



Acoustic and hybrid 3D-printed electrochemical biosensors for the real-time immunodetection of liver cancer cells (HepG2)

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ABSTRACT

This study presents an efficient acoustic and hybrid three-dimensional (3D)-printed electrochemical biosensors for the detection of liver cancer cells. The biosensors function by recognizing the highly expressed tumor marker CD133, which is located on the surface of liver cancer cells. Detection was achieved by recrystallizing a recombinant S-layer fusion protein (rSbpA/ZZ) on the surface of the sensors. The fused ZZ-domain enables immobilization of the anti-CD133 antibody in a defined manner. These highly accessible anti-CD133 antibodies were employed as a sensing layer, thereby enabling the efficient detection of liver cancer cells (HepG2). The recognition of HepG2 cells was investigated *in situ* using a quartz crystal microbalance with dissipation monitoring (QCM-D), which enabled the label-free, real-time detection of living cells on the modified sensor surface under controlled conditions. Furthermore, the hybrid 3D additive printing strategy for biosensors facilitates both rapid development and small-scale manufacturing. The hybrid strategy of combining 3D-printed parts and more traditionally fabricated parts enables the use of optimal materials: a ceramic substrate with noble metals for the sensing element and 3D-printed capillary channels to guide and constrain the clinical sample. Cyclic voltammetry (CV) measurements confirmed the efficiency of the fabricated sensors. Most importantly, these sensors offer low-cost and disposable detection platforms for real-world applications. Thus, as demonstrated in this study, both fabricated acoustic and electrochemical sensing platforms can detect cancer cells and therefore may have further potential in other clinical applications and drug-screening studies.

1. Introduction

Although cancer remains one of the leading cause of death worldwide, the number of patients diagnosed with the four most common cancers (breast, prostate, lung, and colorectal) has decreased since the early 1990s. This reduction is partially attributable to progress in the development of effective diagnostic tools. Currently, hepatocellular carcinoma (HCC) is ranked 6th on the list of cancers linked to deaths, and the number of patients with this type of cancer is increasing because of the transmission of hepatitis B and C viruses (Llovet et al., 2003). Clearly, the early diagnosis of HCC would provide both medical benefits to patients and macro-medical economic benefits. However,

the early diagnosis of HCC is hindered by some technical issues that remain unsolved.

Some existing cancer diagnostic methods, such as polymerase chain reaction (PCR)-based methods and optical fluorescence, enable characterization of nuclear and cytoskeletal markers (Berois et al., 2013; Dumartin et al., 2011; Yin et al., 2012). However, these conventional diagnostic procedures have relatively high limits of detection and are expensive to perform and time consuming to implement. Therefore, the development of new biosensors that are simple, selective and sensitive has attracted increasing attention. The detection of cancer cells in real-time with a minimum number of cells has been previously investigated using methods based on mass-sensitive, electrochemical and micro-

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fluidic techniques (Camps et al., 2005; 2010; Arya et al., 2013; Fu et al., 2013; Weltin et al., 2014; S. Zhang et al., 2014; J. Zhang et al., 2014). Recently, the quartz crystal microbalance with dissipation monitoring (QCM-D) has emerged as an attractive technique in the field of cancer research. QCM-D is a label-free, non-invasive technology that enables the continuous monitoring of cell surface interactions and determination of the kinetics of cell attachment and spreading in real-time (Tan et al., 2008). Nevertheless, QCM-D is still at an early phase of development as a functionalized biosensor able to both recognize and capture target cancer cells with high selectivity and sensitivity.

The available immunoassay systems are currently limited by several factors, including the detection limit, non-specific binding, and the orientation and density of affinity biomolecules (antibodies and antigens) on the biosensor surfaces (Baniukevic et al., 2013; Deng et al., 2016). Therefore, the immobilization of antibodies in the correct orientation on the sensor surface is critical for improving the selectivity and sensitivity of these immunosensors (Prieto-Simocn et al., 2008; Karyakin et al., 2000). Methods used to immobilize antibodies to substrates have included physical adsorption and chemical binding. However, these immobilization techniques are not without problems. Some examples include covalent coupling based on glutaraldehyde or carbodiimide or, alternatively, a self-assembled monolayer containing 11-mercaptoundecanoic acid, protein A, protein G and gold nanoparticles, but all have some limitations. These limitations include (i) time-consuming protocols, (ii) alteration of the biological activity of the antigen-binding sites of the antibodies, and (iii) the need to first split the intact antibody into two fragments (Löfås et al., 1990; Babacan et al., 2000; Oh et al., 2004; Bae et al., 2005; Kausaite-Minkstiniene et al., 2010; Karyakin et al., 2000; Pum et al., 2013). Therefore, finding an alternative strategy to overcome these limitations is important. For this purpose, the recombinant S-layer fusion protein rSbpA/ZZ has been constructed (Fig. S1).

SbpA, the S-layer protein from *Lysinibacillus sphaericus* CCM 2177, has square lattice symmetry with a lattice constant of 14 nm (Pum et al., 2013). rSbpA/ZZ is composed of two copies of the 58-amino-acid-long Fc-binding Z-domain, a synthetic analog of the immunoglobulin G (IgG)-binding B-domain of protein A of *Staphylococcus aureus* (Eliasson et al., 1988; Völlenkle et al., 2004). Staphylococcal protein A has a cell wall-binding region and five domains (C (next to the cell wall), B, A, D, and E). The molecular interactions of staphylococcal protein A with immunoglobulins and the binding sites on the Fc domain of IgG-1, -2, and -4 have been investigated previously (Lowenadler et al., 1987; Nilsson et al., 1987). The protein A-binding site of rSbpA/ZZ enables the efficient capture of antibodies (IgG) and has been used as a specific adsorbent in extracorporeal blood purification (Völlenkle et al., 2004; Ilk et al., 2011; Rothbauer et al., 2015). Moreover, rSbpA/ZZ can self-assemble while retaining its square lattice symmetry in suspension and recrystallize on solid supports (Sleytr et al., 1999; Sara et al., 2005, 2006; Pum et al., 2013, Pum and Sleytr, 2014). Thus, rSbpA/ZZ can be exploited to form an intermediate layer on a gold substrate, thereby providing an effective matrix for binding and capturing antibodies. The high-density and regular arrangement of functional groups in the S-layer lattice generates a relatively uniform and aligned layer (Sleytr et al., 1999, 2014; Völlenkle et al., 2004), and indeed, the S-layer surface has been shown to exhibit extremely low non-specific protein adsorption (Sara and Sleytr, 1987; Rothbauer et al., 2013; Picher et al., 2013). Because of its biological properties, the rSbpA/ZZ can be easily manipulated to make it compatible with biomolecules. Furthermore, rSbpA/ZZ ensures the correct orientation of antibody immobilization and limits protein denaturing (Matis et al., 2008).

The present paper reports on the fabrication of a biosensor to capture liver cancer cells (HepG2) via the recognition of CD133 as a cell surface marker by an anti-CD133 antibody (Scheme 1). Moreover, the lattice of the S-layer protein rSbpA/ZZ on gold sensors is utilized for the first time for the specific immobilization of the anti-CD133 anti-

body. The various steps involved in immunosensor functionalization and capture of cancer cells were characterized by both QCM-D and electrochemical measurements. QCM-D was used to track the formation of functional biomolecular layers on the sensor surface and for the detection of viable cells in real-time. Moreover, HepG2 cells captured on anti-CD133 antibody/rSbpA/ZZ lattice/Au electrodes were confirmed using cyclic voltammetry (CV). Our goal was to investigate liver cancer detection using sensors whose form factors were relatively close to those of manufactured parts. Thus, the final acoustic sensor described here can be exploited in future studies to investigate the cellular response to chemotherapeutics. Moreover, the final electrochemical sensors, which are an assembly of ceramic substrates, pressure-sensitive adhesives and 3D-printed capillaries, represent a functional assay on a device with a remarkable similarity to commercial sensors.

2. Materials and methods

2.1. Materials

HepG2 (human HCC cell line) was obtained from the American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic/antimycotic solution were obtained from PAA (Austria). Guanidine hydrochloride (GHCl), bovine serum albumin (BSA), Tris, phosphate-buffered saline (PBS) tablets, $K_3[Fe(CN)_6]$, KCl, and $CaCl_2$ were purchased from Sigma-Aldrich GmbH (Germany). Mono- and polyclonal anti-CD133 antibodies were purchased from Novusbio (UK). Cell-counting slides were obtained from Nanoentek (USA).

2.2. Preparation of S-layer fusion protein

Two milligrams of lyophilized S-layer fusion protein (rSbpA/ZZ) was reconstituted using the chaotropic agent GHCl (5 M in 50 mM Tris-HCl buffer, pH 7.2) and dialyzed in MilliQ water for one hour at 4 °C. Water was exchanged once after 30 min. After dialysis, the protein solution was centrifuged at 13,000 rpm for 15 min at 4 °C to remove self-assembly products. For recrystallization, the protein concentration was adjusted to 100 µg/ml in Tris buffer (10 mM $CaCl_2$ in 0.5 mM Tris-HCl, pH 9).

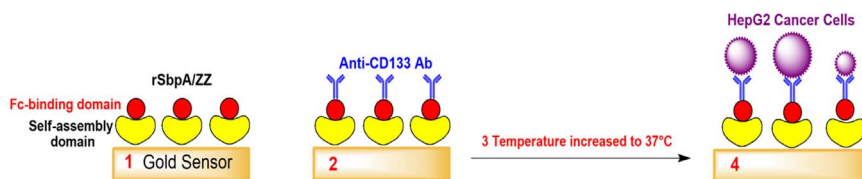
2.3. Cell lines and cell culture

HepG2 cells were cultivated in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution under standard cell culture conditions at 37 °C in a humidified 5% CO_2 and 95% air atmosphere as adherent monolayers in 25-cm² cell culture flasks. After culturing for 48 h, trypsin was added to detach the cells from the flask. Then, the cells were separated from the medium by centrifugation at 1000 rpm for 5 min and counted using an automated cell counter. Subsequently, the separated cells were washed once with PBS and centrifuged again at 1000 rpm for 5 min. The washed cells were re-suspended in PBS and used immediately either for QCM-D or CV measurements.

To count and discriminate between viable and non-viable HepG2 cells, trypan blue was used to selectively stain dead cells blue. Briefly, a suspension of HepG2 cells in PBS was diluted in 0.4% trypan blue solution (1:1) at room temperature. Non-viable cells absorbed the dye and exhibited distinctive blue cytoplasm under the microscope.

2.4. Contact angle (CA) measurements

Gold-coated microscope slides with a 10-nm-thick gold layer were used. Before use, all chips were cleaned by immersion into a 5:1:1 mixture of MilliQ water, NH_3 , and H_2O_2 at 70 °C for ~10 min and then dried under a stream of nitrogen. To remove any organic contaminants from the surface, the gold surfaces were also cleaned by ozone plasma



Scheme 1. The schematic sketch (not drawn to scale) shows the step-by-step modification of the gold sensor to the final functional anti-CD133 antibody/rSbpA/ZZ/Au sensor surface: (1) recrystallization of the S-layer fusion protein rSbpA/ZZ on the gold sensor; (2) immobilization of the anti-CD133 antibody onto rSbpA/ZZ; (3) increasing the temperature from 27 °C to 37 °C; (4) capturing of HepG2 cancer cells.

treatment in a plasma cleaner (Plasma Prep 2, Germany).

The CAs of water on gold surfaces with different coating layers were determined using a goniometer (Easy Drop, Germany), and the data were evaluated with the enclosed analysis software. A drop of MilliQ water (5 μ l) was deposited onto the surface, and the static CA was immediately measured. At least three drops were analyzed at different positions on each surface.

2.5. QCM-D experiments

QCM-D (E4, Q-Sense, Sweden) was used to measure the changes in frequency and energy dissipation. Indeed, the substrates used in this study are commercially available sensor crystals for the E4 system (QSX 301, Q-Sense, AB, Sweden) with a diameter of 14 mm. They were coated with a 100-nm-thick gold film and had a sensitivity constant of 17.7 ng/cm³. The gold sensors were cleaned by immersion in a 5:1:1 mixture of MilliQ water, NH₃, and H₂O₂ at 70 °C for ~10 min. Before each experiment, the sensors were also cleaned by ultraviolet (UV)/ozone treatment.

QCM-D experiments were performed at 27 or 37 $\pm$ 0.02 °C in duplicate. The flow rate for S-layer fusion protein injection was 100 μ l min⁻¹; 50 μ l min⁻¹ was used for all other fluid injection and rinsing steps. QCM-D data were recorded from the 3rd to the 13th odd-numbered overtones. Briefly, a solution of rSbpA/ZZ (0.1 mg/ml) was recrystallized on the gold sensors for at least 3–4 h. After rinsing with Tris buffer, mono- or polyclonal anti-CD133 antibodies (50 μ g/ml) in 0.5-mM Tris buffer (pH 8.0) were immobilized on the S-lattice and incubated for at least 60 min before rinsing with BPS. Subsequently, 1% BSA in PBS was added and incubated for ~90 min. After rinsing with PBS and increasing the temperature to 37 °C, the HepG2 cancer cell samples were injected into the QCM-D chamber for direct analysis. After approximately 1 h, they were rinsed with PBS to remove non-bound cells.

2.6. Electrochemical measurements

Electrochemical CV measurements were performed in duplicate using a potentiostat (Drop Sens, Spain). All measurements were conducted in a 10 mM solution of K₃[Fe(CN)₆] containing 100-mM KCl as the supporting electrolyte, and the CV were recorded from -0.2 to 0.4 V at a scan rate of 0.05 V/s.

Screen-printed gold electrodes were exposed to rSbpA/ZZ solution for at least 3–4 h. After S-protein recrystallization, the electrode was rinsed with Tris buffer to remove excess protein. Anti-CD133 anti-

bodies (50 μ g/ml) in 0.5-mM Tris buffer (pH 8.0) were immobilized on the S-lattice for ~90 min at room temperature and then rinsed with PBS to remove unbound antibodies. Subsequently, 1% BSA in PBS was added and incubated for ~90 min. After rinsing with PBS, the modified electrodes were incubated with different number of HepG2 cells at 37 °C for ~60 min. Finally, the electrodes were rinsed with PBS to remove unbound cells.

3. Results and discussion

3.1. Characterization of the anti-CD133 antibody/rSbpA/ZZ/Au biosensors: CA measurements

In general, an immunosensor is based on specific recognition between an antibody immobilized on a substrate and the target antigen. This interaction can be affected by several factors, such as the surface charge, wettability and hydrophilicity. Clearly, the nature of the surface is important for cell attachment and/or proliferation (Song et al., 2011; Zhang et al., 2014); therefore, the fabricated sensor surface was analyzed by measuring the CA (Fig. 1).

The CA decreased from 63.1 $\pm$ 0.6° for the bare gold surface to 13° upon rSbpA/ZZ protein recrystallization on the gold surface. This decrease in the CA indicated that the surface became much more hydrophilic, which is consistent with the results of prior studies in which S-layer proteins formed water-filled hydrophilic pores with low unspecific adsorption (Sleytr et al., 2014). Charged groups (e.g., carboxyl or amino residues) on the surface of the S-layer lattice likely contribute to increasing the surface wettability. In the next step, anti-CD133 antibodies were immobilized on the rSbpA/ZZ lattice, which was accompanied by an increase of the CA to 47.2 $\pm$ 0.8°. The observed CA increase upon antibody immobilization was also consistent with previous work (Zou et al., 2007; Darain et al., 2009). After rinsing the surface with 1% BSA, the CA decreased slightly to 43.8 $\pm$ 2.5°. The CA value has been previously demonstrated to decrease as the BSA concentration increases (Bialopiotrowicz et al., 2001; Waghmare et al., 2013). However, in the present study, a relatively low concentration of BSA was used, and therefore, no considerable change in the CA was observed. This result indicated that few BSA molecules were captured between the immobilized anti-CD133 antibodies on the rSbpA/ZZ protein lattice. In summary, the generated hydrophilic sensor surface should provide a biocompatible surface and enhance the loading of cells while retaining their bioactivity.

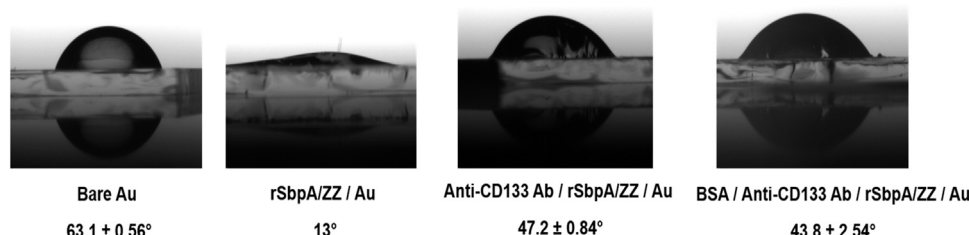


Fig. 1. Image of the water drop on gold and step-by-step modified gold surfaces for the determination of the contact angle (CA) of each terminal layer.

3.2. Formation and characterization of the anti-CD133 antibody/rSbpA/ZZ/Au acoustic sensors: QCM-D measurements

QCM-D is a surface-sensitive microbalance technique based on acoustic wave propagation along a quartz crystal. The molecular adsorption of materials (e.g., single S-layer proteins that self-assemble into a coherent lattice) is reflected by a shift in the resonance frequency (Δf). A negative Δf is usually associated with molecular adsorption events that increase the attached mass on the quartz crystal. However, cell adhesion may result in a completely different Δf shift because QCM-D responds to the particle resonance frequency (Olsson et al., 2009). In brief, the latter depends not only on the mass of the adsorbed material but also on the adhesive bond stiffness. Hence, a negative shift in Δf is observed when cells are tightly immobilized on the sensor surface (Tarantola et al., 2010). Whenever a cell is captured by a long and flexible linker, the stiffness of the adhesive bonds is low, and for the binding of a cell, a positive shift in Δf may even be measured by QCM-D. The specific interaction of a cell captured by a short linker will lead to a Δf value between the two boundary conditions described above. The second measured parameter is the energy dissipation factor (ΔD), which accounts for the energy loss by providing information about the viscoelastic properties of the adsorbed materials before, during, and after the interaction (Rodahl et al., 1995, 1997; Fredriksson et al., 1998). Low dissipation signals reflect the formation of a rigid layer, whereas large signals reflect the formation of a soft layer. Hence, rigid films may strongly couple to the sensor surface and therefore allow tracking of thicker generated layers (Dixon et al., 2008).

In this work, QCM-D was used to monitor the recrystallization of the rSbpA/ZZ S-layer protein on the gold-coated quartz sensors, the subsequent immobilization of anti-CD133 antibodies on the S-lattice at 27 °C, and, finally, the capture of HepG2 cells (3×10^6 cells/ml) at 37 °C (Fig. 2a). The changes in the Δf and ΔD signals occurred simultaneously upon the injection of rSbpA/ZZ and the anti-CD133 antibody and, finally, upon exposure to the cell suspension. The self-assembly of the rSbpA/ZZ protein from solution to form a lattice on the gold sensor led to a rapid decrease in the frequency level to approximately -70 Hz, indicating increased mass (adsorbed materials) on the surface of the sensor and a slight increase in the energy dissipation level (2×10^{-6}). These results indicated that a tightly bound and rigid crystalline layer was formed on the surface of the sensor and are in agreement with previously published works (Damiati et al., 2015a, 2015b). A more pronounced shift in Δf was observed upon injection of the anti-CD133 antibody. The anti-CD133 antibody solution passed over the rSbpA/ZZ protein monolayer, further shifting the frequency (-92 Hz) and increasing the dissipation value (from 2×10^{-6} to 3.6×10^{-6}). These results indicated the binding of the antibody to the functional domains of the rSbpA/ZZ protein. Because of the construction principle of the recombinant S-layer fusion protein, the ZZ-domains on the outermost surface of the S-layer protein lattice were exposed to the aqueous environment. This setting facilitated the immobilization of the anti-CD133 antibody in the correct orientation (Völlenkle et al., 2004; Ilk et al., 2011). The addition of BSA only slightly decreased the frequency signal (-95.5 Hz) and noticeably increased the dissipation signal (7.75×10^{-6}). The almost negligible change in the frequency indicated that few BSA molecules were trapped within the anti-CD133 antibodies bound to the rSbpA/ZZ lattice. The increase in ΔD , however, suggested increased softness in the region where the BSA was located.

3.3. Performance of the QCM-D immunosensor for the detection of HepG2 cells

CD133 is a transmembrane glycoprotein that is considered an important cell surface marker for stem and cancer stem cells in various tissues, such as liver, lung, brain and colon (Weigmann et al., 1997; Miraglia et al., 1997; Ma et al., 2007). The proposed sensor can detect different types of cancer that over-express CD133 on the membrane. In

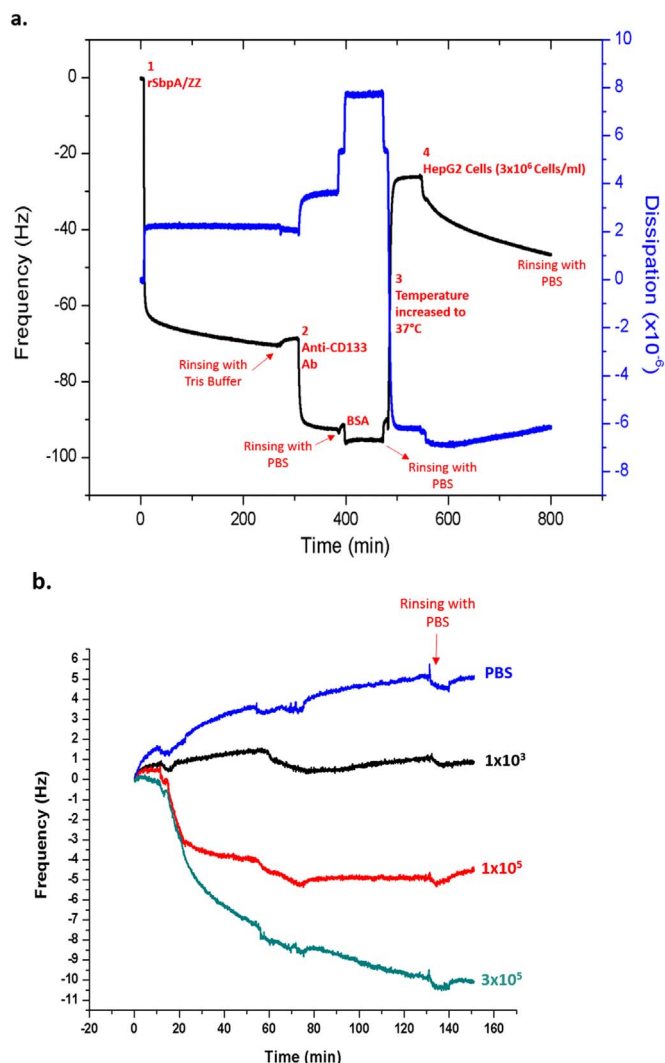


Fig. 2. Representative shift in frequency and dissipation versus time for the formation of the final anti-CD133 antibody/rSbpA/ZZ/Au sensor structure (a) and the subsequent addition of HepG2 cells at different concentrations (Cell viability above 85%) (b). All shown QCM-D data were recorded at the 5th overtone.

this study, the selectivity of the fabricated biosensor is due to CD133 being a marker that is highly expressed on the cell membranes of liver cancer cells, as detected and confirmed by flow cytometry (fluorescence-activated cell sorting [FACS] analysis). The HepG2 liver cancer cell line was chosen as a cell model that expresses the CD133 marker.

QCM-D measurements were performed to detect cell binding under controlled conditions, such as humid and temperature-controlled environments, by monitoring the changes of Δf and ΔD of the quartz crystal. The direct binding event was detected by measuring the decreasing frequency and increasing dissipation at different overtones. The modified QCM-D sensors (anti-CD133 Ab/rSbpA/ZZ/Au) were incubated with suspensions containing different numbers of HepG2 cells (1×10^3 , 1×10^5 , 3×10^5 , 1×10^6 , and 3×10^6 cells/ml) to determine the sensitivity and quantitative range of the immunosensor setup (Fig. 2b). When the number of cells was low (1×10^3 cells/ml), the shift in the Δf signals was not significant. However, the response increased as the number of cells increased from 1×10^5 cells/ml to 3×10^6 cells/ml. Thus, the fabricated sensor was able to detect cancer cells quantitatively within a certain detection range.

Although Δf decreased as the number of HepG2 cells per ml and the immobilized mass on the surface (i.e., HepG2 cells) increased, the sensitivity was lower than expected. Previous studies involving bacterial and cancer cells have reported large changes in the Δf and ΔD

signals when cells adhered to the sensor surface (Tarantola et al., 2010; S Zhang et al., 2014; J Zhang et al., 2014; Li et al., 2015). In the present work, the shifts in frequency and dissipation were less pronounced, although they were dependent on the number of cells. Clearly, the formation of a dense layer of rSbpA/ZZ on the gold sensor separated the cancer cells from the sensor surface. However, whereas the S-layer lattice is a rigid structure, the anti-CD133 antibodies can be considered to be a flexible linker. Therefore, the changes in Δf were less clear than those observed when the cells are tightly immobilized on a surface. However, the values for frequency and dissipation obtained in this study are in good agreement with previously published results for detection of both bacterial and cancer cells (Olsson et al., 2009; Xi et al., 2012; Sandrin et al., 2015; Elmlund et al., 2015; Iturri et al., 2015).

QCM-D is an excellent tool for slicing the cell–substratum interface into different sections by analyzing multiple overtones (Olsson et al., 2009). This is because the depth to which the shear evanescent waves penetrate the fluid or cell material adjacent to the crystal surface constitutes the analysis depth and is strongly dependent on the magnitude of the frequency. If a cell is directly immobilized on the sensor surface, all shear evanescent waves with different frequency magnitudes (overtones at 15, 25, 35, 45, 55, and 65 MHz) penetrate essentially the same biological material. Thus, the shift in frequency should be similar for all measured overtones. The same is true when an S-layer lattice is between the crystal surface and the immobilized cells. The shift in frequency measured at various overtones, however, will differ significantly if the cells are separated by linkers from the sensor surface because the penetration of the shear evanescent waves will depend on the overtones of different biological materials (e.g., captured water, cell membrane, and cytosol). In the present study, the frequency shifts for various overtones differed only slightly, beginning at the recrystallization of the rSbpA/ZZ monolayer (Fig. S2). The immobilization of the cells resulted in no further splitting effect. This result suggests that the cells are tightly bound to the anti-CD133 antibodies without excess space between the S-layer lattice and the cell membranes of the HepG2 cells.

Two important observations were made during the QCM-D measurements. First, when the cell suspension of HepG2 was passed over the modified sensor at 27 °C, neither the frequency nor the dissipation signal changed, whereas at 37 °C, both signals shifted, highlighting the importance of optimizing the temperature to enable efficient cell capture by the antibodies. HepG2 cells were captured by mono- and polyclonal anti-CD133 antibodies, and both showed similar cell-capture efficacy. Second, when the cell viability was approximately 10%, the Δf and ΔD signals did not change (Fig. S3), whereas when the cell viability was above 85%, the signals did change (Fig. 2b). This observation illustrated the ability of QCM-D to detect living cells in real-time and to discriminate between viable and non-viable cells, which could facilitate investigating the impact of chemotherapy drugs on cancer cell viability in further studies.

Determination of cell viability by trypan blue exclusion test was performed. This test is a common method of counting viable/non-viable cells in a suspension. Viable cells with intact cell membranes exhibit highly selective permeability that allows the free passage of small molecules while restricting the passage of others. Trypan blue is a diazo dye that stains non-viable cells blue by penetrating the degradable membrane but does not stain viable cells (Chan et al., 2015). Thus, the functional loss of cell membrane integrity affects not only the permeability but also the recognition of cells by the antibody because of the loss of functional cell surface receptors.

3.4. Formation of the electrochemical disposable sensors

The electrochemical data reported here were gathered using A-AD-GG-101-N sensors (Zimmer and Peacock, UK) (Fig. 3). The bare gold working electrodes were modified to the required biological specificity

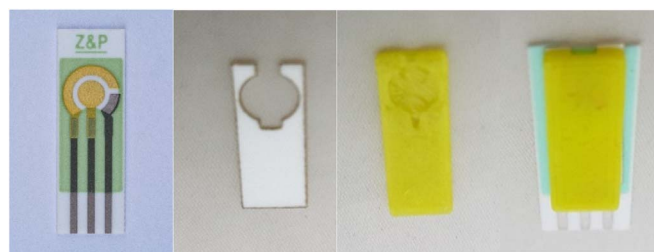


Fig. 3. From left to right (a) The bare sensor (b) The adhesive layer (c) The 3D printed microfluidic chamber (d) The final hand-assembled part.

using the methods described in this paper. The sensors were subsequently modified using a double-sided, pressure-sensitive adhesive (Zimmer and Peacock, 92000041). The adhesive film came with a peel-off film that, when removed, exposed the adhesive. Finally, the microfluidic chamber was designed (dimensions: 1.5×1×7 mm) and 3D printed (the STL file is available for download). The 3D printer was a LulzBot TAZ 6 3D, and the printing filament was co-polyester polymer (Zimmer and Peacock, 92000042). The hydride sensor device was assembled by sandwiching the double-sided adhesive spacer layer between the ceramic substrate and the 3D printed microfluidic chamber.

3.5. Electrochemical characterization of the modified electrode for HepG2 cell detection

Similar to the acoustic sensor, an electrochemical sensor was also developed. Regardless of the substrate surface, the orientation of CD133 antibodies on the rSbpA/ZZ lattice was the same, and both types of modified sensors had the same characteristics, as confirmed by measuring the CAs.

Electrochemical biosensors have been widely reported for the detection of cancer cells because of their inherent sensitivity. Therefore, CV was performed (and the response current *vs.* applied potential plotted) to confirm the changes in the electrochemical properties after each electrode modification step. The bare gold electrode exhibited the highest peak current, which indicated a small electron transfer resistance (Fig. 4a). As expected, upon the self-assembly of the S-layer fusion protein rSbpA/ZZ on the surface of the gold electrode, the peak current decreased from 165 to 87 μ A (47%), reflecting an increase in the electron transfer resistance. Subsequently, after the immobilization of the anti-CD133 antibody and the addition of BSA, the electron transfer was further reduced because the biomolecule layer became tighter, and as a result, the associated electron transfer resistance increased.

Systematic CV measurements were performed to detect the cancer cells. Based on the specific interaction between the anti-CD133 antibody and CD133, which is over-expressed by the cancer cells, the ability of HepG2 cells to bind to the modified immunosensor surface was verified. Voltammetric measurements were performed to convert the interaction between the anti-CD133 antibody and the HepG2 cancer cells into an electronic signal. When a suspension of HepG2 cells was added to the biosensor, the peak current decreased relative to the initial value measured in the absence of cells. The CVs of HepG2 cells were measured, and the results obtained from the blank test (no cells) and those collected with increasing cell numbers (1×10^3 , 1×10^5 , 3×10^5 , 1×10^6 , and 3×10^6 cells/ml) were compared. The peak current response consistently decreased (8%, 16%, 30%, 80%, and 82.2%, respectively) as the number of cells captured on the sensor increased (Fig. 4b).

The high sensitivity and efficiency of electrochemical biosensors for the detection of cancer cells can only be achieved by carefully designing the surface architecture. Previously, the immobilization of the antibodies on the sensor and the binding of the target molecules were

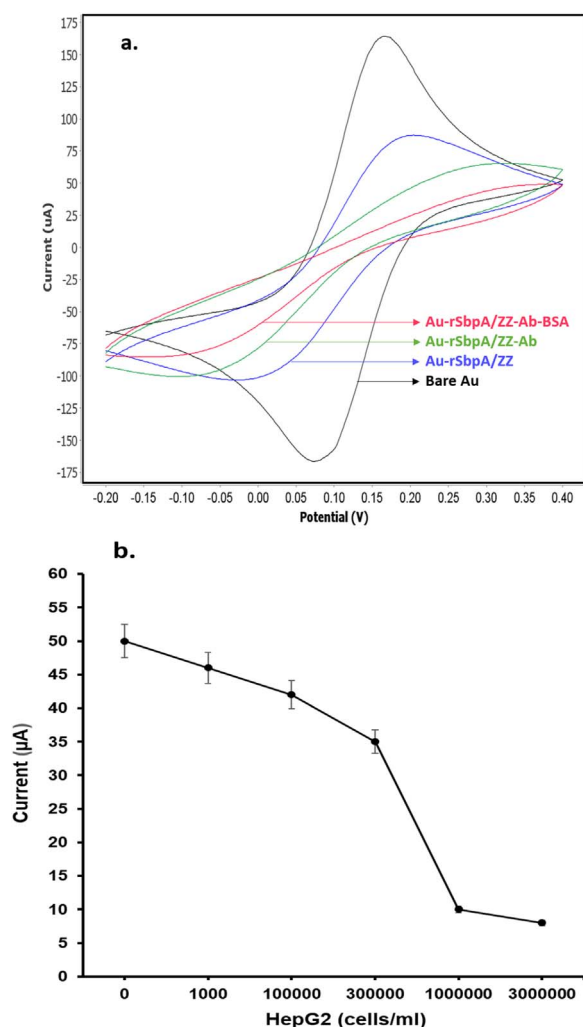


Fig. 4. CV spectra of the functionalized anti-CD133 antibody/rSbpA/ZZ/Au electrode in 10 mM $K_3[Fe(CN)_6]$ containing 100 mM KCl at a scan rate of 50 mV/s (a). Peak current values as a function of number of HepG2 cancer cells, which have been added onto the anti-CD133 antibody/rSbpA/ZZ modified screen-printed gold electrode (b).

performed by incubating a liquid drop and subsequently drying it. This drying process and the resulting pattern may affect the assay performance significantly because of the non-uniformly functionalized surface generated. By contrast, using rSbpA/ZZ enables obtaining a precise orientation of the antibodies because of the binding of the antibodies to the functional ZZ-domains of the monomolecular S-layer protein lattice. Moreover, combining electrochemical techniques and gold sensor fabrication based on rSbpA/ZZ and anti-CD133 antibodies yields a sensor that is low cost and disposable.

4. Conclusion

The present work reports on the novel design and development of bio-inspired immunosensors with acoustical or electrical read-out systems for the label-free detection of cancer cells that over-express CD133. We placed particular emphasis on modifying the gold sensor surface, which was performed by recrystallizing the S-layer fusion protein rSbpA/ZZ. The latter formed a coherent lattice on the gold sensor and provided, as determined by CA measurements, a biocompatible surface able to enhance the loading of cells and the retainment of their bioactivity. Because of the underlying rSbpA/ZZ lattice, the anti-CD133 antibodies could be specifically immobilized in an aligned and spatially precise arrangement, thereby enabling the efficient capture of liver cancer cells (HepG2). The QCM-D technique allowed

monitoring the step-by-step assembly of the sensing biosurface *in situ* and also provided a useful platform for the detection of cancer cells under controlled conditions and the differentiation of viable and non-viable cells in real-time. Based on these findings, the effects of chemotherapeutic drugs on cancer cell viability and cellular response will be investigated in the future. The measured shift in the frequency decreased as the HepG2 cell number increased from 1×10^5 to 3×10^6 cells/ml, indicating that the fabricated biosensor could detect HepG2 cells quantitatively within this range. Moreover, CV measurements obtained while exposing the proposed modified electrode to solutions with different HepG2 cell numbers revealed a consistently decreasing peak current response as the number of cells specifically bound to the sensor increased. This platform, which is based on modified screen-printed electrodes, is a low-cost and robust detection platform. Finally, the obtained results should facilitate the application of these antibody-functionalized rSbpA/ZZ surface modifications for biosensing and the development of a promising platform for clinical diagnosis in the future.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.bios.2017.03.045](https://doi.org/10.1016/j.bios.2017.03.045).

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