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Two-dimensional label-free affinity analysis of tumor-specific CD8 T cells with a biomimetic plasmonic sensor

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ABSTRACT: The screening and analysis of T cells functional avidity for specific tumor-associated antigens is crucial for the development of personalized immunotherapies against cancer. The affinity and kinetics of a T cell receptor (TCR) binding to the peptide-major histocompatibility complex (pMHC), expressed on tumor or antigen-presenting cells, have shown major implications in T cell activation and effector functions. We introduce an innovative methodology for the two-dimensional affinity analysis of TCR-pMHC in a label-free configuration by employing a multi-parametric Surface Plasmon Resonance biosensor (MP-SPR) functionalized with artificial cell membranes. The biomimetic scaffold created with planar lipid bilayers is able to efficiently capture the specific and intact tumor-specific T cells and monitor the formation of the immunological synapse *in situ*. We have achieved excellent limits of detection for in-flow cell capturing, up to two orders of magnitude below the current state-of-the-art for plasmonic sensing. We demonstrate the accuracy and selectivity of our sensor for the analysis of $CD8^+$ T cells bioengineered with TCR of incremental affinities specific for the HLA-A0201/NY-ESO-$I_{157-165}$ pMHC complex. The study confirmed the significance of providing a biomimetic microenvironment, compared to the traditional molecular analysis, and showed fine agreement with previous results employing flow cytometry. Our methodology is reliable and versatile thus it can be applied to more sophisticated photonic and nanoplasmonic technologies for the screening of multiple cell types and boost the development of novel treatments for cancer.

Cancer immunotherapies have been identified as a major biomedical breakthrough of the last years, and they will most likely remain a paramount biomedical landmark in the next decades. This efficient treatment seeks the activation, inhibition or improvement of the immune system to fight cancer. Instead of directly targeting tumor cells, as conventional radiation therapy or chemotherapy, immunotherapy approaches empower the patient's natural immune system to combat them. Most attractive and encouraging advances in cancer immunotherapy have been achived with the so-called Adoptive Cell Therapy (ACT).[1,2] This approach is a highly personalized therapy that involves the identification or genetic bioengineering of the patient's own immune tumor-specific cells (i.e. T cells) to provide an enhanced anti-tumor activity.

Immune T cell activation is essentially triggered by the interaction between the T cell receptor (TCR) and antigenic peptides presented by the major histocompatibility complex (MHC) in either tumor cells or antigen-presenting cells (e.g. dendritic cells).[3,4] It has been shown that the affinity and binding strength of a TCR to its cognate peptide-MHC (pMHC) correlate with T cell effector functions, and thus the cell-mediated immunity.[5,6] Therefore, there is a need for a robust technology that enables rapid identification and study of autologous or engineered T cells expressing TCRs specific for tumor-associated antigens (TAAs).

Conventional means for assessing TCR-pMHC binding parameters are based on molecular kinetic studies employing Surface Plasmon Resonance (SPR) technology.[7–9] These biosensors have become a benchmark technology in research and pharmaceutical laboratories, offering label-free and real-time analysis of biomolecular binding in a user-friendly and automatized manner. However, the SPR affinity studies are commonly performed by flowing recombinant TCR molecules over a sensor chip coated with pMHC monomers – namely, three-dimensional analysis, 3D – which, apart from requiring expensive production, do not take into account the intrinsic cell physiology and microenvironment (e.g. co-receptors, cell membrane restrictions). There is strong evidence that soluble monomeric TCR and pMHC cannot reproduce accurately the immunological synapse occurring in cells, as they predict that only high-affinity interactions might induce T cell activation in clear contrast with the low affinities observed in naturally occurring TCRs.[10,11] Alternatively, the use of reversible

fluorescent pMHC multimers (so called NTAmers) has been proposed to study monomeric TCR-pMHC dissociation rates by directly staining the T cell surface.[5,6,12] Although this approach accounts for the co-receptor contributions on T cell membranes (e.g. CD8) and has been shown to accurately correlate with $CD8^+$ T cell responsiveness, it is limited to determining only the dissociation rates and cannot directly evaluate the TCR-pMHC binding affinity.[12,13] Furthermore, the use of expensive fluorescent dyes and the need of high amount of cloned cells for accurate measurements by flow cytometry truly hampers the throughput of this technique. To overcome these limitations, novel technologies have been used, including mechanically-based micropipette assays (e.g. adhesion frequency or thermal fluctuation analysis)[11] or single-molecule microscopy based on fluorescence resonance energy transfer (FRET).[14] These techniques enable the measurement of TCR-pMHC interaction kinetics at the interface between a live T cell and a surrogate antigen-presenting cell. As both target molecules are embedded in their respective cell membranes, these studies are known as two-dimensional (2D) analysis. The 2D binding measurements have shown an exceptional sensitivity in detecting antigen-specific T cells and more importantly, they have revealed dramatically different kinetic parameters when compared to 3D measurements.[12] However, these technologies are based on complicated manipulation of individual cells (and dedicated sophisticated instruments) or they require fluorescent labelling of TCR and pMHC molecules, which could interfere with the native behavior of cells and considerably hamper their application for rapid and efficient screening of T cells.

In this work, we take advantage of the exceptional properties of plasmonic biosensors for simple, real-time and label-free affinity analysis to develop a multifunctional and biomimetic methodology for the 2D screening of pMHC-TCR interactions. Particularly, we create an artificial cell membrane scaffold directly on the sensing surface, which can be easily functionalized for anchoring tumor-associated pMHC monomers and serve as a specific probe for capturing the target T cells. Artificial membranes formed by planar lipid bilayers have been previously investigated in biomedical engineering and biosensing since they provide a unique platform for the study of cell biology (e.g. protein-membrane interactions, ion channel regulation) and drug discovery research.[15–17] In the field of immunotherapies, planar lipid bilayers have been used to visualize the formation of molecular pMHC clusters during the immunological synapse formation with live T cells and its direct influence in T cell activation and cytokine secretion.[18,19] Also recently, Mooney et al. proved that lipid bilayer scaffolds mimicking antigen-presenting cell membranes could drastically increase the efficiency of *ex vivo* immune cell proliferation for application in adoptive cell transfer therapies.[20] We have developed an innovative procedure combining a functional lipid bilayer interface with a multi-parametric label-free plasmonic biosensor for real-time T cell affinity interrogation. Our multi-parametric SPR biosensor is capable to scan the full angular spectrum of the reflected light, enabling the on-line correction of possible bulk refractive index interferences that might be caused by the whole cell entity. Furthermore, the sensing instrument is equipped with three different wavelength light sources for simultaneous monitoring, which allows precise structural characterization and validation of the functionalization procedures. To demonstrate the advantages of our approach, we analyze the capture efficiency and structural affinity of $CD8^+$ T cells expressing engineered TCR of incremental affinities for the melanoma antigen NY-ESO-I.[21] Our 2D biomimetic sensor enables the identification of tumor-specific $CD8^+$ T cells in a rapid, efficient and accurate manner, therefore we anticipate the implementation of this technique as a valuable step forward in the development of cost-effective personalized therapies against cancer.

EXPERIMENTAL SECTION

Materials: PEGylated alkanethiols (HS-C6-EG4-OH, HS-C11-EG4-OCH2-COOH, and HS-C11-EG3-biotin) were acquired from Prochimia Surfaces (Sopot, Poland). Lipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine POPC, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine DOPS, and biotinylated 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, biotin-DOPE) were acquired from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Octadechanethiol (ODT), octyl glucopyranoside (OG), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccinimide (NHS), ethanolamine, streptavidin, bovine serum albumin (BSA), and all reagents and salts for buffer preparations were acquired from Sigma-Aldrich (Steinhem, Germany). Anti-CD3 antibody was acquired from Abcam (Cambridge, UK) and biotinylated HLA-A0201/NY-ESO-$1_{157-165}$ monomers were obtained from the Tetramer Core Facility (Ludwig Institut for Cancer Research, Lausanne, Switzerland). All other solvents, reagents and materials were acquired from EPFL chemical shop.

Cell culture and sample preparation: For cell capture experiments, SupT1 cells (CRL-1942, ATCC) were employed. $CD8^+$ SupT1 cells were transduced with different TCR sequences (wild-type WT, DMβ, V49I, and T1φ – knockout TCR) as described in previous works.[6,21] Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. For measurements, cells were harvested from the culture flask and medium was exchanged to ice-cold Krebbs-EDTA buffer by centrifugation (1000 rpm, 3 min) and resuspension three times. The cell density was adjusted to 10^6 cells/mL and the different cell samples were prepared by serial dilution.

MP-SPR instrument and methodology: Surface plasmon resonance studies were performed with a commercial multi-parametric SPR (MP-SPR Navi 210A VASA, Bionavis Ltd, Tampere, Finland) equipped with 3 different wavelength lasers (λ = 670 nm, 785 nm, 980 nm). Affinity analyses were performed with the real-time sensorgrams obtained at 670 nm in both measurement chambers. Layer thickness characterization was peformed by MP-SPR peak spectral analysis using the three wavelengths for fitting and iteration. All measurements were carried out by angular scanning (60° – 75°, scan speed 1.5s), with a continuous buffer flow (Krebbs-EDTA buffer, 10 µL/min) and stabilized temperature at 16°C. SPR sensor chips were also acquired from Bionavis Ltd (Tampere, Finland). All sensors were made of glass with approximately 50 nm gold coating (and 10 nm chromium adhesion layer). For SLB experiments, an additional 10-15 nm SiO_2 layer was coated on the gold surface. Prior to use, the chips were consecutively rinsed with acetone, ethanol and water, and cleaned in piranha solution (H_2SO_4:H_2O_2 3:1) for 1 minute. The SPR instrument was thoroughly cleaned and sterilized (EtOH 70%) before use.

Lipid vesicles preparation: Small unilamellar vesicles (SUV) were prepared according to manufacturer instructions (Avanti Polar Lipids Inc.). Briefly, desired lipid compositions were prepared by mixing the different phospholipids dissolved in chloroform: POPC 90% with either 10% DOPS or 10% biotin-DOPE at 1 mg total mass. Chloroform was gently evaporated under vacuum at room temperature overnight. Lipid precipitated was then dissolved in PBS buffer to 1 mg/mL concentration and the lipid suspension was sonicated for 1h at room temperature to achieve small liposomes (50-100 nm).

Sensor functionalization procedures: Conventional SAM modification was carried out *ex situ* by immersing the gold sensor chips in an EtOH solution of (i) COOH/OH-functional PEGylated alkanethiols (molar ratio 1:10, 1 mM), or (ii) Biotin/OH-functional PEGylated alkanethiols (molar ratio 1:10, 1 mM) overnight at room temperature. After rinsing and drying, the chip was placed in the SPR system for further functionalization. SLB formation was carried out in flow on SiO_2-coated sensor chips as follows: 1 mg/mL SUV in PBS buffer for 20 min; 20 mM NaOH cleaning for 5 min; BSA in PBS for 20 min. For HBM formation, the Au-sensor chip was modified *ex situ* by immersing in an EtOH solution of ODT at 5 mM overnight at room temperature. After rinsing and drying, the in-flow procedure follows: 40 mM OG priming in PBS buffer for 10 min, 1 mg/mL lipid vesicles in PBS buffer for 20 min; 20 mM NaOH cleaning for 5 min; BSA in PBS for 20 min. In all three cases, the rest of the functionalization procedures follow equally. For antibody immobilization, COOH-groups were activated with a mixture of 200 µM EDC and 50 µM NHS in MES buffer for 10 min, and readily contacted to 100 µg/mL of anti-CD3 antibody for 20 min. A 1M ethanolamine solution was employed for 5 min to deactivate the unreacted COOH groups. For pMHC immobilization, a layer of streptavidin (100 µg/mL) was first immobilized and then biotinylated-pMHC monomers (200 nM) were subsequently attached.

Cell capture experiment and affinity analysis: After sensor functionalization, 250 µL ice-cold cell samples of different densities (10^2 – 10^5 cells/mL) were sequentially injected into the SPR measurement chambers at 10 µL/min during 25 min and rinsed afterwards for 10 min. Sensorgrams were acquired by monitoring the weighted centroid of the SPR peak in real time, with simultaneous subtraction of the TIR angle displacements, which are related to bulk refractive index changes. Affinity parameters were extracted by fitting the sensorgrams to a 1:1 interaction model for association and dissociation. Data analysis was carried out with GraphPad Prism 7 software.

RESULTS AND DISCUSSION

Multi-parametric surface plasmon resonance (MP-SPR)

Our biosensor is based on a multi-parametric surface plasmon resonance (MP-SPR) system combined with a biomimetic scaffold for enabling live T cell affinity analysis (Figure 1). SPR biosensors are a widely used technique for label-free and real-time monitoring of biomolecular interactions. The basic working principle consists in the excitation of so-called surface plasmon polaritons in a thin metal layer – usually 45-50 nm gold (Au) –, which generate an enhanced electromagnetic field, that is evanescently decaying into the adjacent dielectric medium, and thereby allows to probe minor refractive index variations caused by molecular interactions. This optical method has found applications in diagnostics and environmental monitoring, enabling fast quantification of a wide range of molecules. Also, it is commonly employed in pharmaceutics and drug discovery for kinetic and affinity studies. By analyzing the SPR sensorgrams, it is straightforward to extract the kinetic parameters for the association and dissociation processes (k_{on} and k_{off}, respectively) and the corresponding affinity constant (K_D) (Figure 1C). However, SPR kinetic analyses are generally performed using biomolecules in solution or, in certain occasions, employing immobilized cells to study the interaction of their receptors with target proteins or drugs.[22–24] So far, to our knowledge, no studies have attempted the direct affinity analysis of cell membrane receptors employing live cells as analytes.

The two main challenges for such measurements are the complexity of capturing the whole cell in flow and the bulk refractive index change occurring simultaneously to the molecular binding, which needs to be corrected for an accurate kinetics analysis. In conventional SPR affinity analysis, measuring the interaction of biomolecules in solution, bulk artifacts are mostly due to different buffer compositions and therefore can be subtracted from a background reference channel. In our case, the target analyte (TCR) is not free in the solution but embedded into the whole cell entity, which inherently produces a change of the dielectric refractive index close to the sensor. For subtracting this bulk effect, an intrinsic reference must be considered.

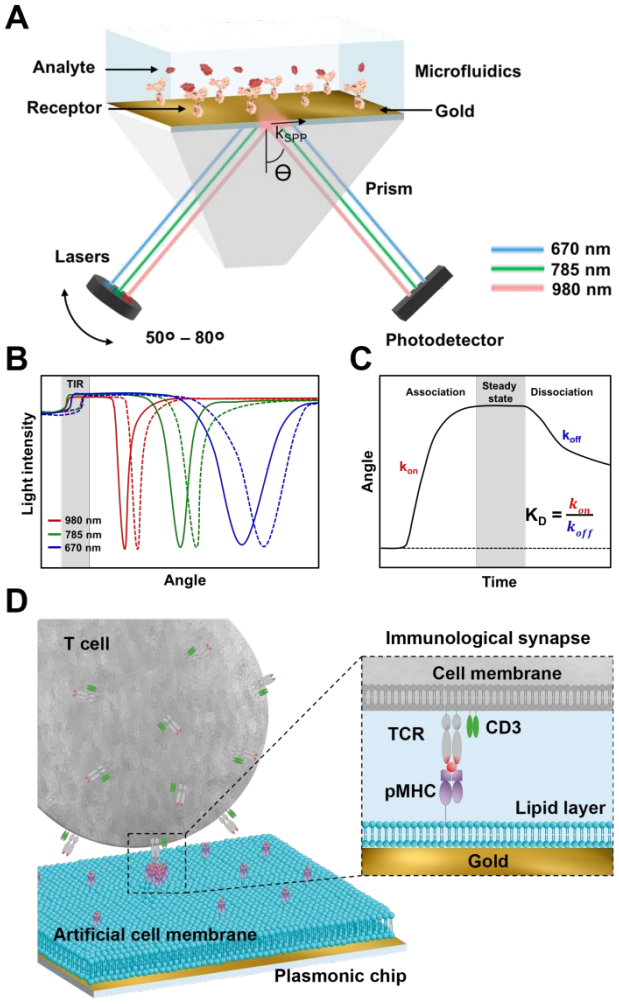

Figure 1. Illustration of the principle combining the multi-parametric Surface Plasmon Resonance (MP-SPR) biosensor with a biomimetic scaffold for live T cell assay. (A) Schematics of a prism-coupling SPR system with three-wavelength lasers and angular scanning working principle. (B) Example of a three-wavelength MP-SPR curve with the total internal reflection (TIR) angle (shaded area) and the SPR dip displacement before and after analyte capture (solid and dashed lines, respectively). (C) Example of a MP-SPR sensorgram with association (capture of analyte), steady state (equilibrium ─ shaded area) and dissociation (release of analyte). (D) Illustration of the biomimetic assay design on a plasmonic chip: a planar lipid bilayer simulates an artificial cell membrane with tumor-specific pMHC tethered on the surface; T cells in flow interact with the biosensor interface through their T cell receptor (TCR).

We employ a multi-parametric SPR (MP-SPR) approach based on the full angular scan of light incidence (50° - 80°). The main advantage of this technique is the real-time monitoring of not only the SPR angular dip displacements – probing the molecular binding on the surface – but also the total internal reflection (TIR) region (Figure 1A and 1B). The TIR spectral shape changes with the bulk refractive index, so that it provides an accurate reference for extracting the intrinsic interferences produced by the whole cell entity.[25] With this method, we can obtain real-time sensorgrams revealing solely the kinetic information of the biomolecular interaction and keeping the target analytes in their natural state and conformation (i.e. embedded in the T cell membrane) (Figure 1D). Additionally, our MP-SPR instrument was equipped with three laser sources (wavelengths: 670 nm, 785 nm, 980 nm) to enable a comprehensive and parallel structural characterization of the metal-dielectric interface properties, such as the layer thickness and the refractive index of the materials deposited on the sensor. These measurements are performed by analyzing the full MP-SPR angular spectra (TIR and SPR dip) before and after each binding step (Figure 1B). Fitting the curves to corresponding models, it is possible to calculate by numerical iteration the approximate values for the thickness and refractive index or each biochemical layer. Technical details are provided in the Supporting Information (SI).

Formation of functional lipid scaffolds on plasmonic surfaces

In order to measure T cell affinities in a two-dimensional (2D) format, the plasmonic sensors were functionalized with different biochemical scaffolds mimicking artificial cell membranes. The 2D affinity analysis requires an intact TCR molecule embedded in the T cell membrane, able to associate and form molecular complexes with co-receptors. In addition, the sensor surface must provide an appropriate microenvironment for efficient cell capturing and attachment, with the specific cell receptors smartly anchored and able to form molecular clusters during the immunological synapse (Figure 1D).

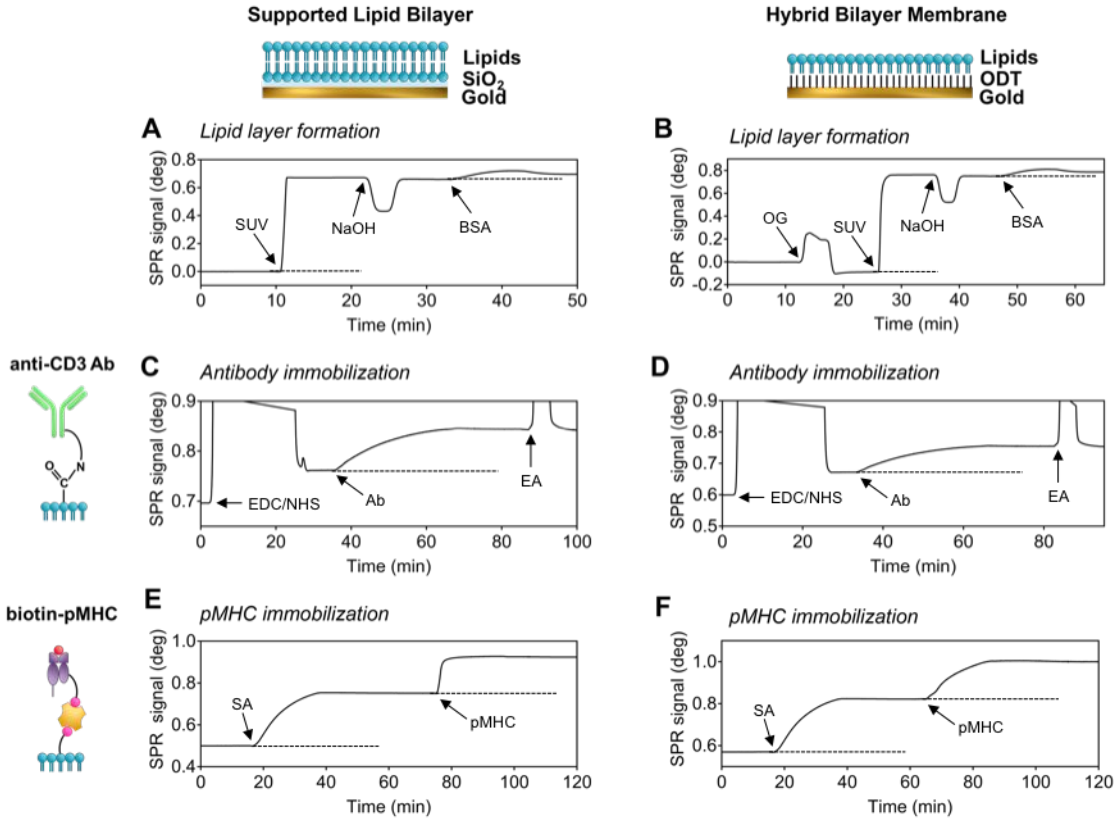

Figure 2. Formation and functionalization of supported lipid bilayer (SLB) and hybrid bilayer membrane (HBM). (A) Formation of SLB by disruption of small unilamellar vesicles (SUVs), rinsing with NaOH and blocking with BSA. **(B)** Formation of HBM by disruption of SUVs on a previously formed alkanethiol SAM and primed with a detergent (octyl β-D-glucopyranoside, OG), rinsing with NaOH and blocking with BSA. **(C-D)** Antibody immobilization by carbodiimide activation of COOH (i.e. EDC/NHS) and blocking with ethanolamine (EA). **(E-F)** Peptide-MHC immobilization through biotin-streptavidin interaction. Dashed lines illustrate the equilibrium signal reached for relevant functionalization steps.

We have studied three sensor interfaces tethered with different bioreceptors: (i) a functional polyethylene glycol (PEG) chemical matrix in the form of alkanethiol self-assembled monolayer (SAM) – used as a reference; (ii) a functional supported lipid bilayer (SLB); and (iii) a functional hybrid bilayer membrane (HBM) (Figure 2). PEGylated alkanethiol SAMs are considered a standard methodology for gold surface modification, providing a hydrophilic chemical matrix with excellent surface coverage and composition flexibility for incorporating different functional groups (-

COOH, -NH_2, -biotin, etc.). This strategy is robust, simple and highly reproducible.[26,27] However, the immobilization of bioreceptors on a SAM renders static attachment and thus provides no mobility, hampering the formation of molecular clusters or specific receptor-ligand assemblies when contacting the T cell membrane.[28] Such mobility can be accomplished with planar supported lipid bilayers, which are known to present high fluidity and allow natural rearrangements within the surface.[18,29,30] SLB can be formed on planar substrates by disruption of small unilamellar vesicles (SUV) upon contact with hydrophilic surfaces, like SiO_2.[31] Indeed, the vast majority of preparation methods, characterization and applications of SLB employ glass substrates. Hence, the common strategy to prepare SLB on plasmonic sensors is by coating the gold surface with a thin layer of SiO_2 (10-15 nm). Although this technique is effective and relatively simple when considering conventional SPR sensors, transferring to more sophisticated plasmonic nanostructured surfaces could be problematic (e.g. reducing sensitivity).[17] The formation of hybrid lipid bilayers could be an alternative solution.[32–34] HBM is built by modifying the gold surface with a hydrophobic alkanethiol SAM (e.g. octadecanethiol, ODT), which mimics the hydrophobic carbon chains of lipids. When vesicles are introduced after a priming step with specific detergent (octyl β-D-glucopyranoside, OG), they disrupt to form the complementary lipid layer on the hydrophobic SAM. The priming step is necessary to assure a well-ordered and clean hydrophobic monolayer formation in aqueous solution conditions.[35]

The large versatility in lipid composition allows preparing SUVs with different mixtures of lipids containing specific functional head groups. Particularly, we employed two different SUV compositions based on phosphatidylcholine (POPC) as the main lipid component (90% molar ratio), combined with either a COOH-functional lipid (10% DOPS) or a biotin-functional lipid (10% biotin-DOPE). On these scaffolds we selectively attached two bioreceptors for cell detection and study: CD3 antibodies (anti-CD3 Ab) were chosen as polyclonal capturing elements and immobilized by covalent amide binding to COOH functional lipid layer, and specific peptide- MHC complex (pMHC) monomers for tumor-specific detection, immobilized via biotin-streptavidin (SA) interaction. All the procedures were monitored and characterized with the MP-SPR biosensor as shown in Figure 2.

The analysis of the sensorgams and angular spectra obtained for both SLB and HBM procedures (Figure 2A and 2B) confirmed and validated the formation of stable, uniform, and planar lipid layers on the sensor surface. It can be observed that the SUV disruption occurs as a cooperative binding event – sharp signal increase –, meaning that upon contact with the surface all lipid vesicles rapidly break down and reassemble in the form of a flat layer. The rinsing step with sodium hydroxide (NaOH) and subsequent blocking step with bovine serum albumin (BSA) resulted in negligible responses proving that neither vesicles nor empty spaces remained on the functionalized sensor surface. To further characterize the formation of both membranes, the layer thickness and refractive index of the interface were determined by the MP-SPR instrument. This measurement was carried out by fitting the angular scans obtained for the three wavelengths (670 nm, 785 nm, 980 nm) to their corresponding models (Au-SiO_2 layers and Au layer, respectively), and resolving the unknown values (layer thickness and refractive index) by numerical iteration (see details in the supporting information (SI)). For the SLB, we measured a thickness of approximately 5-6 nm, while for the HBM the alkanethiol SAM was measured to be around 2 nm and the lipid monolayer 3 nm. These values agree well with expected and reported values for the lengths of the biomolecules forming planar bilayers.[36,37]

Polyclonal detection of human T cells in real time

To demonstrate the feasibility of our biochemical matrices for the capture and detection of T cells, we functionalized the three different scaffolds (PEGylated alkanethiol SAM, SLB, and HBM) with anti-CD3 antibodies (Figure 3). These antibodies target a membrane receptor complex (cluster of differentiation 3) expressed in T cells that serves as a co-receptor associating to the TCR for activation processes. Anti-CD3 antibodies are widely employed as markers and biorecognition elements for the identification of polyclonal T cells with high efficiency and reliability.[20] Antibody immobilization was carried out by covalent binding of their amine terminal groups (e.g. Lysine residues) to the COOH groups exposed on the SAM, SLB, and HBM. This well-known procedure requires the activation of carboxylic groups via carbodiimide intermediate (EDC/NHS) to form a highly stable amide link with the biomolecule (Figures 2C and 2D). Later, the unreacted active groups can be deactivated with ethanolamine. Although it renders to a randomly oriented antibody layer, the simplicity and robustness of this methodology ensure a highly reproducible and well-controlled antibody immobilization. Regarding cells, we used human SupT1 cells (T lymphoblast expressing multiple T lineage markers, i.e CD3, CD8) tranduced with the wild-type TCR specific for the HLA-A0201/NY-ESO-$I_{157-165}$ pMHC complex.

In order to optimize the interface and assay conditions for the cell capture, several factors were considered. Due to the large size of mammalian cells (10-15 μm), we employed a low molar ratio of COOH functional groups for all the biochemical matrices (10%). It has been shown that the relatively low density of antibodies on the surface greatly facilitates the detection of large analytes, such as cells or pathogens.[38] Flow conditions, temperature, and dilution buffers were also thoroughly defined for the analysis. Cell samples were collected from culture media and transferred to ice-cold Krebbs buffer containing 3 mM ethylenediaminetetraacetic acid (EDTA). This buffer guarantees a proper cell dispersion in the solution, avoiding possible cell aggregations, ensures sufficient ionic strength and glucose content, and the low temperature prevents membrane receptor internalization.[39] For cell measurements, 250 μL samples were introduced and flowed over the sensor surface at 10 μL/min during 25 min, followed by subsequent rinsing with the same buffer at the same flow rate. The sensorgrams obtained for cell capture at different cell densities with the three different scaffolds are shown in Figure 3 (B-D). In all three cases, we obtained a limit of detection of approximately 500 cells/mL. This high sensitivity already demonstrates that the designed sensing interfaces could be extremely valuable for direct cell detection, isolation, and analysis. Compared to the state-of-the-art, it is worth mentioning that very few works have reported the real-time detection of mammalian cells with plasmonic biosensors, which exposes the evident difficulty and challenges of such process mainly related to mass transport and the complexity

and delicacy of live cells.[40] To our knowledge, most recent SPR biosensors using cells as analytes do not show detection limits below 10^4 cells/mL.[39,41–43] With our strategy, we were able to detect up to 2 orders of magnitude lower concentrations.

Differences in the MP-SPR sensorgrams revealed contrasting behaviors of cell capturing when employing a conventional static SAM or the planar membrane scaffolds. It can be noticed that both SLB and HBM provided a slight but significant higher sensitivity for cell detection, resulting in larger SPR responses for high cell densities especially. This might be attributed to the preference of cells for attaching to biomimetic matrices and also to antibodies immobilized on fluidic membranes with better ability to rearrange on the surface and accommodate a higher number of cells. Interestingly, distinct sensorgram shapes were observed during the dissociation phase. For SAM measurements, the signal decrease after reaching the steady state was qualitatively higher than for SLB or HBM. This effect could reflect the dissociation and washing of non-immobilized cells on the sensor surface, confirming that more cells are captured by functional artificial membranes and the attachment is more efficient.

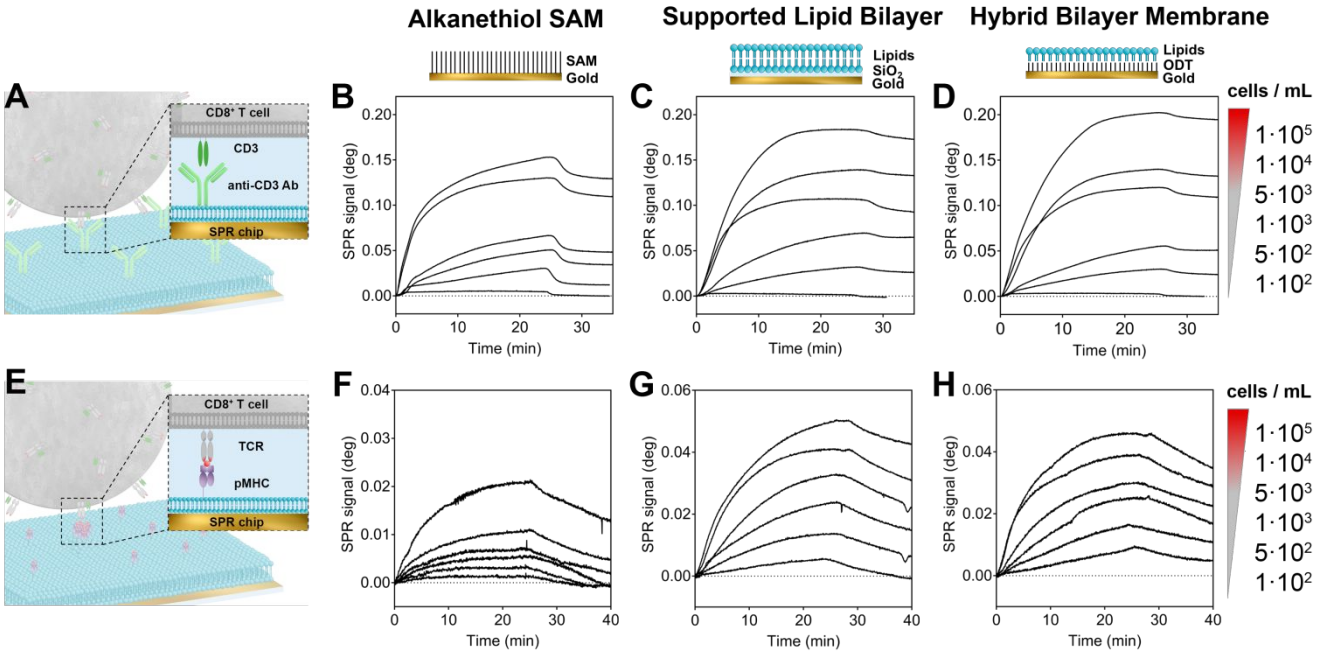

Figure 3. Polyclonal and tumor-specific cell capturing and detection on the different functionalized surfaces: (A) Illustrative scheme of a lipid membrane functionalized with anti-CD3 antibodies capturing a $CD8^+$ T cell. Wild-type $CD8^+$ T cell attachment to anti-CD3 antibodies immobilized on **(B)** an alkanethiol SAM, **(C)** a SLB, and **(D)** a HBM. **(E)** Illustrative scheme of a lipid membrane functionalized with pMHC monomers capturing a $CD8^+$ T cell. Wild-type $CD8^+$ T cell attachment to specific pMHC immobilized on **(F)** an alkanethiol SAM, **(G)** a SLB, and **(H)** a HBM. All sensorgrams show sequential injections of cell samples at different densities: 10^2-10^5 cells/mL.

Tumor-specific detection of human T cells in real time

An attractive and extremely relevant biomedical application for our interface biosensor design is the detection and analysis of tumor-specific $CD8^+$ T cells. The real-time capture of specific T cells in a label-free plasmonic sensor would enable to analyze the kinetics and affinity of intact T cells for tumor-associated antigens, without any chemical or mechanical interference. To accomplish this, instead of functionalizing the biochemical matrices with anti-CD3 antibodies, which target the majority of T cells, we immobilized synthetic biotinylated HLA-A0201 major histocompatibility complex (MHC) monomers loaded with the NY-ESO-$I_{157\text{-}165}$ antigen (Figure 3E). Besides, the $CD8^+$ SupT1 cells were genetically transduced with the wild-type TCR isolated from a melanoma patient and recognizing this specific HLA-A0201/NY-ESO-$I_{157\text{-}165}$ complex.[21] Note that the previous measurements with anti-CD3 antibodies were performed with these same cells. To facilitate pMHC immobilization, the monomers were enzymatically biotinylated on the heavy chain in such a way that the tag does not interfere with the peptide recognition site and enables a simple attachment via biotin-streptavidin interaction. This affinity-based immobilization strategy is widely employed in biosensing and microarray technologies due to its advantages in terms of stability and robustness. In our case, the procedure generally consisted in forming a biotin-functional layer – either with biotinylated PEG alkanethiols for the conventional SAM matrix or by using biotin-DOPE lipid in the SUV composition of the lipid membranes. Then, streptavidin molecules (with 4 biotin binding sites) were attached to the scaffolds and the remaining available sites were used to incorporate the biotinylated pMHC monomers (see Figure 2E and Figure 2F). The biotin density of the three different biochemical matrices was also adjusted to minimize steric hindrance issues for cell capturing, employing only 10% molar ratio of the biotinylated reagents.

Adopting the conditions previously optimized for anti-CD3 detection (e.g. flow conditions, temperature, and buffer), $CD8^+$ SupT1 cell samples at different concentrations were measured with the MP-SPR biosensor (Figure 3 F-H). The first and more significant difference observed when comparing to CD3

targeted assays is the relatively lower signals obtained for tumor-specific capturing (4-6 times lower). This result is not too surprising as the affinities between TCR and pMHC usually are relatively low in nature (i.e. in the μM range). To confirm cell binding to pMHC, we also acquired optical microscope images of gold chips selectively modified with pMHC after incubation with SupT1 cell samples (see SI). The images revealed that the cells effectively contact and bind to the pMHC functionalized spots while no cells attached to the regions without the specific pMHC. It is important to notice though that the dissociation phase in these sensorgrams is clearly different to the polyclonal capturing signals. The interaction between T cells and pMHC-functional layers is reversible, indicating that after association and equilibrium, the immune cells are being released steadily with the continuous flow. This observation agrees with the biological analysis reported in the literature, stating that a relatively quick contact between wild-type TCR and specific peptides expressed in antigen-presenting cells is sufficient for triggering T cell signaling.[6] Yet, both the study of association and dissociation kinetics are crucial to determine T cell responsiveness.

Structural affinity analysis of tumor-specific T cells expressing NY-ESO-I-specific TCR of incremental affinity

After optimizing and evaluating the sensitivity for in-flow cell capturing with our biomimetic scaffolds, we carried out the first label-free affinity analysis of tumor-specific human $CD8^+$ T cells in real time and 2D configuration using a plasmonic biosensor. We characterized the kinetics of several bioengineered T cell variants expressing HLA-A0201/NY-ESO-I-specific TCRs with different affinities. Particularly, we employed the $CD8^+$ SupT1 cells transduced with the wild-type TCR (WT) isolated from a melanoma patient, a high-affinity (DMβ) and a low-affinity receptor (V49I) designed *in silico* and validated by SPR, and used untransduced SupT1 cells (T1φ) as negative control.[6,21] The four cell variants were measured with both biomimetic matrices, the SLB and HBM functionalized with the corresponding pMHC (Figure 4).

At first glance, the MP-SPR sensorgrams already showed clear differences in the cell affinity for the functionalized surfaces. Compared to the WT cells (Figure 4C and 4D), the signals obtained for the DMβ variant reached higher values for all cell concentrations, which can be attributed to a higher number of cells attaching to the sensor (Figure 4A and 4B). Besides, it can be observed that cell dissociation in DMβ sensorgrams occurs at a much slower rate. On the other hand, T1φ cells, which do not express any TCR, did not attach to the surface and therefore MP-SPR signals were negligible (Figure 4G and 4H). These negative-control sensorgrams also demonstrate the excellent specificity and selectivity of our interface design, avoiding undesired cell sedimentation and non-specific binding. Special focus worth to be placed on the V49I experiments (Figure 4E and 4F). This engineered TCR has very low affinity for the HLA-A0201/NY-ESO-I complex and therefore represents a critical challenge for the detection and interrogation of very low affinity T cells, like CD4 T cells. Indeed, 3D affinity analyses of such receptor by conventional SPR usually fail, lacking enough sensitivity for the detection.[21] Our results show that the combined use of MP-SPR sensor, the functional biomimetic interfaces and the whole T cell as analyte enable the detection of the low-affinity lymphocytes and subsequent 2D kinetic analysis. Finally, it should be mentioned that no significant differences were observed between the SLB and the HBM strategies. These results indicate that our hybrid bilayer membrane acts alike the conventional supported bilayers, probably with similar fluidity and providing the cells with a microenvironment that simulates the contact with another cell surface. Our experiments are introducing a novel methodology that can be readily applied to more sophisticated nanoplasmonic sensors, with higher sensitivities, and compatibilities for integration in lab-on-a-chip devices and multiplexing.

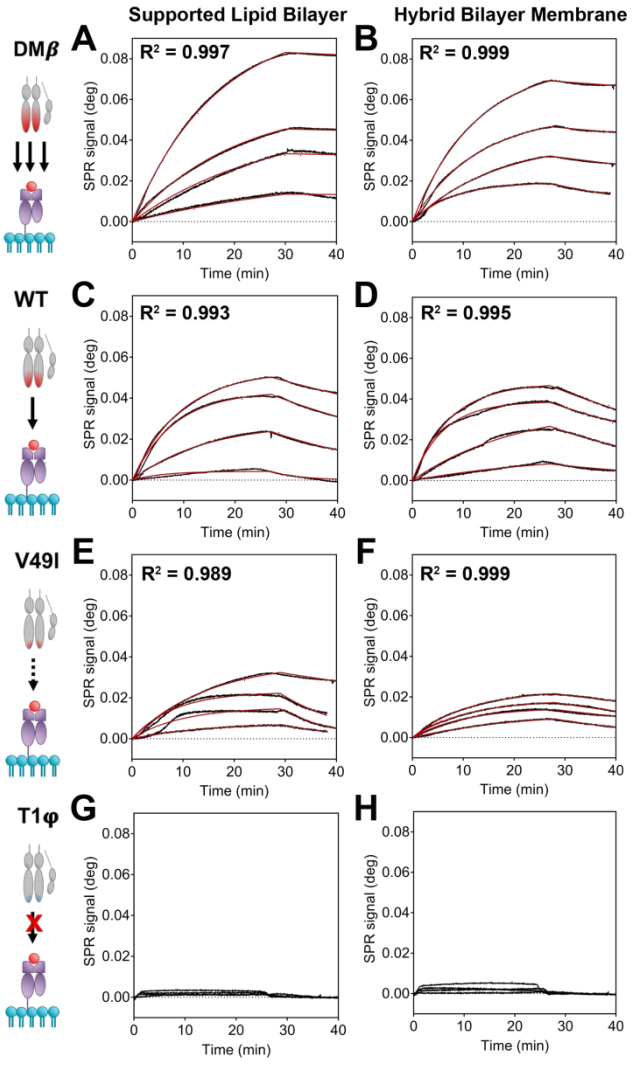

Figure 4. Two-dimensional structural affinity analysis of tumor-specific $CD8^+$ T cells. Cell capture and analysis on pMHC-functionalized SLB and HBM with **(A,B)** DMβ T cells, **(C,D)** Wild-type (WT) T cells, **(E,F)** V49I T cells, and **(G,H)** Negative control tests with T1φ T cells. All sensorgrams show the response of different samples with densities: 10^2, 10^3, 10^4, 10^5 cells/mL (bottom to top). Graphs are plotted with the same axes scale for easier comparison. Red lines correspond to the model fitting for kinetic parameters determination.

To ultimately demonstrate the important capabilities of our technique for 2D structural affinity analysis of tumor-specific immune cells, we determined the kinetic parameters of the

interactions occurring between pMHC-functionalized membranes and the different T cell variants. The MP-SPR sensorgrams were fitted to a Langmuir-based function, assuming a 1:1 specific interaction. In order to confirm and validate our results with standard technologies, we extracted the k_{off} parameter for each set of measurements and compared with previous results obtained with reversible fluorescent pMHC multimer (NTAmer technology using flow cytometry)[6] and 3D molecular SPR analysis[21] (Table 1). As expected, our kinetic analysis significantly differs from the 3D measurements. Instead, the values are comparable to the ones obtained by more novel and reliable methodologies (i.e NTAmers) that take into account the cell membrane organization and contribution. By analyzing the strength of the interaction between molecular complexes (i.e. TCR and pMHC clusters) rather than isolated molecules, we include avidity factors in the measurement, which increases the accuracy of the study.[44] Due to conceptual restrictions in the numerical analysis, the values extracted for the k_{on} and K_D parameters in our case should not be quantitatively compared with other SPR measurements. These parameters highly depend on the exact molar concentration of the target analyte, which we could not determine accurately in our laboratory since we employed the whole live cell as analyte. However, the qualitative study of the sensorgrams agrees with the TCR designs and affinities expected for the different cell variants. The remarkable sensitivity of our device enables the binding parameter analysis of very low affinity T cells that seem to play an important role in immune mediated responses.[45] In this regard, further investigation and evaluation would be necessary to exploit the plasmonic sensing capabilities not only for simple molecular analysis but also for the interrogation and screening of live cells.

Table 1. Kinetic parameters for NY-ESO-1 specific TCR variants

TCR variants	3D SPR analysis[a]	Fluorescent NTAmers[b]	2D MP-SPR analysis	
			SLB	HBM
	k_{off} ($x10^{-2}$ s^{-1})	k_{off} ($x10^{-2}$ s^{-1})	k_{off} ($x10^{-2}$ s^{-1})	k_{off} ($x10^{-2}$ s^{-1})
DMβ	4.5	0.78	0.57	0.63
WT	23.0	4.08	3.65	3.81
V49I	n.a.	21.21	17.83	19.54

Abbreviation: n.a., not applicable

[a] SPR measurements reported in ref. 21

[b] Fluorescent NTAmer measurements reported in ref. 6

CONCLUSIONS AND OUTLOOK

We have introduced a simple but highly efficient strategy for creating functional artificial cell membranes on plasmonic surfaces that enable tumor-specific cell detection, screening, and structural affinity analysis with an excellent sensitivity and reliability. We have demonstrated the direct and label-free interrogation of whole and intact cells for determining the TCR structural affinity for a relevant melanoma antigen. The multi-parametric SPR biosensor enables the determination and evaluation of the 2D kinetic parameters of TCR-pMHC interactions employing whole intact cells as analytes. Functionalizing the plasmonic surface with planar lipid layers tethered with specific pMHCs – simulating the membrane of antigen-presenting cells –, we enhanced the sensitivity for cell capturing, providing in turn an appropriate microenvironment for reliable analysis. This method with its ability to study TCR-pMHC affinities can be a valuable tool in cell therapy design and manufacturing processes. For instance, it can be used to evaluate newly engineered TCR designs with selected cell lines. Due to the high versatility provided by our functionalization strategy, the method can be readily adapted to the analysis of a wide variety of cells, including immune, tumor or stem cells. By selecting suitable membrane receptors, the complete profiling of cell functionality, specificity, and activity can be obtained. In this regard, it would be powerful to eventually extend the method for operation in more complex matrices such as blood. This would for example enable the direct capture and isolation of tumor-specific primary T cells from peripheral blood samples, which could be readily employed for immunotherapy development. Additionally, incorporating single-cell measurement capabilities could enablea more accurate analysis and identification of anti-tumor activity and the possibility to decipher the underlying mechanisms of immunological functions. For such future directions, it will be necessary to carefully optimize surface chemistry, microfluidics, and assay conditions to mitigate non-specific binding as well as introduce new plasmonic sensing schemes to improve the overall device sensitivity. The upcoming synergy between nanotechnologies and bioengineering methods has the potential to revolutionize the field of personalized therapies for cancer, autoimmune disorders, or other critical diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

- Determination of layer thickness and validation of tumor-specific cell binding (PDF).

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ABBREVIATIONS

MP-SPR, multi-parametric surface plasmon resonance; pMHC, peptide major histocompatibility complex; TCR, T cell receptor;

SAM, self-assembled monolayer; SLB, supported lipid bilayer; HBM, hybrid bilayer membrane.

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