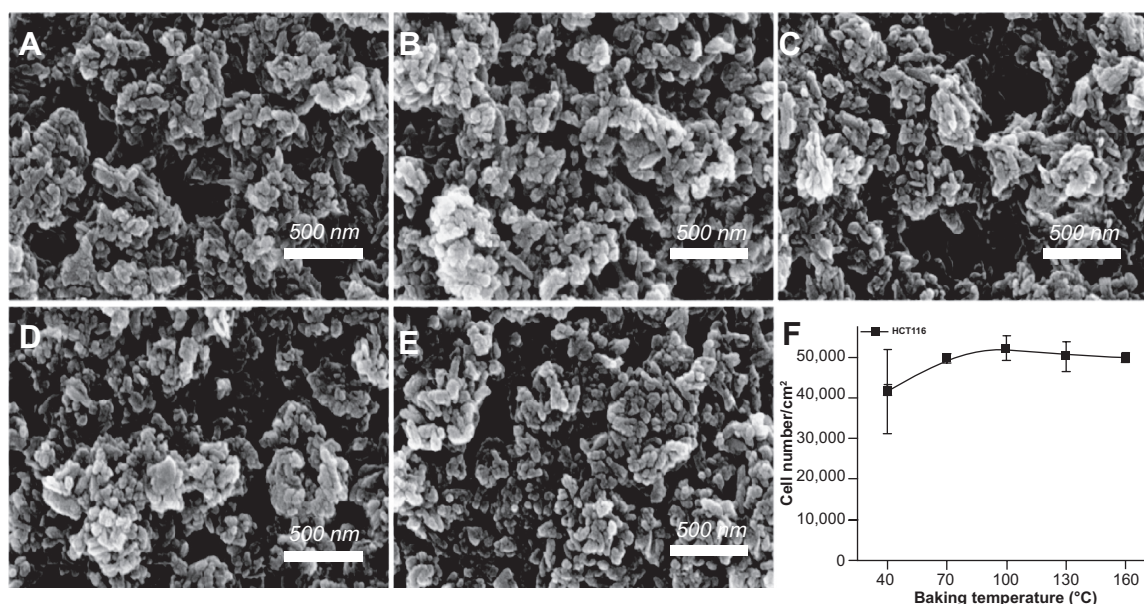


Figure S3 SEM images of HA/CTS NF substrates with different baking temperatures.

Notes: (A) 40°C, (B) 70°C, (C) 100°C, (D) 130°C, and (E) 160°C. (F) Plotted number of cells captured on HA/CTS NF substrates with different baking temperatures. No differences were observed in captured cell numbers when the baking temperature was higher than 70°C for HCT-116 cancer cells.

Abbreviations: SEM, scanning electron microscopy; HA/CTS NF, hydroxyapatite/chitosan nanofilm.

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