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A comparative study on EpCAM antibody immobilization on gold surfaces and microfluidic channels for the detection of circulating tumor cells

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ABSTRACT

Detection of circulating tumor cells (CTCs) from the bloodstream holds great importance to diagnose cancer at early stages. However, CTCs being extremely rare in blood makes them difficult to reach. In this paper, we introduced different surface modification techniques for the enrichment and detection of MCF-7 in microfluidic biosensor applications using gold surface and EpCAM antibody. Mainly, two different mechanisms were employed to immobilize the antibodies; covalent bonding and bioaffinity interaction. Self-assembled monolayers (SAMs) formed on the gold surfaces were treated further for the immobilization of the antibody. The bioaffinity-based studies were performed with streptavidin and biotinylated EpCAM over the SAM coated surfaces. The cell attachment events were monitored using fluorescent microscope. Comparisons were made considering the length and functional end of alkanethiols and the positioning of the antibody. Then, these methods were integrated into a microfluidic channel system. Surface characterizations were performed with X-ray Photoelectron Spectroscopy, Atomic Force Microscopy, and contact angle measurements. The selectivity studies were carried out with EpCAM negative K562 leukaemia cell lines and the experiments were repeated for different types of surfaces, such as glass and polymer. Studies showed that long ($n > 10$) and aromatic ring containing alkanethiols lead to better cell capture events compared to shorter ones. Results obtained from the comparisons are of importance for the gold surface-based microfluidic biosensor designs aimed for CTC detection.

1. Introduction

Cancer is the second leading cause of death worldwide and 90 % of cases are due to the metastasis of cancer [1]. Metastasis occurs when the tumor reaches a certain size and starts to secrete chemicals to hijack the signals that cause new blood vessels to grow towards itself. Once the tumor is connected to a bloodstream, it disseminates cells which often travel to other parts of the body through blood circulation. In many cancer patients, there exists circulating tumor cells (CTCs) which are the result of the primary tumor and travel through the circulatory system. Some of these cells are ultimately capable of forming distant metastases. The detection and quantification of these cells from blood, especially during the initial stages, is quite important for the early diagnosis and accordingly for the treatment of the patient and diseased organs.

The major challenge in the detection and enumeration of the CTCs is their extreme rarity compared to the number of hematopoietic cells present in the blood sample [2]. There are few million leukocytes and a billion red blood cells present in one ml of blood, whereas, CTCs are found in the order of 1–10 cell/ml in patients with metastatic cancer [3].

One approach for the detection of CTCs is to use surface markers on their membranes and target them by specific antibodies. This kind of isolation offers very high specificity and one of the most common surface markers is Epithelial Cellular Adhesion Molecule (EpCAM). This transmembrane protein is highly expressed on a variety of carcinomas such as breast, lung, brain, colon etc. [4]. Moreover, the expression of EpCAM is restricted in healthy epithelial cells which make it particular interest for tumour diagnosis and therapy. Correspondingly, the only FDA approved CTC detection system CellSearch® depends on EpCAM specific cell capturing strategy for evaluating the diagnosis and prognostic effect of metastatic cancer patients.

With the help of the growing demand for efficient and fast automated systems, microfluidic systems have evolved enormously. The

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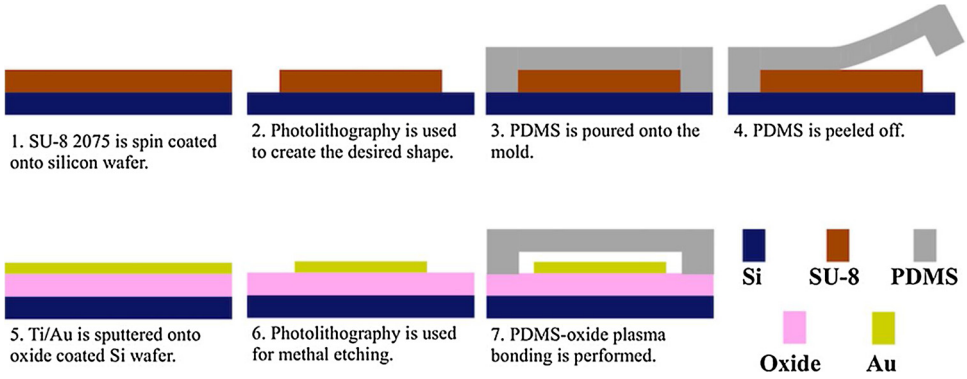


Scheme 1. Fabrication flow of PDMS-oxide based channel device. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

latest advancements and progresses enabled microfluidic devices to be developed for liquid biopsy, including the CTC detection. Studies with microfluidic sensors showed that such systems can detect cancer biomarkers with high sensitivity [5,6]. The employment of such platform provides various advantages over conventional techniques such as eliminating the need for large sample volumes, having tuneable flow conditions, allowing multiplexing detections, etc. Most importantly, the design of these devices can be controlled to provide precision for liquid manipulation [7]. Therefore, studies and optimizations with microfluidics are of importance for the detection of low-abundance biomolecules.

Modifying the chemical and topographical properties of a surface to immobilize antibodies enables CTC capturing in various sensing systems including the aforementioned microfluidic systems. Therefore, the biosensor development strongly depends on the optimization of the surface functionalization. Self-Assembled Monolayers (SAMs) is one of the most preferred approaches in literature [8]. In the SAM approach, ordered molecular structures are formed spontaneously by the chemisorption of the head group with a specific affinity to a substrate [9,10]. The terminal functional group of the molecule can be used to anchor different biomolecules by weak interactions, covalent bonds, etc. A well-known phenomenon to employ is the strong gold-thiol interaction when using gold surfaces [11]. Using molecules with thiol ends enable them to be attached on gold surfaces without having to perform any additional step. In this sense, cysteamine and cystamine are amongst very commonly used molecules [12–14]. SAM molecules can also be mixed, as previously done by Cancino and Machado [15], however, the idea of making these types of combinations was introduced to the literature long ago by Whitesides et al. [16,17]. One crucial thing to consider, either mixing the alkanethiols or not, is that the efficiency of a sensor system highly depends on their physical and chemical characteristics [18].

In this study, SAMs of various molecules with different functional groups, chain lengths and shapes were formed on plain gold surfaces and investigated to achieve specific and maximum antibody-CTC interaction. EpCAM antibodies were immobilized on the SAM formed surfaces employing both covalent bonding through carbodiimide crosslinking method with EDC and NHS and bioaffinity based immobilization using streptavidin and biotinylated EpCAM. After incubating the prepared surfaces with MCF-7 breast cancer cells, K562 leukaemia cells and WBCs, surfaces were examined with a fluorescent microscope and comparisons were made in terms of the number of captured cells. Next, these strategies were implemented to the gold microfluidic channels produced. To the best of our knowledge, there is no systematic study for the comparison of CTC capturing efficiencies concerning SAM molecules with different physical properties under continuous flow in microfluidic channels. Although there exist studies with such comparisons, their implementation to the microfluidic channels may shed light to various other applications. Results obtained from the comparisons are crucial for the improvement of antibody immobilization techniques that can be used for CTC capture in microfluidic biosensor designs.

2. Materials and methods

2.1. Materials

Plain gold (1,1,1) surfaces and microfluidic gold channels were fabricated in METU MEMS Center (Ankara, Turkey). Both the CTC capture experiments and surface characterizations were carried out with gold surfaces (1 cm x 1 cm) coated on silicon with 30 nm thick titanium and 300 nm gold layers. For microfluidic channel tests, 650 μm Si wafer was coated with 500 μm oxide, 20 μm Ti and 200 μm Au according to the gold channel patterned mask design. SU-8 2075 was spin coated over silicon wafer patterned with soft lithography and used as a master mold. PDMS was poured over the mold and cured at 70 °C for 2 h and peeled off. PDMS and oxide layer bonding was performed with plasma oxidation at 15 W for 20 s to obtain the final microfluidic channel device (Scheme 1).

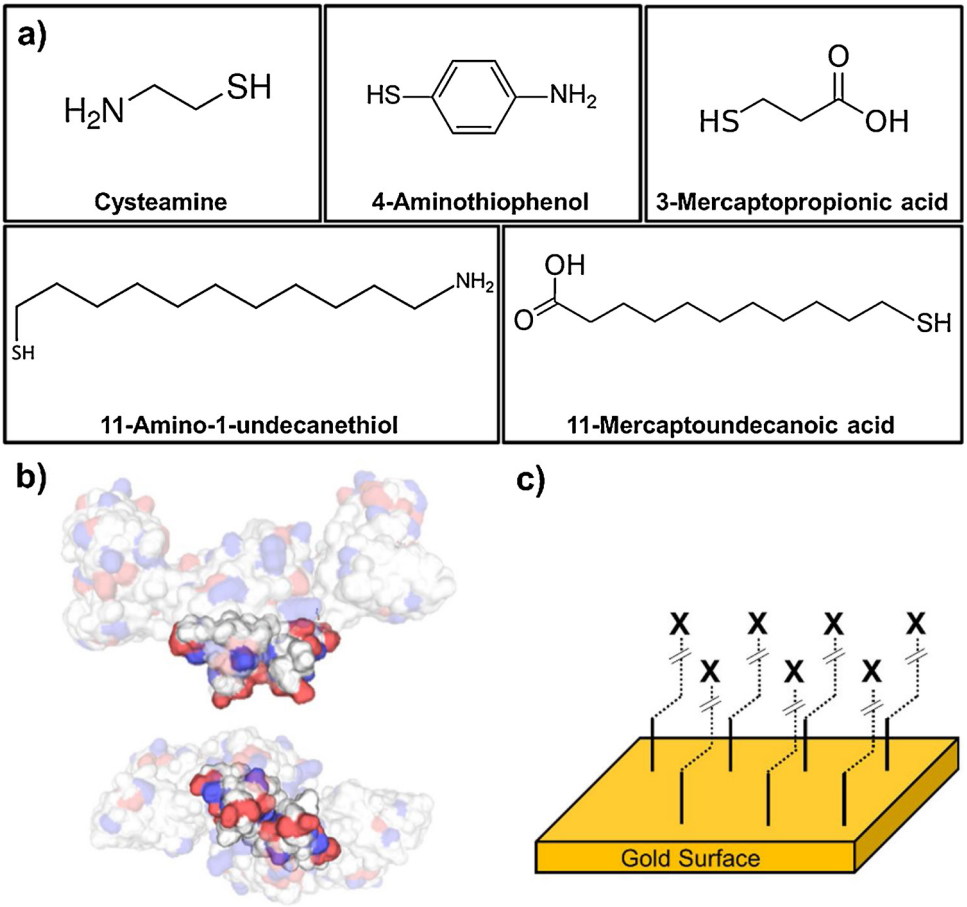
11-Mercaptoundecanoic acid (11-MUA) (%98), 11-Amino-1-undecanethiol hydrochloride (11-AUT) (99 %), 4-Aminothiophenol (4-ATP) (97 %), Cysteamine (Cys) (95 %), 3-Mercaptopropionic acid (3-MPA) (≥99 %), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC)(≥98.0 %), N-Hydroxysuccinimide (NHS) (98 %), Bovine Serum Albumin (BSA)(≥98 %), Ethylenediamine (≥99 %), Streptavidin from streptomycesavidinii, Phosphate buffered saline (PBS, 10× concentrate), MES monohydrate (≥%99,5), Fluorescein diacetate (FDA), were purchased from Sigma-Aldrich. The anti-EpCAM and Biotinylated anti-EpCAM was purchased from Abcam, UK.

2.2. The CTC model

For the CTC model, MCF-7 breast cancer cell line was selected due to its relatively high EpCAM expression level and K562 leukaemia cell line was used as the EpCAM negative model. Cells were purchased from Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ) and cultured in Mikro Biyosistemler Inc. The cells were cultured in RPMI 1640 medium and stained with fluorescein diacetate according to the manufacturer's instructions for the fluorescence microscope imaging.

2.3. Self-Assembled Monolayer (SAM) formation on the gold surfaces

All bare gold surfaces were first cleaned with piranha solution; a mixture of concentrated sulfuric acid (H_2SO_4) and 30 % hydrogen peroxide (H_2O_2) (3:1, v/v). Then, they were rinsed with deionized water, followed by ethanol and dried with nitrogen gas. For the microfluidic studies, the channel surface was cleaned with ethanol with



Scheme 2. a) Chemical structures of SAM molecules used in this study, b) crystal structure of EpCAM where reds indicate negative and blues indicate positively charged sections (obtained from swissmodel.expasy.org) c) representative illustration for bare gold and SAM formation (X = amine of carboxyl free end).

5.5 ml/h flow rate. Besides the cleaning process, all the channel experiment steps were performed under 0.5 ml/h flow rate.

Once the surfaces were cleaned, they were soaked in freshly prepared alkanethiol solutions in ethanol at 5 mM concentrations and incubated overnight (~16 h) at room temperature for SAM formation. Then, they were rinsed with ethanol to remove the feebly bound molecules. The molecules used for SAM formation and their free functional groups are provided in Scheme 2. Cys, 4-ATP and 11-AUT have amine (NH_2) functional groups and 3-MPA and 11-MUA have carboxyl (COOH) functional groups.

2.4. EpCAM antibody immobilization of the SAM formed gold surfaces

Each protocol explained under this section includes detailed information regarding the immobilization steps (Scheme 2). To get the highest efficiency from an antibody the paratope, which is the antigen-binding site, should be freely available on the surface to enable the epitope of the antigen to bind. If this orientation can be controlled, better analyte binding will improve the sensitivity of the sensor. It is, on the other hand, not possible to make a direct conclusion about the positioning of the antibody. In order to make sure of the orientation of the antibody, site-specific chemistries such as periodate oxidation of antibody glycan or surfaces exhibiting orientation-prone molecular features such as protein A, protein G, anti Fc antibodies, various recombinant proteins etc. should be employed. Although these techniques would allow for better positioning, the surface properties of the antibodies may still contain fraction of binding sites that are not available for antigen binding. The positioning phenomena has been investigated and examined in many studies and review articles [19–22], especially in depth by O'Kennedy et al. [19] and it was concluded that the immobilization of the antibody through non-antigen binding regions, such as the Fc region, maximizes the functionality and allows a rather correct orientation. Although orientation specific immobilization techniques include the usage of specific proteins etc. it is also possible to do it in a simpler fashion by adjusting the pKa of the surface and pH of the buffer solution [21]. These phenomena are explained in detail in the Results section. In any case, antibodies tend to lose their binding capacity when they are immobilized on a surface, whether chemically or through adsorption, due to blockage of binding sites resulting from proximity effects or inaccessibility, incorrect orientation, etc. [23–25]. Even though it is rather risky in terms of orientation, covalent attachment of antibodies is employed widely and is believed to improve the performance. It improves the stability which accordingly promotes the overall performance of the system [26]. Biotinylation of antibodies and immobilization through avidin or streptavidin have also been employed in various studies, yet the risk of non-efficient binding still remains as long as there isn't a non-directed chemical linkage [27]. In this study instead of orientation specific immobilization techniques the effects of rather simpler and direct methods were investigated. In this sense, the surfaces were coated both with amine and carboxyl terminated SAMs and the effect of functional groups were investigated as well as the length of the SAM and inclusion of aromatic rings. A comparison was made also between covalent approaches and bioaffinity interaction using biotin and streptavidin. Finally, and most importantly, the methods worked best were applied to the microfluidic channels to observe the cell binding effects under flow.

Protocol 1. In order to activate carboxyl ended SAM surfaces, a solution of 10 mM NHS and 10 mM EDC were prepared in 0.1 M MES

buffer at pH 5.0. Surfaces were incubated in this solution for 45 min at room temperature. Then, the surfaces were rinsed with PBS solution and incubated with 10 μg/ml anti-EpCAM antibodies in PBS for 2 h at room temperature for covalent binding of anti-EpCAM antibodies onto the SAM modified surface. The gold surfaces then rinsed with PBS and incubated with 1 % BSA for 15 min at room temperature to block the free carboxyl ends without any antibodies attached.

Protocol 2. In order to activate carboxyl end of the antibodies, a solution of 10 mM NHS and 10 mM EDC were prepared in 0.1 M MES Buffer at pH 5.0 and mixed with 10 μg/ml anti-EpCAM antibodies in PBS solution (1:1, v/v). This solution was incubated for 45 min. Then, it was poured on the gold surfaces and immobilized 2 h at room temperature for covalent binding of EpCAM antibodies onto the SAM modified surface. The gold surfaces were rinsed with PBS and incubated with 1 % BSA for 15 min at room temperature free amine ends without any antibodies attached.

Protocol 3. For the bioaffinity method, carboxyl ended SAM surfaces were activated with the solution of 10 mM NHS and 10 mM EDC which prepared in 0.1 M MES buffer at pH 5.0. Then, the surfaces were treated with streptavidin solution (10 μg/ml) and incubated for 45 min at room temperature. Then, 10 μg/ml biotinylated EpCAM antibodies in PBS were incubated on the surface for 2 h at room temperature. The gold surfaces were rinsed with PBS and incubated with 1 % BSA for 15 min at room temperature to block nonspecific sites.

Protocol 4. In this method, antibody and selected alkanethiols were exposed to each other in an external environment and then incubated with the cleaned gold surface. Although there exist studies were this technique was employed, in this case, no successful result was obtained [28].

2.5. Characterization of the surfaces

X-Ray Photoelectron Spectroscopy (XPS): XPS measurements were performed with PHI 5000 Versa Probe by Physical Electronics in METU Central Laboratory. Before taking XPS measurements, the surfaces were rinsed with PBS buffer and distilled water dried under nitrogen stream. AugerScan program was used for the determination of relative atomic concentration by subtracted spectrum backgrounds with Shirley method. The neutral (C-C) carbon C1s peak at 284.6 eV was used as the charge reference for the XPS spectra.

Contact Angle Measurement: Surface contact angle measurements were taken for each of the functionalized surfaces with Attension Theta by Biolin Scientific in METU Central Laboratory. Surfaces were rinsed with PBS buffer and distilled water before the analysis and measurements were taken using the sessile drop method. For each surface, the contact angle was measured at five different locations and the arithmetic average was taken.

Atomic Force Microscopy: AFM characterization was performed to obtain information regarding the surface roughness and thickness after each functionalization step. Images were taken with VeecoMultiMode V, from Veeco Instruments Inc., in METU Central Laboratory in dry state. Surfaces were rinsed with PBS buffer and distilled water and dried with nitrogen gas. The roughness and root mean square values were calculated using the Gwyddion program.

2.6. CTC capture studies

Once the antibodies were immobilized on the surfaces either through covalent binding or bioaffinity interaction, the CTC capture studies were performed. To image and track this process, a fully automated bright-field microscope was used (Olympus SZX12, Olympus Inc.,) To be able to see the cells, they were previously stained with fluorescein diacetate; capturing images of fluorescent signals emitted by fluorescently labelled cells immobilized on the surfaces was therefore made possible. The cells removed from the flask were prepared in RPMI 1640 and the total amount was determined using the cell counter Bio-Rad TC20. The cells were washed by centrifuging and re-suspending in PBS buffer for three times. The final concentration was determined as 5×10^5 cells/ml.

The images recorded in each experiment were analysed using Fiji software program and the mean values of the number of cells captured were calculated from 12 frames by repeating the same experiment for 3 times and counting 4 different frames from each experiment.

2.7. Real blood studies

Blood samples were taken in tubes containing anticoagulant ethylenediamine tetra acetic acid (EDTA) from healthy donors as approved by the Institutional Review Board of METU Medical Center with the informed consent from all donors. Whole blood was immediately processed and lysed with red blood cell lysis solution (10x, MACS, MiltenyiBiotec) according to the manufacturer's instructions. The remaining white blood cells were then precipitated by centrifugation and washed with PBS Solution.

3. Results

Different strategies were performed to immobilize antibodies on the SAM formed surfaces in order to find the optimum surface modification strategy. These strategies were namely the carbodiimide crosslinking with EDC/NHS and the bioaffinity interaction using biotin and streptavidin. The studies were performed both on gold surfaces and in microfluidic channels produced in the clean room (Figure S1).

3.1. Characterization of the surfaces

Atomic Force Microscopy (AFM) imaging was performed to visualize the surface topography of the SAM formed and antibody attached surfaces (Supplementary Info). The root mean square (RMS) roughness values were found to be 0.50 ± 0.06 nm, 0.93 ± 0.02 nm and 0.48 ± 0.05 nm for SAMs created with 11-AUT, 11-MUA and 4-ATP, respectively. These RMS roughness values increased to 1.21 ± 0.03 nm, 2.38 ± 0.02 nm and 10.72 ± 0.06 nm, respectively, after the antibodies were immobilized on the surfaces (Figure S8). This increase ensures that antibodies were successfully bound to the free ends of alkanethiols.

EpCAM antibody attached aromatic hydrocarbon (4-ATP) causes more RMS roughness than other brush-like linear alkanethiols because of the benzene ring in its structure. The structural difference between 11-AUT and 11-MUA can lead to distinctive results since the presence of the amine functional group influences the SAM due to the hydrogen bonding between amino groups [29].

To investigate the elemental composition of each surface modification strategy, X-RAY Photoelectron Spectroscopy (XPS) measurements were performed and the intensities of the atomic concentrations were determined by numerical integration of the relative peaks. The formation of the SAMs using different alkanethiols was confirmed by tracking the changes of the C1s, N1s and O1 values. Because 11-MUA does not contain any nitrogen, no N1s peak was observed, which is the only case where no nitrogen was present prior to antibody immobilization. The presence of nitrogen confirms the existence of antibody [22]. Table 1 shows the elemental compositions obtained after each SAM and EpCAM immobilization steps. It should be noted that previously published studies showed that the percentage of N is always smaller than that of O and C in the XPS results, which is in good agreement with our findings. For instance, in a study by Myung et al., the XPS results for EpCAM immobilized surfaces yielded 26.2 % C1s, 39.2 % O1s and only 2.6 % N1s [30]. This indicated an N/C ratio of 0.1 and a C/O ratio of 0.7. Obtained spectra are provided in Figures S9-S15.

Changes in the surface free energies after the SAM formation of alkanethiols and the immobilization of EpCAM antibody were investigated with contact angle measurements. The water contact angle on the bare gold surface was measured as 45°. When SAM of 11-MUA

Table 1
XPS measurement results of the elemental compositions for each surface modification.

Elemental Compositions	Au4f	C1s	O1	N1s
4-ATP	50.1	43.6	< 0.1	6.3
4-ATP + EpCAM	26.6	59.3	4.2	9.9
11-AUT	22.8	62.6	4	10.6
11-AUT + EpCAM	24.5	64.4	2.8	8.4
11-MUA	20.3	72.5	7.2	—
11-MUA + EpCAM	2.4	46.7	19.5	5.1
11-MUA + Strep + B.EpCAM	9.8	57.7	21.6	10.9

was formed, this angle decreased down to 41° ± 2. After antibodies were immobilized on 11-MUA formed surfaces, the angle increased to 43° ± 2 but when streptavidin and biotinylated antibody was used, the surface became more hydrophilic and the angle was measured as 41° because of polar ureido-tetrahydrothiophene rings of biotin [31]. In the cases of amine terminated alkanethiol, the angle was increased with the formation of SAMs because of the creation of hydrophobic groups on the surface. Namely, for 11-AUT and 4-ATP, the angles were measured as 53° ± 1 and 52° ± 2, respectively. When the EpCAM antibodies were immobilized, the angles further increased for both of the surfaces functionalized with 11-AUT and 4-ATP and became 51° ± 4 and 53° ± 3, respectively. So, when carboxyl groups were created on the surface, the hydrophilic character was increased. This increase is in good agreement with the literature [11,32,33].

3.2. Fluorescent microscope images of CTC capture

Human EpCAM contain 314 amino acids, of which 242 are in the extracellular domain [34], but the corresponding highly EpCAM specific monoclonal antibody, clone VU-1D9 is unknown. Some residues can be buried in the tertiary structure, covered by the side chains whereas some can be homogeneously distributed outer surface of the antibody [35]. Carboxylic acid residues exist on the outer surface of the antibody and are ready to go through amidation reactions with external amine groups [36]. An immobilized antibody can exist in four possible positions; side-, tail-, head- or flat-on. It is also shown in many studies that covalent immobilization provides longevity and high sensitivity [21]. The classical and less sophisticated covalent approaches include the employment of amine groups present on the lysine amino acid side-chain of the antibody, which results in a random positioning [22]. They can also be immobilized through carboxyl groups present on the aspartate and glutamate amino acids [37,38]. Antibodies experience a physisorption step prior to covalent binding, at which state the pKa of the surface and pH of the buffer solution become rather crucial. Therefore, it might be possible to favour a specific position by properly selecting the right conditions [21]. A study on the effect of pH on the antibody immobilization and orientation showed that in case of using carboxyl functionalized surface, below pH 5.0 the surface attraction is significantly reduced and at lower values the negative surface charge can be maintained. The study concluded that the electrostatic interaction was necessary for successful antibody immobilization on 2D carboxyl surface and that using EDC/NHS or EDC/sulfo-NHS yielded much better activation results compared to only EDC [39]. It was also shown in another study that electrostatic repulsion between antibody and polystyrene microparticles at basic pH inhibited the access of the antibody to the microparticle's surface and promoted the antigen-accessible orientation which resulted in high antigen recognition capacity [40]. These findings strengthened the possibility that the adjustment of the pH at which the reaction takes place might to some extend help providing a preferred orientation to the antibody.

Before the characterization of the SAM formed surfaces, optical imaging was performed with a fluorescent microscope to eliminate the strategies for further analysis. SAM was formed on bare gold surfaces using different molecules and EpCAM antibody was immobilized covalently employing carbodiimide crosslinking process. The experiments were carried out firstly with carboxyl group terminated 3-MPA and 11-MUA which were selected as models to determine the optimum concentration for the maximum cell adhesion on surfaces prepared with alkanethiols of different lengths. Incubation periods were selected as 2, 7, 16, 24 and 48 h but no significant difference was observed from the cell attachment assays after 16 h, which in agreement with a previous study by Asiaei et al. [41]. Concentration tests were also performed for EDC/NHS at different MES buffer pH values (4.5, 5 and 5.5) and optimum values were used in the experiments. Amine functional groups were formed on the surface when Cys, 4-ATP and 11-AUT were used to create SAM. Fig. 1a, b and c show the cells attached when amine terminated end SAM was formed.

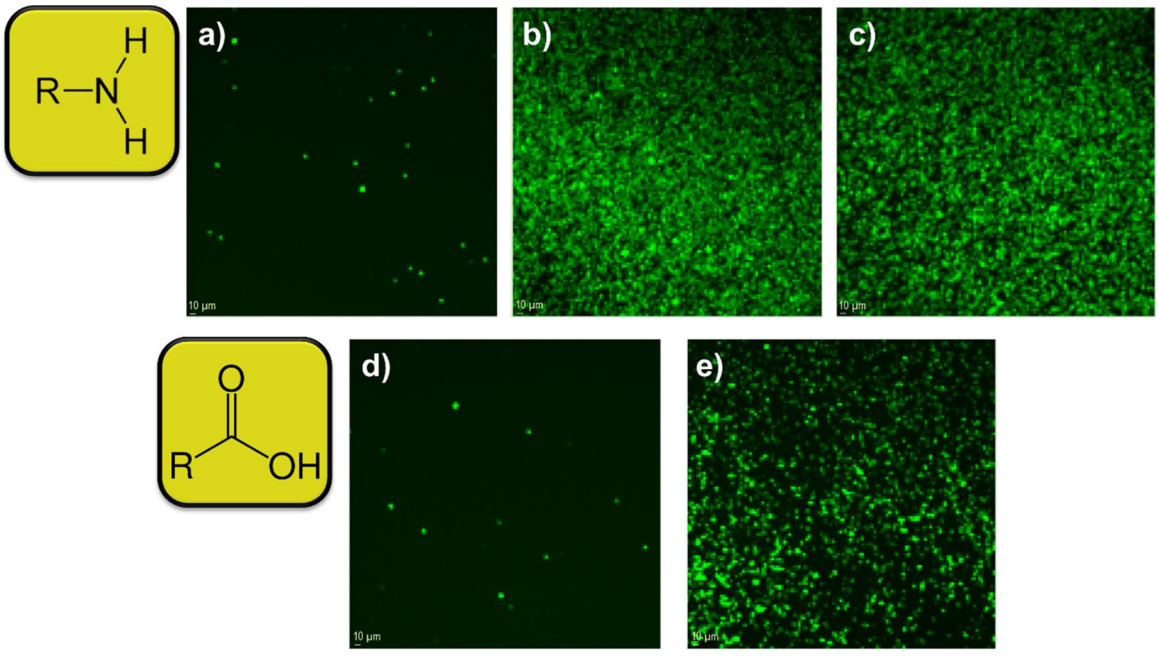


Fig. 1. The fluorescent microscope images of attached CTCs on surfaces treated with a) Cys, b) 4-ATP and c) 11-AUT, d) 3-MPA and e) 11-MUA to create SAM.

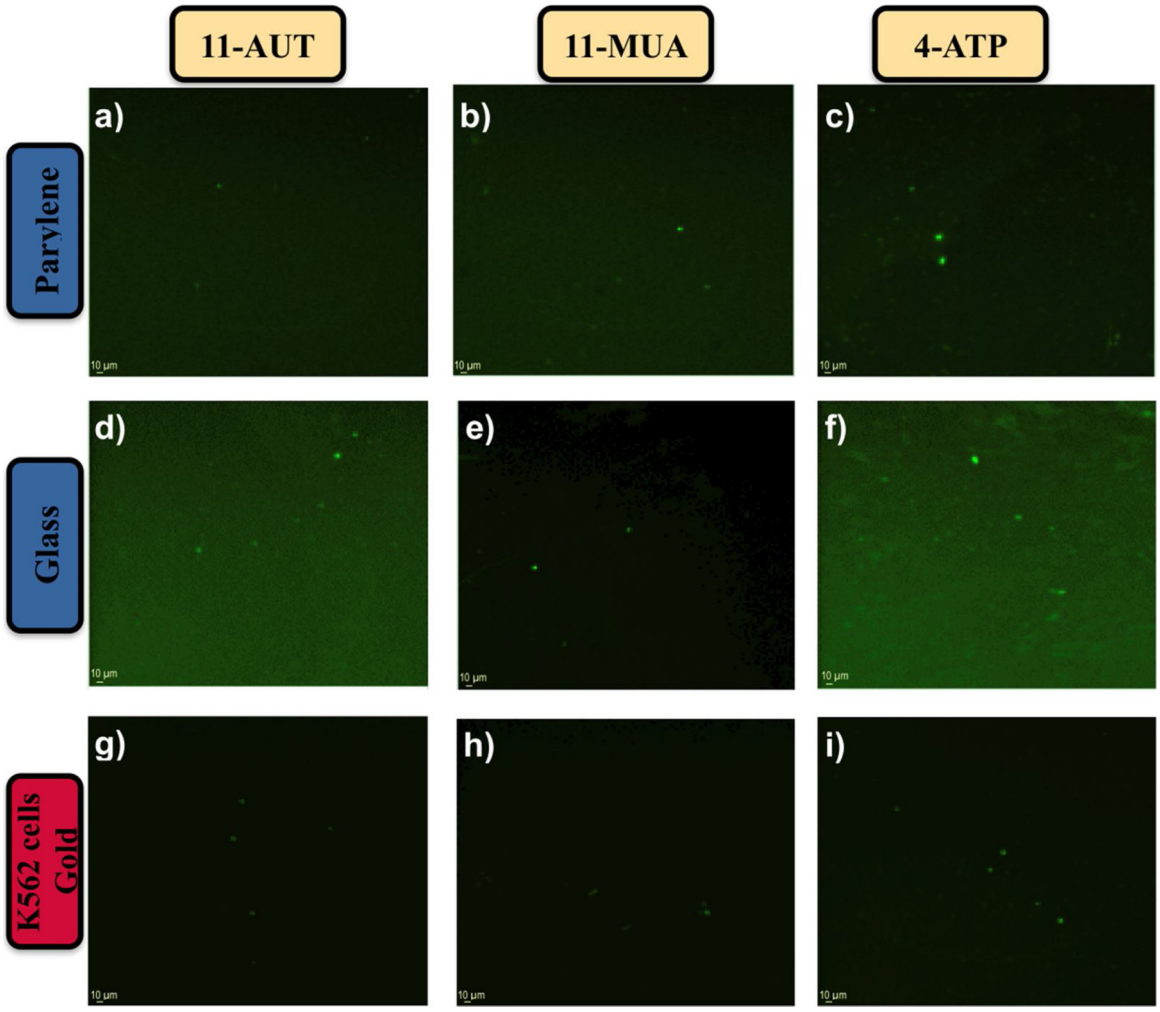


Fig. 2. The fluorescent microscope images of attached CTCs on parylene surfaces where SAM of a) 11-AUT, b) 11-MUA and c) 4-ATP were created, and on glass surfaces where SAM of d) 11-AUT, e) 11-MUA and f) 4-ATP were created and attached K562 leukaemia cells on gold surfaces treated with g) 11-AUT, h) 11-MUA and i) 4-ATP.

The results were found as 618 ± 21.5 cell/frame for Cys, 1974 ± 95 cell/frame for 4-ATP and 1937 ± 165 cell/frame for 11-AUT (Fig. 1a-c, Figure S3). Both 4-ATP and 11-AUT SAMs resulted in a large amount of CTC capture whereas in the case of Cys there was less number of cells attached. The reason for this insufficient capture is believed to be due to the steric hindrance effect. As Cys molecule is a very short molecule consisting of two carbon atoms, it both limits the immobilized antibody amount and accordingly the captured CTC amount. Because it is a short-chain alkanethiol, molecules stay quite close to one another and form a dense structure. This phenomenon was also mentioned in several articles published earlier [13,42,43]. Long and short chain molecules are usually mixed to eliminate this effect [7,44]. As a result of this, steric hindrance is minimized because the anchor molecules became more available [8]. On the other hand, the CTC capture in the other two cases, especially in 4-ATP, were quite high. Because 4-ATP molecule contains a phenol ring, the activated sites of the molecule stay far away from each other compared to the distance that occurs in the case of Cys. Also, it was shown that aromatic ring containing thiols permit high ordering via π-π interactions, which also increase the stability and rigidity of the SAM compared to alkyl based ones [45]. This prevents the formation of a heterogeneous surface and leads to maximum CTC capture amongst three amine terminated molecules. 11-AUT also yielded a high amount of CTC capture because long-chained alkanethiols create strong Van der Waals forces between their chains and causes to dense and well-ordered brush-like structures. It was also proved in a study that the binding kinetics was faster on SAM created with long-chain molecules compared to the short-chain ones. The short-chain SAMs were resulted in increased packing density [11]. The same behaviour was observed when CTC capture was compared for the 3-MPA and 11-MUA treated surfaces. Fig. 1d and e show the cells attached when carboxyl terminated SAM was formed. The captured cell amount was found as 153 ± 29 cell/frame for 3-MPA and as 1851 ± 115 cell/frame for 11-MUA (Figure S3). Similarly, in the case of 3-MPA because it is a short-chained molecule, extremely poor CTC capture was observed. Another crucial point here is that when 11-MUA and 11-AUT were compared, 11-AUT resulted in better capture (1851 ± 115 and 1937 ± 165 cell/frame, respectively). When a surface is functionalized with amine groups, it becomes positively charged, lacks hydrophobicity and would be ionic in nature. Such a surface can be used to couple negatively charged biomolecules ionically. Because there would be no hydrophobic areas on the surface, the immobilization of hydrophobic molecules would be precluded. It should, therefore, be used with bifunctional crosslinkers, such as carbodiimide used in this study, to couple functional groups. The pH of the buffer solution is crucial during this coupling step. The charged state of both amine and carboxyl groups existing on the amino acids on the antibody depend on this pH value. When the pH of the solution is greater than the pKa of the functional groups, that group becomes protonated and vice versa. Since the pH of the buffer solution was 4.8–5.0 in this study, the amine groups created on the surface become protonated (NH_3^+). This way,

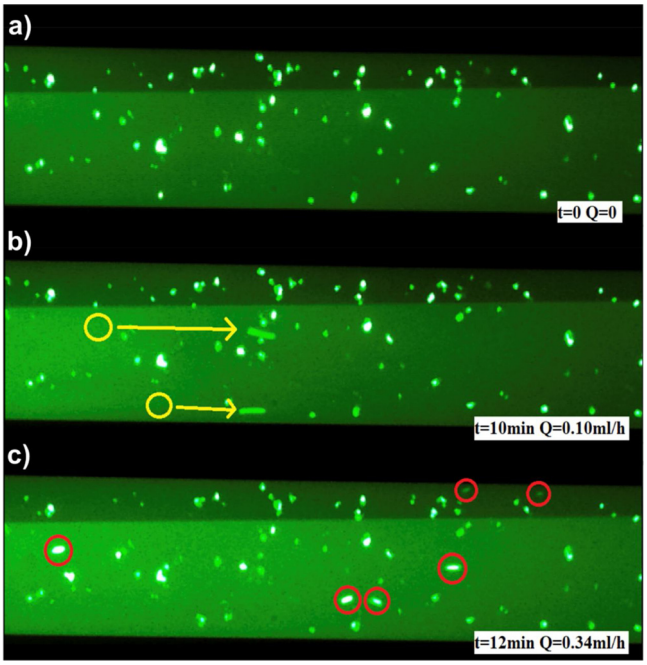


Fig. 3. Fluorescent microscope image of the attached CTC on 4-ATP modified and EpCAM antibody covered surface. Yellow circles are the cells that were detached from the surface and the red circles shows the cells that are travelling with the flow that enters to frame. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the surface might have attracted the more negatively charged side of the antibody. When the EpCAM molecule is examined, although both charges exist throughout the whole structure, the rather negatively charged parts appear to be on the non-antigen binding end. This might be the reason for observing better cell capture behaviour with amine terminated surfaces.

From this point on, the experiments were continued with molecules that provided the most efficient CTC capture, namely 11-AUT, 11-MUA and 4-ATP. The short-chain molecules Cys and 3-MPA were eliminated because the cell attachment was not sufficient enough.

Control tests were carried out using different surfaces, such as parylene and glass to see whether SAM still forms and CTC capture works or not. The reason why parylene and glass were selected was that these are the most commonly used materials both in our group and in many BioMEMS applications. Both surfaces were prepared following the very same steps as done in the gold ones.

Fig. 2a-f shows the results obtained from parylene and glass surfaces. As observed, there was almost no CTC capture by both surfaces, indicating that the SAM formation relies only on the gold-thiol interaction. Further control experiments were carried out using a different type of cell, namely K562 leukaemia cell, for which the EpCAM antibody should have no specificity. Gold surfaces were prepared following the same steps, using 11-AUT, 11-MUA and 4-ATP. Fig. 2g-i shows that K562 cells were not captured by the EpCAM antibodies. This proves that there exists a specific interaction between the immobilized antibodies and the CTCs. No experiment with mixed cells (breast cancer cell + leukaemia cell) was carried out since the main equipment used in this study was a fluorescent microscope and the image would not indicate which cells were attached on the surface. Therefore, to show the specificity of the system, this step was performed separately.

For further cell capture studies, real blood samples were used to see whether the system works with complex solutions. For this study, 11-MUA, 11-AUT and 4-ATP SAM surfaces were prepared and tested with the white blood cells remaining from the blood sample after the red blood cell lysis. The fluorescent microscope images showed that there was no cell capture, indicating that the prepared surfaces worked fine (Figure S4).

In addition to the covalently bonded systems, bioaffinity method provides an alternative strategy since the affinity of streptavidin for biotin is known to be one of the strongest non-covalent interaction while promoting site-directed antibody immobilization [22]. For this study, the surface was first coated with 11-MUA followed by the attachment of streptavidin covalently using EDC/NHS. Then, the biotinylated EpCAM antibodies were immobilized on the surface through streptavidin-biotin interaction and the cell attachment experiment was conducted (Figure S2). The bioaffinity method was tested with 2 mM and 5 mM 11-MUA concentrations. Compared to the covalent method, the bioaffinity method provides more cell attachment on low concentration SAM surfaces. The possible reason for this could be the non-homogeneous immobilization of the streptavidin molecules on the crowded SAM layer [46]. Therefore, when using the streptavidin-biotin interaction technique, lower SAM concentrations were employed [28,47,48]. The cell attachment on 2 mM 11-MUA was found as 1712 ± 65 cell/frame (Figure S3).

The microfluidic studies were performed once the experiments with gold surfaces were completed and 11-MUA, 4-ATP and 11-AUT were selected for their high CTC capture performances. The concentration of the cell solution was adjusted as 2×10^5 cells/ml to prevent any cluster formation. It should be noted that the cell capture efficiencies in the microfluidic channel experiments do not represent the maximum possible cell capture. The images taken during the experiment only represent the cells that entered the channel at a specific time and those that were detached from the surface.

The number of cells present in the microchannel was counted during the incubation period (N_i). Then, the flow was slowly increased to 0.10 ml/h (6 mbar) to remove the unbounded cells, and the remaining number was counted (N_0). The cell capture efficiency was measured from the following formula:

$$\text{Cell capture efficiency \%} = \frac{\text{The number of cells present in the channel after washing (No)}}{\text{The number of cells present in the channel during incubation (Ni)}} \times 100$$

For the surfaces prepared with 4-ATP, stationary 75 cells/frame were counted during the initial phase. Later, the flow was increased to 0.10 ml/h and two of the cells were detached. When the flow was further increased up to 0.34 ml/h, 73 cells were still on the surface, indicating 97 % cell capture efficiency (Fig. 3).

For the channel studies with 11-MUA modified surfaces, the number of cells entered to the frame were counted as 104 cells/frame. After 10 min, the flow was increased to 0.20 ml/h and the cells did not change their location, besides one cell debris (Figure S5). Even when the pressure was increased to 0.34 ml/h, no cell detachment was observed. For the surfaces prepared with another amine-terminated molecule 11-AUT, 43 cells/frame were counted at the beginning. The cells remained the same after increasing the flow to 0.34 ml/h (Figure S6).

Next, the effect of covalent bonding was compared to bioaffinity interaction in the microfluidic channel under flow. The surfaces inside the channels were prepared as previously described. The cells that entered the channel were counted as 345 cells/frame. The flow was then slowly increased to 0.10 ml/h, and 13 were detached from the surface, corresponding to 96 % capture efficiency. When the flow was further increased to 0.34 ml/h, more cells were detached, and their number was decreased to 198 cells/frame. This final 57 % capture efficiency shows that bioaffinity interaction cannot endure underflow, which makes it a poor choice to be used in microfluidic applications (Figure S7).

4. Conclusion

In this study, the cell capture efficiency of both different alkanethiols and different antibody immobilization methodologies were compared first on plain gold surfaces then in gold microfluidic channels under flow. For this purpose, MCF-7 cells were employed as a CTC model and EpCAM antibody was used. Surface characterizations were made with AFM, XPS and contact angle measurements. To investigate the selectivity and specificity of the functionalized surfaces, K562 cell line, an EpCAM negative CTC model, and different types of surfaces (glass and parylene) were used. Studies showed that short alkanethiols ($n < 8$) generated less well-organized SAM whereas the crystalline-like order could be achieved either with long chains ($n > 10$) or with phenolic groups. Alkanethiols containing aromatic rings permit high intermolecular interactions, leading to better cell capture events compared to that of alkanethiols with same chain length but without benzene ring. It was both suggested earlier and found in this study that the higher packing density of SAM caused steric hindrance and prevented the capture of the incoming cells by the antibodies. The critical issue in the microfluidic channel studies for effective cell capturing was the flow rate. The high throughput is essential to improve the performance of biosensors. It was found that the bioaffinity method limited achieving high flow rates. Compared to covalent bonding, where negligible number of cells was detached under high flow rates, the streptavidin-biotin interaction resulted in rather poor efficiencies, therefore leading to inadequate cell capture.

Our aim was firstly to keep the immobilization techniques as simple as possible, without including any rather complicated orientation specific approach [22], and secondly to apply these simple techniques to the microfluidic channels and observe the behaviours under flow. The findings obtained in the study can shed light on various microfluidics-based biosensor applications since there is a growing demand both for microfluidic lab-on-a-chip systems and for CTC detections. The methods discussed in this paper can be employed in other relevant CTC capture analysis for further studies.

Credit author statement

Didem Cetin conducted all the experiments and analysis including surface functionalization, antibody immobilization, cell capture studies with plain surfaces and microfluidic channels, surface characterizations and contributed to writing the manuscript. Meltem Okan designed the experimental setups, selected the alkanethiols, interpreted the results and wrote and organized the manuscript. Erhan Bat coordinated the study and interpreted the results. Haluk Kulah coordinated the study and the project and organized the manuscript.

Declaration of Competing Interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2020.110808.

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