Nanochips assisted peptide screening for clinical development of CAR-T cell immunotherapy

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Article

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ABSTRACT:

Immunotherapy using CAR-T cells is a new paradigm technology for cancer treatment. To avoid severe side effects and tumor escape variants observed for conventional CAR-T cells approach, adaptor CAR technologies are under development, where intermediate target modules redirect immune cells against cancer cells. In this work, silicon nanowire field effect transistors are used to assist in the development of target modules for an optimized CAR-T cell operation. Focusing on a library of seven variants of E5B9 peptide that is used as CAR peptide epitope, we performed multiplexed binding tests in serum using nanosensor chips. Peptides have been immobilized onto the sensor to compare the signals of transistor upon titration with anti-E5B9 antibodies. Correlation analysis of binding affinities and sensitivities enabled a selection of best candidates for the interaction between CAR and target modules. Finally, cytotoxic functionality of CAR-T cells in combination with the selected target modules were successfully proven. Our results open the perspective for the nanobiosensorics to go beyond the early diagnostics in the field of clinical cancer research, and paves the way towards personalization and efficient monitoring of the immunotherapeutic treatment, where the quantitative analysis with the standard techniques is not an option.

Key words: CAR-T cells, UniCAR-T cells, cancer, silicon nanowires, nanosensor, field effect transistor, ELISA, immunotherapy

1. INTRODUCTION

Cancer affects our society globally. According to the most recent cancer statistics [1], approximately 19 million new cases of cancer and around 10 million deaths were documented in 2020. Surgery, radiotherapy and chemotherapy are the most common types of cancer treatments available nowadays [2]. In recent years, immunotherapy, especially T cells genetically modified with chimeric antigen receptor (CARs) are considered as milestone technology for cancer treatment [3, 4]. After remarkable remissions observed in clinical trial patients, anti-CD19 and anti-CD269 CAR-T cells received approval from Health Canada and U.S Food and Drug Administration (FDA) [5] for treatment of hematologic malignancies in 2017. Genetically modified CAR-T cells express specific receptors (CARs) on their surface for an effective tumor addressing. Upon binding on tumor cells, CARs induce a signal cascade resulting in T cell activation and thus elimination of cancer cells. Despite the demonstrated clinical success, CAR-T cell therapy can be accompanied by severe side effects including cytokine release syndrome, neurologic toxicity, on-target/off-tumor reactions, and anaphylaxis [6-8].

While reactivity of conventional CAR-T cells cannot be controlled in the human body, the recently established adaptor universal (Uni)CAR platform enables the on/off switch of CAR-T cell activity providing an improved safety management [9-13]. This system consists of two components: (I) T cells expressing UniCARs, that comprise the extracellular anti-E5B9 single-chain fragment variable (scFv) derived from the anti-E5B9 monoclonal antibody (mAb), the transmembrane domain and intracellular signaling domains, and (II) target modules (TMs) that consist of an antigen binding moiety equipped with the peptide epitope E5B9 (Figure 1A). TMs play a very important role in this concept as they simultaneously bind to tumor antigens and the UniCAR-T cell and mediate tumor lysis. As the TMs have usually a short biological half-life [14], the UniCARs will, thus, switch off after stopping the TM infusion. This opens an instrument of a control over the therapy, which is absent in the conventional CAR therapy approach.

Note, the strength and persistence of the interaction between UniCAR-T cells and tumor cells is of paramount importance and its optimization is valuable for an advanced therapeutic outcome. It depends on the recognition of the E5B9 peptide epitope (as part of the TM) by the extracellular UniCAR anti-E5B9 scFv. Furthermore, as the UniCAR platform is subject of current clinical trials (NCT04633148, NCT04230265 [15]), the optimal dosing of TMs and UniCAR-T cells in human blood are also of high interest. Despite responding to therapy, first clinical trial patients showed very low concentrations of TMs in the blood that could so far not be detected using conventional techniques [15]. Taking into account the potentially high amount of molecular complexes to be tested, and the low concentrations expected in blood

post-injections, the use of compact tools capable of multiplexed detection with exceptional sensitivity is indispensable for such studies.

Nanobioelectronics is an emerging field considered for future clinical applications, offering highly sensitive, label-free, rapid and reagent-saving analytical tools. As a part of this field - nanobiosensors, have shown their efficiency in ultrasensitive detection of low concentrations of biomolecules [16-26] and single cells [27-30]. While the major focuses in the community are dedicated to the delivery and characterization of new efficient transducer materials for point-of-care biosensors [31-34], there is a limited number of reports with the clinical applications of such nanodevices [27, 35-40]. We attribute it to the necessary complexity of the system, need for a long-term process monitoring, and thus, a stable performance of the sensor. Particularly, when reviewing the application of nanobiosensors for cancer research, the major contributions have been done mainly in the early diagnosis of cancer with more than 70% of articles published in the last decade (see Figure 1B). Next 20-25% of works included the monitoring of the patient response to the treatment through exhaled breath [41], various cell signaling agents [42-44], and growth factors [45, 46]; counting circulating cancer cells [47-49]; detecting mutation in cancer [50-53]; tracking cancer progression [54, 55]; and quantifying anti-cancer drug residual in patient [56, 57]. Meanwhile, application of nanoelectronics in the development of the cancer therapy remains unexplored with only a handful of quality works and lack of breakthrough proof-of-concept. Works in this direction largely demonstrate monitoring of living cancer cell response for e.g. drug screening or change in treatment condition [58-61] and detection of drug-resistance gene/cell [62, 63] [64]. Still, there is confidence that the nanosensors can accelerate the progress of many clinical applications, beyond traditionally considered diagnostics and point-of-care. Thus, works related to the use of nanobioelectronics for the development of personalized cancer therapies and monitoring of therapy efficiency are still expected. To the best of our knowledge, the utilization of a purely nanoelectronic biosensor in immunotherapy is completely absent.

In this work, we go beyond the traditional use of the nanobiosensors in the diagnostics area and explore their ability to assist in clinical development and optimization of the UniCAR-T cell therapy, at a proof-of-concept level. For these purposes, we employ an array of top down fabricated silicon nanowire (SiNW) ion-sensitive field-effect transistors (ISFET) – responding to changes in the distribution of the surface charge within Debye layer, mainly affecting the charge carriers in the conductance channel [64]. SiNW based devices have outstanding relevance for this specific task due to several arguments: (i) their long term stability as biosensor device even upon multiple use, (ii) lower stand-by energy consumption, and (iii) compatibility to a technology based on complementary metal oxide semiconductor (CMOS) and thus higher commercialization potential [65]. Although the use of SiNW FETs as

biosensors have been reported for more than a decade ago [66, 67] , their applications still did not enter the clinics.

In the following, we applied the nanoscopic FET sensor to analyze the interaction between the peptide epitope E5B9 and the anti-E5B9 scFv, used for optimization of the UniCAR approach. Success at this stage creates a tool for detection of UniCAR-T cells or TMs in the blood or sera of patients, receiving UniCAR-T cell therapy. This aspect is of high interest for continuous monitoring of the UniCAR approach efficiency.

To evaluate the SiNW FET sensor-based technology and to identify the most suitable E5B9 peptide to redirect UniCAR-T cells, the interaction between seven E5B9 peptide variants (Figure 1C) and the corresponding anti-E5B9 mAb was investigated using the SiNW FET sensors in comparison to ELISA. Therefore, the multiple peptides have been immobilized onto the sensing area (Figure 1D), and threshold voltage shift of the transistor was recorded upon titration of anti-E5B9 mAb in order to pre-select the optimal epitope for UniCAR-T cell redirection. Finally, in vitro cytotoxicity models were used to test whether TMs equipped with different E5B9 variants are able to redirect UniCAR-T cells for elimination of prostate stem cell antigen (PSCA)-expressing tumor cells [77].

2. RESULTS AND DISCUSSION

2.1. *Fabrication of nanowire based FET sensors*

A sensor chip consisting of 16 *n*-doped ($5 \times 10^{18} \text{ cm}^{-3}$ phosphorous) SiNW FET devices (Figure 2A) was fabricated on an 8-inch silicon-on-insulator (SOI) wafer, as described in previous works [16, 17, 68-71] and **Materials and Methods** (Figure 2A left panel). The width of a single wire is about 50 nm; assembly into a honeycomb pattern results in the total sensing area of about $45 \mu\text{m}^2$. Compared to an array of straight wires, the patterned assembly of nanowires (Figure 2A right panel), offers large sensing area, better electrical characteristics, and higher mechanical stability, making it preferable for biosensing applications [70, 72, 73]. Nanowires and reference electrode are directly exposed to the environment, while the remaining elements of the chip are isolated using SU-8 photoresist. During measurement, a liquid is placed in the contact with the nanowires, covering the reference electrode and the SiNW channel. Attachment of the biomolecules to the surface of the silicon changes the surface potential at the nanowires, thus, influencing the conductance in the nanowire structure [74-76]. For the case of *n*-typed silicon channel an increase in conductance is expected with positive gate voltage bias, while a decrease is anticipated with negative gate voltage as electrons are the main carriers in *n*-doped silicon. Figures 2B show typical transfer characteristics of the SiNW FET measured in deionized water. The source-drain current (I_{SD}) increases when rising the

source-drain voltage (V_{SD}) with different gate bias (V_G). In addition, the source-drain current also escalated as higher positive gate voltage is applied which clearly indicates n-type behavior of SiNW FET. Due to heavily doped channel with phosphorus, inversion mode of n-type FET has been converted to accumulation mode. As a result, the device is normally in an ON-state at the gate bias $V_G=0$ V (Figure 2B), which is advantageous for low power sensor application.

2.2. Functionalization of the silicon surface

Figure 1A represents a scheme of the UniCAR approach: genetically modified UniCAR-T cells interact with the tumor cells via TMs [77] [78, 79]. TMs consist of two components: the peptide epitope E5B9 (red sphere) recognized by UniCAR-T cells and a binding domain that binds to a certain target antigen overexpressed on the surface of tumor cells (orange triangles). The interaction between effector and target cells depends on the affinity of the UniCAR anti-E5B9 scFv towards the peptide epitope E5B9 as part of the TM and can influence the functionality of the UniCAR system. Even a minor change in the amino acid sequence can, in principle, cause a reduction or increase of binding ability and thus, affect the efficiency of the UniCAR-T cell-based therapy. Therefore, a fast and simple screening method for evaluation of the peptide recognition could be valuable to identify the optimal peptide sequence allowing a well-balanced interaction between UniCAR-T cells and TMs resulting in efficient UniCAR-T cell activation and tumor cell lysis. In this study, we have generated different E5B9 peptide epitope variants and analyzed them via the SiNW FET technology.

Here, the SiNW sensors **mimic the interaction of the peptide epitope E5B9 with the anti-E5B9 mAb** used for construction of the UniCAR to explore how alterations in E5B9 structure may influence the efficiency of the UniCAR system. Seven different variants of the peptide E5B9 were covalently bound to the SiNW surface (see **Materials and Methods**, and Figure 1C, D). The original sequence of E5B9 derived from the human La/SS-B protein includes ten amino acids (**KPLPEVTDEY**)[80], as highlighted in black in Figure 1C. In this work, we investigated two groups of E5B9 variants namely M and A group. The M-peptides (**M1**, **M2**, **M3**) have one or two exchanged amino acids in the main sequence while the A-peptides (**A1**, **A2**) have additional functional groups at the N- or C-terminus which might be useful for modification with chelators [81]. In addition, **M0** and **A0** were synthesized to resemble the wild-type E5B9 sequence and act as a reference in each group.

The process of covalently attaching these peptides to SiNW consists of three steps [82, 83] and is briefly described in Figure 2D (step 1 to 3) followed by a blocking step and anti-E5B9 mAb detection. Each step is verified by several methods including sessile drop contact angle measurement (Figure 2E), fluorescent microscopy (Figure 2F) and FET electrical measurements of the subthreshold voltage shift (Figure 2G). Briefly, the silicon nanowire chips were first treated with oxygen plasma and left in 3-triethoxysilylpropyl succinic anhydride

(TESPSA) vapor for four hours as described in [82]. Measuring the transfer curve of SiNW FET, we observed a respective 'positive' shift of about ~110mV compared to the bare sensor (Figure 2G), indicating a change in surface potential at the nanowires. Next, we attached the peptide sequences at the silicon nanowire, incubating a drop of 0.1 mg/mL peptide solution in PBS, at room temperature. Peptides are expected to covalently bind to the SiNW surface after one hour in contact, due to a ring opening reaction forming an amide bond [33, 82, 84]. In this context, all peptides bear in addition to the free N-terminus another primary amino group at the side-chain of the lysine residue. Even though the pK_a value of ϵ -amino group might be higher than that of the α -amino group [83], thus favouring the reaction via the α -amino group at pH 7.4, amide bond formation probably occurs via both amino groups [84]. We then removed any unbound peptides by washing the sensor with PBS solution containing 0.05% TWEEN 20 and blocked remaining binding sites of TESPSA linker by ethanolamine. The contact angle results (Figure 2E) and fluorescent staining using anti-E5B9 mAbs and fluorescent-labeled-secondary Abs (Figure 2F) demonstrated the successful functionalization of the silicon surface (**SI 2,3**).

2.3. Detection of anti-E5B9 mAb binding using SiNW FET devices

Next, we studied the response of each sensor to the presence of the anti- E5B9 mAb in PBS. A serial dilution of mAb was prepared in 1x PBS (0.01 M, pH=7.4) ranging from 0.1 fg/mL to 0.1 ng/mL. For each measurement, a 10 μ L-drop of mAb was incubated on functionalized devices for 15 minutes. In a ISFET biosensor, the biological sensing activities must take place within Debye length so that the newly established electric field does not screen out by moving charge carriers in high ionic solution [33]. Typical physiological samples such as PBS and blood or serum result in a Debye length λ_D of around 0.7 nm, which is much shorter than the size of regular biomolecules (5-10 nm for I_{gG} antibody) [85]. The incubation for Ab binding was made in 1x PBS to maintain the peptide's function, however, all electrical measurements were conducted in 0.01x PBS in which the Debye length λ_D is ~7.61 nm [86], sufficient to sense the binding of the mAb to the immobilized peptides (original E5B9 sequence with molecule weight ~1.3kDa results in size <1 nm [87]). Figure 3B exemplarily shows the transfer characteristics (I_{SD} - V_G) of **A0**-functionalized SiNW FET after incubation with different concentrations of anti-E5B9 mAb. The binding of charged species to the surface of the nanowire acts similar to applying a gate potential, modulating the conductance through the SiNW via depletion of enriching the charge carriers in the silicon channels. Note that all measurements were recorded continuously until steady state is reached and no drifting was observed afterwards, thus, the transfer curve shift is purely a contribution of anti-E5B9 mAb binding. After repetitive measurements of all peptides (each point is repeated 5 times), we observed no change in the slope of transfer characteristics but only the shift of the transfer characteristics. Therefore, we extract the threshold gate voltage (V_T) at a fixed current through the source and drain $I_{SD}=10^{-$

⁷A, which can be translated as shift of transfer curve, to construct the calibration for further analysis (Figure 3A). Note that the V_T change is detectable even at a concentration as low as one fg/mL. For better comparison between different peptide variants, Figure 3A brings all calibration curves to the same origin in the same scale. The graph shows that the V_T change of all variants follows a general binding assay curve in which the binding is quickly saturated after one or two orders of magnitudes. Nevertheless, the sensitivity between the peptides is significantly dissimilar (Figure 3C), because all peptides have distinct affinities to the same mAb, which is discussed more carefully below. In summary, current results show that functionalized SiNW sensors have potential for studying interaction between different modified E5B9 peptides and anti-E5B9 mAb.

2.4. Binding of anti-E5B9 mAb to E5B9 peptides: SiNW FET versus ELISA

We further confirm the binding data of the different E5B9 peptides to mAb obtained by SiNW FET sensors and compare these measurements with a traditional enzyme-linked immunosorbent assay (ELISA) (Figure 3A for FET and **SI 4** for ELISA). While the reactivity of the biomolecules is directly observed by the change in electrical signal in the SiNW FET sensor, it is traced by an optical signal with addition of a labeled secondary Ab in ELISA (**Material and Methods**). Note that the working concentration used for testing in SiNW FET are five orders of magnitude lower than that in ELISA. In Figure 3C and Table 1, a comparison of performance parameters between the nanosensor and ELISA approaches is presented for all peptides, where we evaluate sensitivity, working range and limit of detection (LOD) for each test. Knowing that the anti-5B9 mAb is a typical immunoglobulin (Ig)G Ab with a molecular weight of approximately 150,000 g/mol, the detection limit of SiNW FET can go down to a few attomolar levels (10^{-17} M) (Figure 3C lower panel, Table 1) for most of the peptides, except for **M3**. In contrast, ELISA typically has a detection limit in the picomolar level (10^{-12} M). Overall, these data emphasize the superiority of SiNW FET over ELISA in terms of LOD and analyte consumption for applications where the detection of low levels of the analyte is necessary. Exemplarily, plasma levels of UniCAR TMs were below the limit of detection of the applied assay in patients participating in the ongoing clinical phase I trial of the UniCAR system, which showed very promising responses [15]. In this regard, for the detection of low concentrated TMs in patients the high sensitivity of the sensor can be beneficial or even required. The possibility to detect low concentrated TMs using the sensitive sensor technology paves the way towards future therapy monitoring.

Nanosensor technology can also play a role in treatment development. Regarding to peptide screening application, dissimilarity in nanosensor's response to the same target anti-E5B9 mAb reflecting different binding activity of the seven peptides, can help us to evaluate their suitability for TM construction. Binding studies between biomolecules are usually conducted

through titration of one molecule to its counterpart until the equilibrium state is reached and is quantified by the dissociation constant (K_d), or concentration that give half-maximum signal in ELISA. Pearson correlation analysis shows that the sensitivity of SiNW FET give better correlation to K_d and other parameters obtained from ELISA (SI 5). Therefore, we believe that the sensitivity extracted from the steepest slope of the binding curve are representative to demonstrate binding capability of the mAb to the peptides in case of SiNW FET.

In this study, we exchange one or two glutamic acid(s) in the E5B9-sequence of M -peptides or add a functional group in A-peptides to probe the alteration in the affinity of the anti-E5B9 mAb to the peptide variants. According to the sensitivity results in Figure 3C, we expect higher affinities of the mAb to the **M0** and **M1** peptides than to the other mutated E5B9 variants (**M2**, **M3**). Thus, exchange of only one amino acid (**M1**, D to G) (Figure 1B) does not significantly alter binding ability of the anti-E5B9 mAb towards its epitope. However, replacement of two amino acids (**M2**, **M3**) reduced the sensitivity significantly showing that these amino acids are critical for the paratope-epitope interactions. On the other hand, the sensitivity of **A1** and **A2** are comparable to **A0**, which suggests that attachment of different functional groups does not significantly change the binding efficiency of the peptide. These are very useful information during the construction of TMs for UniCAR platform, which can now be provided using nanosensor.

2.5. Influence of human blood serum on SiNW FET

The concept of UniCAR-T cell therapy comprises that autologous T cells are isolated from the blood of patients, genetically modified *ex vivo* with the UniCARs and after their expansion adoptively transferred back into the patient. The TMs are administered by permanent intravenous infusion over several weeks to maintain effective concentrations of these rapidly eliminated molecules. To better monitor therapeutic effects but also possible side effects, it is of high interest to evaluate both UniCAR-T cell and TMs concentration in patient's blood during the treatment. Keeping in mind that TM-UniCAR interactions under physiological conditions will take place in human blood, we challenged our nanowire-based FET technique with the reactivity of TM elements on SiNW FET and anti-E5B9 mAb in human blood serum. For the proof-of-concept, we perform the study in the M group with mutant variants (**M0**, **M1**, **M2** and **M3**) since this group of peptides includes the best and least-sensitive peptides and has the strongest correlation between ELISA and SiNW FET assays (Figure S5 B).

Blood serum contains many proteins, which in turn considerably change the background signal (Figure S6). Although, signal contribution from anti-E5B9 mAb is easily distinguishable (Figure S6), the high concentration of albumin protein in blood serum [88] might have negative effect on sensing response (Figure S7). While removing the unwanted protein is not an option (Figure S8), highly sensitive nanosensors allow us to conduct all further experiments in 10-time-diluted

serum (0.1x serum) where less variation in anti-E5B9 mAb detection is observed but still work in a much lower concentration range compare to conventional technique. All calibration curves are constructed by subtracting the measured signal to the signal of blank serum sample. The results are summarized in Figure 4. The results of **M0**, **M1** and **M3** in human serum is comparable to those in PBS (Figure 4A-B), e.g. **M0** and **M1** still give the best sensitivity, while **M3** reveals weak binding, leading to undetectable signal. Interestingly, the **M2** peptide, which have an average sensitivity of 52.7 mV/dec in PBS, shows significant sensitivity reduction in serum down to only 18 mV/dec (Figure 4B). Figure 4 C shows matrix of Pearson correlation results between the curves obtained for different peptides in different environments. Here, we observed high correlation (>99% confidence interval) between serum and PBS data of **M0** and **M1** which suggest similar activity even in different environment. On the other hand, **M2** binding curve in serum is less correlated to its curve in PBS, which suggests that the binding between the peptides and T cells in a natural environment might be affected, compared to the synthetic buffer. Finally, almost no correlation was observed in **M3** for the two samples, which suggest that the result for this peptide is mostly random noise, and the binding is undetectable.

2.6. Cytotoxic functionality of UniCAR-T cells

To investigate whether TMs equipped with the **M0** or mutated E5B9 peptide epitope **M1**, **M2** or **M3** can be recognized by UniCARs and are able to redirect UniCAR-T cells to eliminate PSCA-expressing prostate cancer cells, we constructed PSCA-specific TMs differing with respect to their E5B9 sequence (**M1**, **M2**, **M3**) (Figure 5 A-B). These results were further compared with the TMs functionality in-vitro using PSCA-TM-M0 comprising the E5B9 **M0** sequence, published elsewhere [55-57]. Firstly, binding capabilities of the novel TMs were analyzed by ELISA and flow cytometry. Figure 5 C shows the binding curves of anti- E5B9 mAb to PSCA-specific TMs determined by ELISA. Similar to previous ELISA measurements using the E5B9 peptides, the anti- E5B9 mAb showed a binding affinity towards PSCA-TM-M0 and PSCA-TM-M1 in the picomolar range, whereas towards the PSCA-TM-M2 and PSCA-TM-M3 lower binding affinity in the nanomolar range was detected. Furthermore, binding capability of the TMs to PC3 and UniCAR-T cells and the availability of the E5B9 peptide of bound TMs were analyzed by flow cytometry. As demonstrated in Figure S9, all of the TMs were able to bind their target antigen PSCA on the surface of the prostate cancer cell line PC3, confirming that in general all constructed PSCA-specific TMs possess a functional PSCA binding domain. Even more interestingly, all E5B9 peptide variants of cell-bound TMs were accessible for the anti-E5B9 mAb whereas M0 or M1 showed highest efficiency compared to M2 and M3. On the other hand, both the TM with the M0-peptide sequence and the TM with the M1-peptide sequence showed effective binding to UniCAR-T cells. However, the binding of the PSCA-TM-M2 and PSCA-TM-M3 could not be detected on UniCAR-T cells via the His-tag, which could

be most likely explained by a reduced binding affinity of the UniCAR anti-E5B9 scFv towards these mutated E5B9 variants. In agreement with the results obtained using SiNW FET and ELISA measurements, we have demonstrated that mutated E5B9 peptide epitope variants differ with respect to their availability for anti-E5B9 mAb and that the binding affinity of the anti-E5B9 mAb towards E5B9 M2 and M3 was clearly reduced.

Secondly, the functionality of the novel TMs with respect to their capability to redirect UniCAR-T cells to kill tumor cells (Figure 5 D) was analyzed using a chromium release assay. Therefore, UniCAR-T cells were co-cultured with PSCA-expressing prostate cancer cell line PC3 in the presence or absence of TMs. As shown in Figure 5 E, the TM with M0 or M1 peptide sequence activated UniCAR-T cells to specifically kill PSCA-expressing PC3 cells. Significant specific tumor cell lysis with high efficiency in the picomolar range (Figure 5 E) was measured. The killing capability of UniCAR-T cells redirected by the TM with M2 or M3 peptide sequence was considerably worse. In the presence of both TMs with either M2 or M3, no specific tumor lysis was observed. These data are in line with the previous study showing that the binding of the TMs with M2 or M3 could not be detected on UniCAR-T cells. Obviously, the affinity of the UniCAR anti-E5B9 scFv is not sufficient to appropriately bind the E5B9 peptide M2 or M3 which prevents the redirection of UniCAR-T cell against tumor cells. In agreement with SiNW FET results, we conclude that Ab affinity towards the TMs with the peptide mutants M2 or M3 was drastically reduced.

3. CONCLUSION

In summary, here we present the possibility of using SiNW FET in the development of anticancer immunotherapy using UniCAR-T cell approach for both research and clinical applications. We demonstrate that SiNW sensor chip can be applied to analyze the interaction of the anti-E5B9 mAb with the corresponding E5B9 peptide epitope. Namely, we show at the proof-of-concept level that the sensors can assist in the selection and design of the optimal UniCAR TM peptide epitope, necessary to reach a well-balanced redirection of UniCAR-T cells for an efficient tumor killing.

We observe different sensitivity to anti-E5B9 mAb detection in nanosensor functionalized with different peptide candidates and predict that the higher the sensitivity, the better binding effect to the UniCAR-T cells, and thus better tumor killing effect. Keeping that in mind, we constructed PSCA-specific TM based on representative group of E5B9 mutated variants (M1-M3) and successfully verified efficiency of these TMs. Finally, we compare our results with ELISA data which point out the outperformance of the FET devices in terms of limit of detection, working volume, measurement time and simplicity in sample preparation. Under optimal condition, the

detection limit can go down to an attomolar level, which is around 4-5 orders of magnitude lower than LOD in ELISA. Such extreme limit of detection enables the possibility to detect low concentrated clinical specimens and, thus, simplify the pretreatment of the patient sample.

While early diagnosis impact greatly on patient survival, monitoring the patient response to the therapy is equally important, which require timely adjustment of treatment strategy [89]. In this respect, we demonstrated that SiNW FET can be applied in clinical development of the therapeutic approaches and reached the comparable sensitivity, as well as LOD, when exposed to human blood serum. This potentially extends the use of electronic nanobiosensors for monitoring UniCAR system components in patient's blood.

Finally, all achieved results makes us confident that the bionanoelectronic technology is capable to make tremendous contribution for the clinical cancer research beyond the early diagnostics by contributing in personalization and efficient monitoring of the immunotherapeutic treatment, where the quantitative analysis with the conventional methodology is not achievable.

4. MATERIALS AND METHODS

4.1. Materials

E5B9 peptide variants **A0-A2** were purchased from Biosyntan Ltd. Peptides **M0, M1-M3** were synthesized in house as described in detail in **SI 12**. A phosphate-buffered saline (PBS) solution (pH 7.4) was prepared from dry tablets (Sigma Aldrich, Cat# P4417) and laboratory de-ionized water. Human blood serum is obtained from the University hospital Carl Gustav Carus Dresden.

4.2. Device fabrication

Si nanowire devices were fabricated by conventional top-down CMOS process on an 8 inch SOI wafer consisting of a 100 nm top Si layer on a 400 nm buried oxide (BOX) layer. The top Si layer was doped with $5 \times 10^{18} \text{ cm}^{-3}$ phosphorous ions by ion implantation to make the n^+ -channel. The active Si area including the nanowire channel region and the source/drain region was defined using a combination of a stepper and a plasma etcher. Additional phosphorus ions were implanted to the source/drain region with a concentration of over 10^{20} cm^{-3} . The honeycomb structure of nanowire channel was formed by electron beam lithography and plasma reactive ion etching (ICP-RIE). As a gate dielectric layer, 5 nm SiO_2 was thermally grown on the nanowire channel using a wet oxidation process. Afterwards, the source/drain electrode area was patterned by photolithography and etched with HF to remove the grown oxide layer. 100 nm Pt were deposited on a 20 nm Ti adhesion layer to form the source/drain and reference electrodes which was completed after the lift-off process. Finally, a 2 μm -thick

SU-8 passivation layer was coated on the devices except for the nanowire channels and electrode pad (including the center square) region.

4.3. Surface modification

The SiNW surfaces were modified with respective peptides via anhydride silane conjugation followed by ethanolamine passivation (Fig.2B). Before silane deposition, SiNW sensors were cleaned with isopropanol, dried under a stream of nitrogen and placed in plasma treatment at 100W for 20 seconds. The use of 3-triethoxysilylpropyl succinic anhydride (TESPSA) for biomolecule immobilization is explained in detail in ⁵⁸. In short, TESPSA (Gelest Inc., USA) was left for evaporation together with the sensors in a vacuum desiccator for 4 hours under heating of IR-lamp. The sensors were then rinsed in isopropanol and dried to remove any unbound silane molecules. To completely dehydrate the surface, the sensors were cured in an oven at 120°C for one hour. After cooling down the sensors to room temperature, the peptides were immobilized on SiNW by placing a drop of PBS solution containing 0.1 mg/mL of peptides onto the sensor for another hour at room temperature. We used PBS and TWEEN 20 (Sigma Aldrich, Cat# P9416) 0.05v% to wash out any unbound peptide molecules. The surface was then saturated with 0.3v% ethanolaminesolution (Sigma Aldrich, Cat# 398136) to minimize unspecific binding. We submerged the sensors in PBS for storage in a fridge at 4°C prior to measurement.

4.4. SiNW FET measurement

After removing from the storage, we rinsed the sensor with DI water and dried them under a stream of nitrogen. Different concentrations of mAb were prepared by diluting the stock with PBS solution. Starting with the lowest concentration, we incubated the sensor with a 10 μ L drop of solution containing mAb for 15 minutes. 0.01x PBS was used to rinse the sensor and for measurement. Diluted PBS was chosen to increase the Debye length of the sensor. A drop of 0.01x PBS was placed in the center of the sensor, bridging the pseudo-reference electrode and the SiNW, while transfer characteristics of SiNW FET was captured. The electrical measurement was performed using a micro-probe-station and a source-meter-unit (Keithley 2604B) which was controlled by a Matlab program.

4.5. ELISA test

The analysis of the binding affinity between the E5B9 peptide derivatives and the anti-E5B9 monoclonal antibody (mAb) was tested with enzyme-linked immunosorbent assay (ELISA). ELISA test was prepared on 96-well plate (Sigma Aldrich, Cat# A9044-2ML). Each of the peptides was tested in triplicate for each of the anti-La mAb concentrations used (1 ng/mL to

5000 ng/mL). Positive and negative controls were also performed in triplicate. After each incubation step, the wells were washed 3 times with washing buffer (PBS + 0.05% Tween 20). ELISA plates were coated with the appropriate peptide at a concentration of 10 ng/mL and incubated at 5°C overnight. To avoid unspecific absorption, the remaining sites were blocked with blocking solution (PBS + 5% w/w milk powder) for 1 h at room temperature. After blocking, the plates were incubated with anti- β 5B9 mAb for 1 h at 37°C. Then, HRP-coupled detection antibody (Sigma Aldrich, Cat# CLS3590-100EA) (1:1000 v/v in PBS) was added to each well and was incubated for 30 minutes at 37°C. TMB substrate solution was added to each well and allowed to react for 5 min, then the reaction was stopped by adding stop solution (0.5 M H₂SO₄). The optical density was measured at 450 nm with an ELISA plate reader M200 (Tecan Trading AG, Switzerland).

4.6. Data analysis

Data analysis was performed with OriginPro 2017 software (Origin Lab Corp.). For each measurement, the transfer curve was recorded. The calibration curves were constructed by extracting the gate voltages (V_G) that result a current equal to 10^{-7} A through the channel ($I_{sd}=10^{-7}$ A). To make the data comparable between sensors, we normalized the data by calculating the absolute signal difference of each measurement to the value of blank sample. Michaelis Menten equation was used to fit the data and calculate dissociation constant K_d . We calculate the limit of detection and sensitivity of the method by linear fitting the linear range in the calibration curve in logarithm scale. To calculate the limit of detection we used the following equation $\log(C_{LOD}) = \frac{3.3s_B - b}{a} = \gamma \rightarrow C_{LOD} = 10^\gamma$ where the linear fitting is defined as $y = ax + b$.

4.7. Cytotoxicity Assay

Cell lines. PC3 prostate cancer cell lines and 3T3 cell line were obtained from the ATCC and used without further authentication. PC3 cell lines was transduced to express PSCA as described elsewhere [1] and was cultured in RPMI 1640 medium (Sigma Aldrich, Cat# R0883-24X500ML), supplemented with 100 U/ml Penicillin/Streptomycin (Sigma Aldrich, Cat# P0781-100ML), 1 % non-essential amino-acids (Sigma Aldrich, Cat# M7145-100ML), 1 mM Sodiumpyruvate (Biochrom, Cat# L0473), 2 mM N-acetyl-L-alanyl-L-glutamine (Biochrom GmbH, Cat# K0302) and 10 % FCS (Sigma Aldrich, Cat# F0804-500ML). 3T3 cells were cultured in DMEM medium (Life Technologies, Cat# 10569010), supplemented with 100 U/ml Penicillin/Streptomycin, 1 % non-essential amino-acids and 10 % FCS. All cells were kept at 37 °C and 5 % CO₂ and were cultured twice a week.

Cloning of novel TMs. Cloning of the PSCA-TM-M0 was performed as described previously [2]. For cloning of the TMs with mutated E5B9 tag (PSCA-TM-M1, PSCA-TM-M2, PSCA-TM-M3), the M0 E5B9 sequence in the pSecTag2B vector encoding the PSCA-TM-M0 was replaced by dsDNA-oligos (synthesized by Eurofins Scientific SE) encoding the respective mutated E5B9 tags using the enzymes *XhoI* (Thermo Fisher, Cat# ER0692) and *NotI* (Thermo Fisher, Cat# ER0592). In order to establish cell lines that stably express the respective TMs, the open reading frame of the TMs was then cloned into the lentiviral p6NST50 vector using the *XbaI/NheI* (Thermo Fisher, Cat# ER0682 and Cat# ER0972) and *KspAI(HpaI)/MssI(PmeI)* (Thermo Fisher, Cat# ER1031 and Cat# ER1341) restriction sites.

Expression and purification of TMs. 3T3 cell lines expressing the TMs were generated with lentiviral gene transfer [3]. TMs were purified from cell culture supernatants via their C-terminal His-tag using Ni-NTA spin columns (Qiagen GmbH, Cat#31014). SDS-PAGE and subsequent Coomassie Brilliant Blue G250 staining [4] or Western Blot analysis [3] were used to determine TM concentration and purity.

Generation of UniCAR T cells. Buffy Coats were provided by the German Red Cross (Dresden, Germany) after obtaining consent from voluntary donors. The research with human T cells was approved by the local ethics committee of the Medical Faculty Carl Gustav Carus, Technical University Dresden (EK27022006). Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood via density gradient centrifugation using Pancoll separation solution (PanBiotech, Cat#P04-601000). T cells were subsequently isolated from PBMCs using the human Pan T Cell Isolation Kit (Miltenyi Biotech GmbH, Cat#130-096-535). Following isolation, T cells were transduced with the UniCAR 28/ζ construct using lentiviral particles which were generated as described elsewhere [5]. For the transduction, T cells were first activated using T Cell TransAct™ (Miltenyi Biotech GmbH, Cat#130-111-160). After 24 h, T cells were transduced with a multiplicity of infection (MOI) of 2 using a 2 h spin infection. This procedure was repeated after another 24 h. Activation and transduction steps were performed in TexMACS™ Medium (Miltenyi Biotech GmbH, Cat# 170-076-307). Transduced T cells were expanded over the weekend and used in the following week.

Flow cytometry. For binding analysis, 2×10^5 cells were incubated with 1 µg/50 µl of TM for 1 h. Binding of the TMs was detected either via their His-tag or the E5B9-tag. For the former, cells were incubated with anti-His-PE Ab (Miltenyi Biotech GmbH, Cat# 130-120-718) for 30 min. For the latter, cells were incubated with 1 µg/50 µl of the anti-La E5B9 mAb for 1 h, followed by a 30 min incubation with Goat-anti Mouse IgG Fc Cross-Adsorbed-PE secondary ab (Thermo Fisher, Cat# 31861). For the binding of the TMs to UniCAR T cells, 2×10^5 cells

were consecutively incubated with 1 µg/50 µl of TM for 1 h and anti-His-PE Ab for 30 min. To distinguish between living and dead cells, 1 µg/ml popidium iodide/PBS solution (Thermo Fisher, Cat#P3566) was added shortly before the flow cytometric measurement. For the acquisition and analysis of the data, a MACSQuant Analyzer 10 and the MACSQuantify software (Miltenyi Biotech GmbH) were used.

ELISA. For ELISA measurements, the BD OptiEIA™ Reagent Set B (BD Biosciences, Cat# 550534) was used. 96-well plates (Sigma Aldrich, Cat# CLS3590-100EA) were coated over night with 1 µg of TM per well at 4 °C. After blocking, TMs were incubated with anti-La E5B9 mAb in different concentrations and anti-La E5B9 mAb was subsequently detected with an anti-mouse IgG-HRP Ab (Sigma Aldrich, Cat# A9044-2ML). After applying the substrate solution, OD at 450 nm was determined (NanoQuant infinite M200 Pro, Tecan Group AG) and K_d values were calculated using GraphPad Prism 9 software (GraphPad Prism Inc.).

Chromium release assay. Killing of tumor cells with redirected UniCAR T cells was analyzed via chromium release assay as described previously [6]. Shortly, UniCAR T cells were incubated with 5×10^3 ^{51}Cr -labeled tumor target cells at an effector-to-target cell (E:T) ratio of 5:1 with or without TMs in different concentrations. ^{51}Cr release into the co-culture supernatant was measured after 24 h incubation using a MicroBeta² Microplate Counter (PerkinElmer LAS GmbH).

Statistical analysis: was performed using the GraphPad Prism 9 software (GraphPad Prism Inc.). One-way ANOVA with post hoc Dunnett multiple comparison test was used, with p values below 0.0332 being considered significant (* $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$ with respect to samples w/o TM).

ASSOCIATED CONTENTS

The Supporting Information is available free of charge at: _____

In-house synthesis of M group peptide sequences (SI1); contact angle measurement (SI2); fluorescence microscope test (SI3); Calibration curve of ELISA for 7 peptide derivatives (SI4); Pearson correlation test (SI5); Response of SiNW FET to serum sample (SI6); Comparison of BSA solution and serum (SI7); TWEEN test (SI8); Cytometry analysis of TM binding to PC3 cells, anti-La E5B9 and UniCar-T cells (SI9)

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Notes

The authors declare no competing financial interest.

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Figures

Figure 1: (A) Concept of UniCAR-T cell therapy approach: The target module (TM) consists of an anti-tumor binding domain (orange) equipped with the peptide sequence E5B9 (red) that is recognized by UniCAR T-cell. UniCAR-T cells are only activated after their cross-linkage with tumor cell by the TMs. (B) 10-year research landscape of nano-electronic application in cancer research. The color of the circle indicates the main method that was applied in the study while the radius represents number of publications released each year in the relevant topic. (C) Library of seven variants of the E5B9 peptide as candidates for constructing TMs in UniCAR system. The red color highlights the components differ from the original E5B9 sequence. (D) Concept of the present work: different peptide variants were immobilized on active region of SiNW FETs and used to sense against anti-E5B9 mAb to screen for most effective peptide.

Figure 2: (A) Overview of our SiNW FET sensor: microscopic image and SEM images of a chip containing 16 SiNW pair electrodes and a square reference electrode in the center. (B) Electrical characterization of a typical SiNW FET shows that the device has n-type characteristics and an 'ON' state even at $V_G = 0\text{V}$. (C) Illustration of anti-E5B9 mAb testing with different E5B9 variants. (D) Sensor surface functionalization process: (1) introduction of hydroxyl group on bare SiNW surface by plasma cleaning; (2) creation of a monolayer of TESPSA onto SiNW through evaporation in vacuum desiccator; (3) immobilization of peptides onto the surface by reaction of amino-group forming amide bond; (4) saturation of the surface to reduce non-specific binding with ethanolamine and (5) incubation with anti-E5B9 mAb solution for 15 minutes before measurement. (E) Contact angle measurement of a bare silicon substrate after each functionalization step. (F) Fluorescence microscopy image showing a glass slide covered with photoresist hole-pattern and then followed the described functionalization steps. Note that the photoresist was removed after silanization process and fluorescence signal in the last image was mostly from the Alexa Fluor 488 IgG secondary Abs bound to the anti-E5B9 mAb. (G) Measurement of transfer curve of the SiNW FET after each step of modification. Note that all the tests (E-G) were done with M0 E5B9 peptide.

Figure 3: (A) Calibration of anti-E5B9 Abs by SiNW FET with different immobilized E5B9 peptides. The data were normalized relatively to the blank sample measured on each sensor and fitted using Michaelis Menten function. (B) Exemplary transfer characteristics of A0 functionalized SiNW FET measured at $V_{SD}=0.1$ V in $0.01\times$ PBS after incubation with increasing concentration of mAb in $1\times$ PBS. (C) Comparison of sensor characteristics between SiNW FET and ELISA method. Upper panel presents the sensitivity of each measurement. Lower panel illustrates limit of detection (circle) and linear range (bars) of SiNW FET (blue) and ELISA (red).

Table 1: Summary of test performance during titration of mAb to immobilized peptides in SiNW FET and ELISA method. Sensitivities are given in mV and OD per decade of concentration.

	SiNW FET		ELISA		
	Sensitivity (mV/decade)	LOD (M)	K_d (M)	Sensitivity (OD/dec)	LOD (M)
M0	121.5 ± 22.8	1.28×10^{-18}	3.40×10^{-11}	0.270 ± 0.005	4.32×10^{-12}
M1	145.0 ± 15.0	5.38×10^{-20}	3.49×10^{-11}	0.220 ± 0.015	5.54×10^{-12}
M2	52.7 ± 13.3	1.09×10^{-18}	3.12×10^{-9}	0.218 ± 0.030	6.77×10^{-10}
M3	3.4 ± 0.3	1.05×10^{-12}	1.47×10^{-9}	0.028 ± 0.003	1.58×10^{-9}
A0	41.8 ± 2.5	1.50×10^{-17}	2.00×10^{-8}	0.110 ± 0.005	3.16×10^{-9}
A1	38.0 ± 3.7	1.25×10^{-15}	2.23×10^{-11}	0.160 ± 0.008	4.24×10^{-12}
A2	31.8 ± 4.8	6.92×10^{-18}	1.89×10^{-11}	0.140 ± 0.021	4.19×10^{-12}

Figure 4: Result of measurement conducted in human blood serum. (A) Calibration of anti-5B9 Abs in PBS (gray, dash line) and in serum (color, solid line) on different peptides; (B) comparison of sensor performance between tests done in PBS (gray) and serum (color) (inset: illustration of linear range (bars) and limit of detection (squares) of each measurement); (C) Matrix shows Pearson correlation coefficients and significant levels between titration curves of different E5B9-peptide variants in different samples. The closer the coefficient to 1 or -1, the stronger the correlation between that data. The blue boxes highlight the comparison results between PBS and serum sample, while green boxes feature comparison between wild type E5B9 peptide to other peptide variants in the same samples (serum/PBS).

Figure 5: (A) Schematic illustration of UniCAR-T cells cross-linked to prostate cancer cells via an anti-PSCA target module (TM) equipped with different E5B9 peptide variants. (B) Construction of the wildtype PSCA-TM-M0 and the novel PSCA-TMs with mutated E5B9 peptide epitope (M1-M3). (C) Binding analysis by ELISA. ELISA-plates were coated with 1 μ g of TM per well and incubated with different concentrations of the anti-E5B9 mAb. Binding was detected with anti-mouse IgG-HRP through measuring of OD₄₅₀. Mean of OD₄₅₀ $\pm$ SEM for three independent experiments is shown. (D, E) In a chromium release killing assay, UniCAR-T cells were incubated with ⁵¹Cr-labeled PC3-PSCA tumor cells at a ratio of 5:1 either in the absence (w/o) or (D) presence of 250 nM PSCA-TM or (E) in the presence of different concentrations of the indicated PSCA-TMs. ⁵¹Cr release was measured after 24 h incubation. In the diagrams, mean specific lysis $\pm$ SEM for three independent experiments with three different T cell donors is shown. (* p < 0.0332, ** p < 0.0021, *** p < 0.0002 with respect to samples w/o TM, One-way-ANOVA followed by Dunnett's post hoc test). (M0: E5B9 wild type, M1-M3: E5B9 mutant variants).

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