Title

Precise surface functionalization of PLGA particles for human T cell modulation

Journal

Nature Protocols, 18(11)

ISSN

1754-2189

Authors

Hadley, Pierce

Chen, Yuanzhou

Cline, Lariana

et al.

Publication Date

2023-11-01

DOI

10.1038/s41596-023-00887-8

Peer reviewed

HHS Public Access

Author manuscript

Nat Protoc. Author manuscript; available in PMC 2024 January 09.

Published in final edited form as:

Nat Protoc. 2023 November ; 18(11): 3289–3321. doi:10.1038/s41596-023-00887-8.

Precise surface functionalization of PLGA particles for human T cell modulation

Pierce Hadley[1,2,3,4,5], **Yuanzhou Chen**[3,6], **Lariana Cline**[3], **Zhiyuan Han**[3], **Qizhi Tang**[4,5,7], **Xiao Huang**[3,6,8,✉], **Tejal Desai**[2,3,4,6,9,✉]

[1]Medical Scientist Training Program, University of California, San Francisco, CA, USA.

[2]UC Berkeley–UCSF Graduate Program in Bioengineering, UCSF Mission Bay Campus, San Francisco, CA, USA.

[3]Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, CA, USA.

[4]Diabetes Center, University of California, San Francisco, CA, USA.

[5]Gladstone–UCSF Institute of Genomic Immunology, San Francisco, CA, USA.

[6]Cell Design Institute and Center for Synthetic Immunology, University of California, San Francisco, CA, USA.

[7]Department of Surgery, University of California, San Francisco, CA, USA.

[8]School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA, USA.

[9]School of Engineering, Brown University, Providence, RI, USA.

Abstract

The biofunctionalization of synthetic materials has extensive utility for biomedical applications, but approaches to bioconjugation typically show insufficient efficiency and controllability. We recently developed an approach by building synthetic DNA scaffolds on biomaterial surfaces that enables the precise control of cargo density and ratio, thus improving the assembly and organization of functional cargos. We used this approach to show that the modulation and phenotypic adaptation of immune cells can be regulated using our precisely functionalized

✉**Correspondence and requests for materials** should be addressed to Xiao Huang or Tejal Desai. xh324@drexel.edu; tejal_desai@brown.edu.

Author contributions

P.H. and X.H. designed the experiments and drafted the manuscript. P.H. and L.C. contributed to the experiments in Figs. 2–5. Y.C. and Z.H. contributed to the experiments in Fig. 2. P.H. analyzed the data. All authors contributed to the editing of the manuscript.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Competing interests

T.D. and X.H. are inventors of a pending patent related to the technology described within this manuscript (WO2020014270A1). Q.T. is a co-founder, shareholder and scientific advisor of Sonoma Biotherapeutics. P.H., Y.C., Z.H. and L.C. declare no competing interests.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-023-00887-8.

biomaterials. Here, we describe the three key procedures, including the fabrication of polymeric particles engrafted with short DNA scaffolds, the attachment of functional cargos with complementary DNA strands, and the surface assembly control and quantification. We also explain the critical checkpoints needed to ensure the overall quality and expected characteristics of the biological product. We provide additional experimental design considerations for modifying the approach by varying the material composition, size or cargo types. As an example, we cover the use of the protocol for human primary T cell activation and for the identification of parameters that affect ex vivo T cell manufacturing. The protocol requires users with diverse expertise ranging from synthetic materials to bioconjugation chemistry to immunology. The fabrication procedures and validation assays to design high-fidelity DNA-scaffolded biomaterials typically require 8 d.

Introduction

Synthetic materials have been widely engineered to present biomolecules to engage cellular receptors and control cell behaviors for disease modulation[1–4]. In particular, immunotherapies show potential as treatment options for conditions including some types of cancers and autoimmune diseases[5–9]. In both clinical use and preclinical models, these treatments are mostly administered as in vivo immunomodulatory agents, such as antigens, antibodies and cytokines, or as cellular therapies involving ex vivo stimulation and/or engineering to control disease[10–16]. Immune cells in fact respond to signals from cell–cell synapses and the extracellular space to determine their phenotype, fate and behaviors[17–19]. Therefore, methods capable of precisely controlling the signals presented to immune cells may enable the engineering of cell therapeutic products with improved therapeutic efficacy or other benefits[3,20,21].

Although the immobilization of stimulatory ligands on biomaterial surfaces can mimic the natural signals for immune cell programming[22–24], the efficient and controllable conjugation of multiple ligands on synthetic surfaces is a major challenge of traditional chemical approaches[25–28]. Thus, we developed a synthetic short DNA-scaffold strategy for surface biofunctionalization[20]. This plug-and-play approach can precisely control the density and ratios of multiple functionalities with rapid surface assembly. This biofunctionalization approach can be used in various applications and requires the careful assembly of synthetic materials, oligonucleotides and proteins. Here, we provide the step-by-step description to fabricating DNA-scaffolded particles, engineering complementary DNA (cDNA)-conjugated biomolecules and applying these materials to activate human primary T cells ex vivo. This protocol further provides detailed methods and quality control assays to ensure a high fidelity of functional biomaterials and an optimal activation of human T cells.

Applications

We initially tested the approach to present agonistic αCD3 and αCD28 antibodies onto biodegradable polymeric microparticles composed of poly-lactic-co-glycolic acid (PLGA). These immune cell-engaging particles (ICEps) activate T cell receptor and co-stimulatory receptors for human T cell ex vivo expansion, which is a key step for manufacturing T cell-based therapies[5,29]. Due to the biodegradable and biocompatible properties of ICEps,

they did not need to be removed from ex vivo cultures compared with using commercially available magnetic particles (e.g., Dynabeads)[20]. The quantitative control of αCD3 and αCD28 antibodies showed an impact on both T cell expansion fold and phenotypic outcomes—in terms of differentiation fate and exhaustion—which are critical aspects for therapeutic uses[20,30–32]. In addition, these materials can be administered in vivo to control immune cell activities and can be tailored for both localized delivery—such as intratumor or subcutaneous injection—and systemic delivery through intravenous administration[20,33]. For example, logic-gated CAR-T cells have been engineered to recognize dual antigens to minimize 'off-tumor' toxicity, and we engineered microparticles presenting synthetic antigens to prime these T cells to target tumor-specific antigens[34]. With the intratumoral injection of antigen-functionalized microparticles, we were able to restrict the activation of these logic-gated CAR-T cells locally to minimize systemic toxicity[20]. While this protocol will focus primarily on the quality control of ICEp fabrication and the uses in vitro, readers are encouraged to consult the original report on this technology for additional details on in vivo use[20].

The customizability of this approach facilitates a wide range of other applications where the precision control of multiple biomolecules is needed, for example, targeted drug delivery, gene engineering and tissue remodeling[35–38]. An effective intracellular delivery of gene regulatory-or-editing molecules must overcome various barriers at the tissue, cell and intracellular (e.g., endosomes and lysosomes) levels, which can be facilitated using different biological functionalities[1,2,39]. Similarly, the precision density control of ligands for cellular receptors involved in tissue remodeling—for example, integrin and adhesion signaling—can provide avenues for tissue engineering[35]. The approach is also adaptable for drug loading within the particle core, and polymers with different degradation profiles can be leveraged for controlled release[33,40]. Particle size can be varied across multiple length scales, enabling systemic delivery or localized retention[41,42]. Through the joint engineering of the DNA scaffold and underlying polymer, this approach can be reformulated to fit multiple biological challenges, and thus displays unprecedented levels of control for cell modulation and therapeutic applications.

Development of the protocol

This protocol describes the fabrication of (1) DNA-scaffolded PLGA particles, (2) bioconjugation of biomolecules (e.g., antibodies) with cDNA, (3) cDNA–biomolecule conjugate assembly onto DNA scaffolds and (4) primary human T cell activation and phenotyping using ICEp (Fig. 1). The high controllability of surface functionalization requires a dense layer of DNA scaffolds built on the particle surface, which depends on the efficient conjugation of the PLGA–poly(ethyleneglycol)–maleimide (PLGA–PEG–mal) with thiolated DNA (thiol–DNA) and is susceptible to poor reagent quality and improper reaction conditions (Fig. 2). Thus, we developed a framework for testing PLGA–PEG–DNA conjugation efficiency among different lots of precursor materials and correlated this with the DNA-scaffold density of the resultant particles. After validating successful polymer conjugation, PLGA–PEG–DNA batches bearing different DNA sequences can be mixed at select ratios, which will reflect the final DNA-scaffold ratio on particles.

Generating cDNA–biomolecule conjugates requires careful design to preserve the activity of the biomolecule during conjugation and surface attachment[43–45] (Fig. 3). For example, in antibody conjugation, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) is used to selectively reduce hinge-region disulfide bonds to free thiol groups for thiol–DNA conjugation[46]. The TCEP molar excess and the reaction duration are important parameters for maintaining antibody function[47]. After DNA conjugation, a critical concern is the removal of unreacted DNA, which can compete for surface loading in later steps and thus requires affinity-based chromatography methods for purification due to electrostatic interactions between DNA and antibody. After the rapid surface assembly of purified antibody–DNA (Ab–DNA) conjugates, a flow cytometry-based method is provided to quantify the particle surface loading (Fig. 4).

When using ICEps for T cell activation, we found that culture seeding conditions, including the cell density, particle-to-cell ratio and surface ratio of stimulatory biomolecules all influence T cell expansion and the resultant phenotype (Fig. 5). For example, in our original report, we enriched either memory or effector T cell fates through the control of particle compositions including the ratiometric control of agonistic αCD3 and αCD28 antibodies on the particle surfaces[20]. Here, we intend to highlight the influence of these parameters on T cell activation and manufacturing so that they can be taken into consideration for related research. While the focus is on using 2 μm (mean diameter) ICEps for T cell activation, this fabrication protocol is compatible with multiple particle size scales; thus, we have provided protocol modifications throughout.

Comparison with other methods

An often-used approach for surface functionalization is through covalent conjugation between functional groups on the synthetic material and the biomolecule using a bifunctional linker (e.g., PEG linker with *N*-hydroxysuccinimide (NHS) and mal groups at the end sites)[26,27,48]. However, the efficiency of this method is severely limited by surface steric hinderance and the instability of the functional groups[49–53]. While orthogonal chemistries provide an additional dimension of control for immobilizing multiple biomolecules species, they still suffer from the same limitations inherent to covalent surface attachment strategies[49,50]. Further, it becomes increasingly difficult to tune the surface stoichiometry of multiple biomolecule species as characteristics of the biomolecule heavily influence their attachment—including molecular weight and charge[20,54]. In comparison, our DNA hybridization-based approach reaches the theoretical surface saturation limit while simultaneously maintaining independent control over the loading of each biomolecule species. Another surface functionalization approach to load multiple cargos is to use streptavidin handles[21,22]. We previously found that the ratiometric control is largely affected by the molecular weight and charge of the cargo, where the species with highest surface affinity always outcompeted the others. Also, the maximal density of smaller molecules may be bottlenecked by the size of streptavidin.

Limitations

There are three areas of limitations in adapting DNA-scaffolded materials: (1) the broad skillset and equipment required to combine synthetic materials, bioconjugation methods and biological applications; (2) the variations in precursor material quality; and (3) the many steps involved throughout the whole protocol. We have adapted existing technologies commonly available in biological research laboratories for characterizing fabricated materials (e.g., gel electrophoresis, Nanodrop spectrophotometers, flow cytometers, etc.). Most polymers, linkers and synthetic DNAs are commercially sourced to facilitate user adoption. For precursor quality, we have identified that PLGA–PEG–mal was the main source of quality variation, possibly due to reactant impurities remaining in the purchased polymer; therefore, we have included methods for evaluating this precursor quality.

Experimental design

Particle size

This protocol can be adapted for fabricating spherical particles across varying size scales while maintaining the functionality of the DNA approach (Fig. 2g,h). Here, PLGA–PEG–DNA serves as the sole surfactant, which correlates well with particle size control. Different quantification methods are needed for size quantification; micron-scale requires microscopy whereas nano-scale requires either Zetasizer or Nanosight. The protocol exhibits minimal batch-to-batch variation, although large particle sizes are associated with greater size distribution variance, which has been reported with probe-sonication methods[55]. Thus, alternative methods for better size control could be evaluated for compatibility with the DNA-scaffolding method, including postfabrication size filtration, differential centrifugation or even alternatives to probe sonication such as microfluidic droplet generators or electrospray fabrication.

Surface density control

There are two methods for controlling the surface density of biomolecules: varying the DNA-scaffold density during particle fabrication or limiting the input quantity of cDNA–biomolecule conjugates during hybridization[20]. The first method was demonstrated previously by varying the molar excess of thiol–DNA to PLGA–PEG–mal during polymer–DNA conjugation, while keeping the input amount of PLGA–PEG–mal constant for particle fabrication[20]. The second method of density control involves titrating the cDNA–biomolecule below the surface saturation level (Fig. 4a–c), which is more convenient as it shares the same particle formulation and is used within this protocol.

Surface ratiometric control

Ratiometric control of biomolecules is achieved similarly to density control during either particle fabrication or surface hybridization. The surface ratios of scaffold DNA sequences are controlled by the input mixtures of PLGA–PEG–DNAs during particle fabrication. Thus, the addition of excess cDNA–biomolecules will present the biomolecules in a ratio defined by the scaffold DNA ratio (Fig. 4f–h). By contrast, the hybridization method involves

inputting a predefined ratio of cDNA–biomolecules below the saturation level of each respective scaffold-DNA sequence, allowing the input biomolecule stoichiometry to define the surface ratio outcome (Fig. 4d,e).

Particle core loading

While not described within the procedural section, an additional functionality of our material is the capacity for core-loading biomolecules and tracking dyes. Fluorescent dye can be preconjugated to PLGA (e.g., AlexaFluors) and mixed during particle fabrication for in vitro or in vivo tracking. Biomolecules of interest can be loaded into the core for slow release via a double-emulsion procedure[40,56].

Particle biodegradability

We have adopted PLGA due to its biocompatibility and tunable degradation, as degradation rates can be controlled by varying the chain lengths or lactic-to-glycolic acid ratios[40,56]. Different polymers with varying stabilities can alter the release rates of core-loaded biomolecules[57,58]; we have shown that other polymers, such as poly-lactic acid, are also compatible with the DNA-approach technology but requires additional optimization.

Protein–DNA conjugation

There are many protein bioconjugation chemistries available, which should be balanced with conjugation efficiency, cost and maintenance of biomolecule activity[43,44,48,59,60]. Alternatively, a protein tag (e.g., SNAP-tag) can be incorporated at an optimal site of the protein to link with the functional group of the DNA[61,62]. To note, it is necessary to validate protein bioactivity post-conjugation through assays relevant to the biological function.

Protein–DNA storage

The purification procedure for removing unreacted DNA typically results in low Ab–DNA concentration, which reduces its stability. Further, long-term storage in solution is not advisable due to the risk of protein degradation[63–65]. Lyophilization has been used to improve long-term protein storage and is also used here to facilitate increased protein concentrations after resuspension—this can improve stability and minimize particle hybridization volumes as described later in ‘Particle surface loading of antibody’. Biomolecules that are unstable or sensitive to freezing will require protein-specific bioactivity assays to verify minimal bioactivity loss and to decide whether lyophilization is appropriate. Previously, spin-concentrator columns were used to increase protein concentration, but this resulted in substantial protein loss onto the concentrator membrane, and this was more apparent when using DNAs labeled with charged fluorescent dyes.

T cell sourcing and expansion using ICEp

Peripheral blood mononuclear cells can be isolated from whole-blood or leukapheresis products and can be used without further purification or processed in a variety of ways to collect desired T cell fractions[29,66,67]. Cells can be separated on a variety of markers

using commercially available positive or negative selection binding kits. To further enhance population purity and/or collect T cell subsets, such as regulatory T cells or naive T cells, FACS can be used.

Isolated cells can be stimulated using a combination of T cell receptor and co-stimulatory activating proteins and growth factors. The former is provided via ICEps presenting agonistic αCD3 and αCD28 antibodies, while mitogenic cytokines (e.g., IL-2) are provided as soluble supplementation in the medium. For the latter, while we are providing cytokine in the media, we and others have identified advantages for surface delivery of growth factors, which is compatible with ICEp technology[20,22,68]. Various cell-culture parameters using ICEp can influence overall expansion and should be optimized for each cell type and experimental timeline, including: (1) choice of the culture plate, (2) cell seeding and maintenance densities, (3) cytokine concentrations and (4) the particle-to-cell ratio[69]. Following expansion, T cells may be analyzed using flow cytometry.

Critical controls

There are numerous controls that are important in (1) determining PLGA and DNA quality, (2) surface loading of biomolecules onto the PLGA–DNA scaffold and (3) biomolecule activity after DNA conjugation. Determining the quality of the precursors for PLGA–PEG–DNA fabrication requires gel electrophoresis; thus, we suggest using commercially synthesized oligos to serve as an unreacted DNA band control. This serves to identify the unreacted DNA fraction within the PLGA–PEG–DNA lanes, enabling the calculation of DNA consumption during conjugation, which is used as a proxy for PLGA conjugation efficiency.

For quantifying particle biomolecule loading, it is necessary to have a fluorescent standard ladder when using a plate spectrophotometer or, when using flow cytometry, have both unhybridized and saturated single-color particle controls. The fluorescent biomolecule used in either case should match the biomolecule hybridized onto particles. For flow cytometry, batch-to-batch variation in particle size could result in dissimilar fluorescence intensities, thus control and experimental particles should come from the same common stock.

For the biological activity of Ab–DNA conjugates, cell-staining titrations should be compared with unmodified antibody controls and measured via flow cytometry to detect changes over time or between conjugation batches. To minimize variation, a large batch of Ab–DNA should be aliquoted and either frozen or lyophilized immediately after conjugation. Smaller aliquots from this stock could serve as standards when comparing with new conjugations. Similarly, when loading particle with biomolecule–DNA conjugates, it may be beneficial to hybridize a large batch of particles and lyophilize them in aliquots for each future experiment.

Materials

Biological materials

- Peripheral blood mononuclear cells are isolated from leukapheresis products collected from healthy donors (StemCell Technologies)

  CAUTION For working with primary human blood products, the appropriate approvals, trainings, and safety procedures should be followed according to institutional guidelines.

Reagents

PLGA–PEG–DNA synthesis

- PLGA10k–PEG5k–mal (Akina, cat. no. AI053)
- 3′ Thiol–DNA (Integrated DNA Technologies, large-scale custom synthesis)
- 500 mM TCEP (Sigma, cat. no. 646547)
- Glen Gel-Pak 0.2 desalting column (Glen, cat. no. 61-5002-05)
- Glen Gel-Pak 1.0 desalting column (Glen, cat. no. 61-5010-05)
- 1 M Tris–HCl pH 8.0 (Fisher, cat. no. AAJ22638AP)
- 500 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 (Fisher, cat. no. 50-841-658)
- 3 M sodium acetate (EMD, cat. no. 127-09-3)
- Ethanol, 200 proof (EtOH; VWR, cat. no. TX89125-172SFU)

  CAUTION Ethanol is flammable. Keep away from open flame.

- *N,N*-dimethylformamide (DMF; Sigma-Aldrich, cat. no. D158550)

  CAUTION DMF is toxic. Handle in a fumehood with proper personal protective equipment (PPE).

- Triethylamine (Et_3N; Sigma-Aldrich, 471283)

  CAUTION triethylamine is volatile. Handle in a fumehood with proper PPE.

- 2× TBE–urea sample buffer (Thermo, cat. no. LC6876)
- 10× TBE buffer (Biorad, cat. no. 1610733)
- 15% TBE–urea gel (Thermo, cat. no. EC68855BOX)
- 10,000× Sybr Gold (Life Technologies Corporation, cat. no. S11494)
- Hydrochloric acid (Sigma, cat. no. 320331)

  CAUTION Hydrochloric acid is corrosive and can cause severe damage on contact. Wear proper PPE when handling.

- Sodium hydroxide (Sigma, cat. no. 71690)

CAUTION Sodium hydroxide is corrosive and can cause severe damage on contact. Wear proper PPE when handling.

- Nitrogen gas

PLGA particle fabrication

- Purified, deionized water (Purification system; Sartorius, Arium Mini)
- PLGA 50:50 38K–54K (Unmodified PLGA; Sigma, cat. no. 719900)
- Ethyl acetate (EtOAc; Sigma, cat. no. 319902)

  CAUTION EtOAc is volatile. Handle in a fumehood with appropriate PPE.
- Sodium citrate (Sigma, cat. no. S1804)
- Sodium chloride (Sigma, cat. no. S9625)
- Magnesium chloride (Sigma, cat. no. M8266)
- Poly(vinyl alcohol) 31K (PVA; Sigma, cat. no. 81381)
- 40 μm filter (Fisher, cat. no. 22-363-547)
- Tween 20 (Sigma, cat. no. P9416)
- Liquid nitrogen

  CAUTION Liquid nitrogen is a cryogenic fluid. Handle with appropriate PPE.

Particle DNA loading and size quantification

- Razor blade (Fisher, cat. no. 12-640)
- 3′ amine-modified fluorescent DNA (Biosearch, custom synthesis)
- Dimethyl sulfoxide (DMSO; Sigma, cat. no. D2438)

  CAUTION DMSO is hazardous. Handle with appropriate PPE.
- 10× Phosphate-buffered saline (PBS; Calbiochem, cat. no. 6506)
- Microscope slides, frosted (Corning, cat. no. 2948)
- Coverslip 22 × 22 (Fisher, cat. no. 50-365-603)
- Clear nail polish (Fisher, cat. no. 50949071)
- Disposable cuvette (VWR cat. no. 47743-834)

Antibody conjugation with DNA

- Anti-human CD3, Clone: OKT-3 (BioXCell, cat. no. BE0001-2, RRID: AB_1107632)
- Anti-human CD28, Clone: 9.3 (BioXCell, cat. no. BE0248, RRID: AB_2687729)
- Mal–dPEG4–NHS linker (Quanta Biodesign, cat. no. 10214)

- HEPES (Fisher, cat. no. BP310-500)
- 3′ amine-modified dyeless DNA (Integrated DNA Technologies, custom synthesis)

Antibody–DNA purification

- Disposable 2 mL resin gravity column kit (Thermo, cat. no. 29920)
- Protein-G resin beads (Genscript, cat. no. L00209)
- Glycine (Sigma, cat. no. G8898)
- 10K MWCO dialysis column, 50 mL (Thermo, cat. no. 88404)
- microBCA kit (Thermo, cat. no. 90358)
- 1 M triethylammonium acetate buffer (TEAA; Sigma, cat. no. 90358)
- Sodium phosphate monobasic (sodium phosphate; Sigma, cat. no. S8282)
- Sodium azide (Thermo, cat. no. 190380050)
- Tris–glycine 4–20% Gel (Thermo, cat. no. XP04205BOX)
- Tris–3-(*N*-morpholino)propanesulfonic acid–sodium dodecyl sulfate (SDS) running buffer powder (GenScript, cat. no. M00138)

Preparation of antibodies for surface loading quantification

- AlexaFluor488 carboxylic acid, succinimidyl ester (NHS-AF488; Thermo, cat. no. A20000)
- AlexaFluor647 carboxylic acid, succinimidyl ester (NHS-AF647; Thermo cat. no. A20006)
- Zeba-spin desalting column (Thermo, cat. no. 87766)

T cell isolation and culture

- RPMI + GlutaMAX (Thermo, cat. no. 61870036)
- FBS (OmegaScientific, cat. no. HS-20)
- Penicillin (1×10^4 U/mL) + streptomycin (10 mg/mL) (Thermo, cat. no. 15140-122)
- 1 M HEPES (Thermo, cat. no. 15630130)
- 100 mM sodium pyruvate (Thermo, cat. no. 11360070)
- Human CD3/CD28 Dynabeads (Thermo, cat. no. 111.31D)
- EasySep CD4+ T cell enrichment kit (StemCell, cat. no. 17952)
- EasySep CD8+ T cell enrichment kit (StemCell, cat. no. 17953)
- Fixation/permeabilization kit (Thermo, cat. no. 00-5523-00)
- Fixable viability dye eF780 (Thermo, cat. no. 65-0865-14)

- Anti-human CD45RA-BV605 (Biolegend, cat. no. 304134)
- Anti-human CCR7-BV711 (BD, cat. no. 566602)
- Anti-human LAG-3 PerCP-eF710 (Invitrogen, cat. no. 46-2239-42)
- Anti-human PD-1 BV421 (BD, cat. no. 562516)
- Anti-human TIM-3-PE-CF594 (BD, cat. no. 565560)
- Anti-human CD27-PE (BD, cat. no. 557330)
- Human IL-2 (Teceleukin; Roche Ro 23-6019)
- 0.5 M EDTA, sterile (Thermo, cat. no. 15575-038)
- 1× PBS, pH 7.4, calcium/magnesium free (Thermo, cat. no. 10010-023)

Equipment

Plasticware

- 2.0 mL Eppendorf tube (Eppendorf, cat. no. 022363352)
- 15 mL conical tube (Fisher, cat. no. 1495949B)
- 50 mL conical tube (Fisher, cat. no. 14-959-49A)
- Disposable spatula (VWR, cat. no. 80081-188)
- Disposable weigh boat (Ted Pella, cat. no. 20158)
- 10 mL Luer-lock syringe (VWR, cat. no. 76124-664)
- 10 mL serological pipette (Fisher, cat. no. 1367827F)
- 96-Flat-well plate; cell-culture treated (Thermo, cat. no. 167008)
- Cryovials (Sigma, cat. no. CLS430659)
- 0.22 μM PES stericup media filter (Sigma, cat. no. S2GPU02RE)
- 96-Well flat-bottom plate; black (Fisher, cat. no. 14245177)

Equipment related to particle fabrication

- Vortex (VWR, cat. no. 97043-562)
- Benchtop centrifuge (Beckman, Allegra-6R)
- Benchtop microcentrifuge (Fisher, accuSpin Micro 17)
- Benchtop pH meter (Fisher, Accumet AB150)
- Nanodrop One UV–visible spectrophotometer (Thermo, cat. no. 13-400-518)
- Analytical balance scale (Fisher, cat. no. 01-913-921)
- SpeedVac concentrator (Thermo, SPD121P)
- Refrigerated vapor trap (Thermo, RVT400)

- Dry-vacuum pump (Sigma-Aldrich, cat. no. Z411906)
- Orbital shaker (VWR, cat. no. 490000-128)
- Parafilm (Genesee, cat. no. 16-100)
- Pyrex glassware 250 mL beaker (Fisher, cat. no. 02-555-25B)
- Ultrasonic waterbath (VWR 75T)
- Magnetic stir plate (Cimarec Model #SP131325)
- Micro stir bar (Fischer, cat. no. 1451364SIX
- Probe sonicator (Sonicator, Qsonica S-4000)
- Lyophilization chamber (SP Scientific Advantage Plus ES-53)
- Spinning disk confocal (CSU-22 and Nikon Ti)
- Microplate spectrophotometer (Molecular Devices SpectraMax M5)

Equipment related to protein bioconjugation

- Gel laser scanner (GE Typhoon FLA 9000)
- Gel image doc (Azure c150 Gel Doc)
- Heating block (Fisher, cat. no. 11-718-8)
- Gel electrophoresis power supply (Thermo, cat. no. PS0300)
- Gel electrophoresis cell (Thermo, cat. no. EI0001)
- Serological pipette controller (Fisher, cat. no. NC0165100)
- Tube racks (Thermo, cat. no. 8850)

Equipment for T cell isolation and culture

- Incubator (37 °C, 5% CO_2)
- Heated waterbath (Fisher Isotemp 205)
- Flow cytometer (BD LSR-Fortessa, Thermo Attune)
- Inverted bright-light microscope (Leica DM4000M)
- Hemacytometer (Sigma, cat. no. Z359629)
- 50 mL EasySep magnet (StemCell, cat. no. 18002)
- Coolcell (Corning, cat. no. CLS432002)
- Liquid nitrogen cryotank

Software

- FlowJo version 10 (https://www.flowjo.com/solutions/flowjo)
- GraphPad Prism version 9 (https://www.graphpad.com/)

- Excel (https://www.microsoft.com/en-us/microsoft-365/excel)
- ImageJ (https://imagej.nih.gov/ij/ and https://imagej.nih.gov/nih-image/manual/tech.html)

Reagent setup

DNA reagents (thiol–DNA and cDNA)

1. Calculate volume needed to resuspend DNA to 500 μM.
2. Resuspend using 10 mM HEPES, pH 7.0.
3. Allow 30 min to resuspend, vertexing occasionally.
4. Store at −20 °C.

5× particle fabrication buffer, 50 mM sodium citrate, 1.5 M sodium chloride, 10 mM magnesium chloride, pH 3.0

1. Weigh out 735 mg of sodium citrate, 4.38 g of sodium chloride and 47.61 mg of magnesium chloride.
2. Add into a container with 50 mL of deionized water and mix.
3. Measure pH using a pH meter and adjust to pH 3.0 using concentrated hydrochloric acid.
4. Transfer to conical tubes and store at room temperature (20–25 °C).

2× DNA hybridization buffer, 600 mM sodium chloride, 2 mM magnesium chloride, 0.02% Tween 20, pH 7.0

1. Weigh out 1.75 g of sodium chloride and 9.52 mg of magnesium chloride.
2. Add into a container with 50 mL deionized water.
3. Add 10 μL of Tween 20, using a dilution in water if the stock is too viscous to accurately measure.
4. Thoroughly mix and measure pH using a pH meter and adjust to pH 7.0. 5. Store at room temperature.

5× Protein G binding buffer, 100 mM sodium phosphate, 0.75 M sodium chloride, pH 7.0

1. Weigh out 3.0 g of sodium phosphate monobasic and 10.88 g sodium chloride.
2. Add into a container with 250 mL of deionized water.
3. Thoroughly mix and measure the pH using a pH meter and adjust to pH 7.0.
4. Store at room temperature.

Protein G acidic elution buffer, 0.1 M glycine, pH 2.7

1. Weigh out 375.35 mg of glycine and add to a container with 50 mL deionized water.
2. Thoroughly mix and measure the pH using a pH meter and adjust to pH 7.0 using concentrated hydrochloric acid.
3. Store at room temperature.

Protein G basic elution buffer, 0.1 M glycine, pH 10.0

1. Weigh out 375.35 mg of glycine and add to a container with 50 mL deionized water.
2. Thoroughly mix and measure the pH using a pH meter and adjust to pH 7.0 using concentrated sodium hydroxide.
3. Store at room temperature.

10× Protein G acidic neutralization buffer, 1 M Tris–HCl, pH 8.5

1. Measure out 50 mL of 1 M Tris–HCl in a secondary container.
2. Measure the pH using a pH meter and adjust to pH 8.5 using concentrated sodium hydroxide.
3. Store at room temperature.

10× Protein G basic neutralization buffer, 1 M Tris–HCl, pH 6.5

1. Measure out 50 mL of 1 M Tris–HCl in a secondary container.
2. Measure the pH using a pH meter and adjust to pH 6.5 using concentrated hydrochloric acid.
3. Store at room temperature.

PBS–FBS wash buffer, 1× Ca^{2+}/Mg^{2+}-free PBS, 3% (vol/vol) FBS, 1 mM EDTA

1. In a sterile biosafety cabinet (BSC), combine 15 mL of heat-inactivated FBS, 484 mL of 1× PBS and 1 mL of 0.5 M EDTA in a container and mix.
2. Mix and sterile-filter the solution using a 0.22 μM filter.
3. Store at 4 °C.

T cell medium

1. In a sterile BSC, combine 435 mL of RPMI 1640 + GlutaMAX, 50 mL of heat-inactivated FBS, 5 mL of 1 M HEPES, 5 mL of 100 mM sodium pyruvate and 5 mL of combined penicillin (1×10^4 U/mL) + streptomycin (10 mg/mL).
2. Mix and sterile-filter the solution using a 0.22 μM filter and store at 4 °C.

3. Before experimental use, aliquot 50 mL of media into a separate container and add 25 μL of human IL-2 (hIL-2) (2×10^5 U/mL stock) to a final concentration of 100 U/mL.

4. Media containing hIL-2 (complete T cell media) can be used for T cell culturing and should be used within 1 week and stored at 4 °C.

Freezing medium, 10% (vol/vol) DMSO in FBS

1. In a sterile BSC, combine 22.5 mL of heat-inactivated FBS and 2.5 mL of DMSO.

2. Sterile-filter using a 0.22 μM filter and store at 4 °C.

Procedure

PLGA–PEG–DNA conjugate synthesis

TIMING 2 d—CRITICAL The following describes the synthesis of 500 nmol PLGA–PEG–DNA using commercially synthesized PLGA–PEG–mal and thiol–DNA precursors. For validated DNA-sequence options, see Table 1. Repeat the procedure for each desired oligo sequence.

1. Use a micropipette to transfer 500 nmol of thiol–DNA into a 1.5 mL microcentrifuge tube (DNA tube).

2. Add 100 μL of 500 mM TCEP (100× molar excess to thiol–DNA) to reduce any interstrand disulfide bonds and incubate for 1.5 h at 37 °C.

3. Prepare a Glen size-exclusion desalting column that is appropriately sized for the DNA-tube volume using 10 mM EDTA in 10 mM Tris–HCl (1× TE, pH 7.5) for buffer exchange washes, per the manufacturer's instructions.

4. Buffer exchange the TCEP-reduced thiol–DNA into 1× TE (pH 7.5) using the prepared Glen column to collect the DNA-containing flow-through.

   CRITICAL STEP The exchange buffer should not contain any chemical groups that react with the selected conjugation chemistry. EDTA prevents disulfide reformation following reduction.

5. For DNA precipitation, aliquot the thiol–DNA into 1.5 mL tubes (precipitation tubes) with ~400 μL per tube. To each 400 μL tube, add 50 μL of 3 M sodium acetate (pH 5.0) and 1.3 mL of ethanol (200 proof); thoroughly mix and vortex after each addition. Cool tubes at −20°C for 30 min.

6. Centrifuge the precipitation tubes at 18,000*g* for 10 min at 4 °C. Remove the supernatant and either air dry or use a pressurized air line to further dry the DNA pellet.

7. Resuspend the DNA pellet in one precipitation tube with 200 μL of TE. Combine this volume into another precipitation tube and repeat until all tubes are resuspended in a total of 200 μL (targeting ~2.5 mM DNA if DNA loss was minimal during the preceding steps).

CRITICAL STEP DNA should be resuspended in less than 200 μL to be compatible with the optimized reaction conditions later. Adjust this volume appropriately and reoptimize if needed.

8. Measure the absorbance at 260 nm (A_{260}) of a diluted sample of DNA using a Nanodrop. Reference Table 1 for the relevant extinction coefficients and calculate the stock concentration using Beer's law:

$$\text{Stock concentration} = [(\text{dilution factor}) \times (absorbance)]/[(\text{extinction coefficient}) \times (\text{path length})]\,.$$

where Nanodrop path length is 1 cm and the extinction coefficients used here are in M/cm.

9. Create a reaction template in Excel to facilitate reagent calculations for synthesizing the PLGA–PEG–DNA. Refer to Table 2 for the necessary equations and constants for constructing the template. An example template is provided in Supplementary Table 1.

CRITICAL STEP The PLGA–PEG–mal:DNA ratio should be optimized for each new polymer lot.

CRITICAL STEP Use the PLGA–PEG–mal molecular number average instead of weight average due to the distribution of different polymer chain lengths. The number average here is specific to our PLGA–PEG–mal lot.

10. Allow the PLGA–PEG–mal container to warm to room temperature before opening.

CRITICAL STEP Allowing the container to warm to room temperature before opening to avoid water condensation, which can hydrolyze the functional group.

11. Weigh the calculated amount of PLGA–PEG–mal and add DMF to achieve a 30 mg/mL solution.

12. Add the solutions to a 15 mL tube in the following order, referring to the volumes in the reaction template: (1) extra TE buffer, (2) DNA solution, (3) triethylamine, (4) extra DMF and (5) PLGA–PEG–mal DMF solution. Vortex to mix.

13. Wrap the top of the tube with parafilm and shake overnight using an orbital shaker at room temperature.

14. Use nitrogen or other inert gas line to back-fill the stock container of PLGA–PEG–mal.

15. Wrap the container with parafilm before putting back into −20 °C storage.

16. The next day, briefly vortex the PLGA–PEG–DNA reaction tube and aliquot into 1.5 mL tubes with ~500 μL into each tube. As ratiometric particles may be desired, it is recommended to premix PLGA–PEG–DNA bearing different sequences at a specified ratio before drying, ensuring that 100 nmol of total PLGA–PEG–DNA is aliquoted per tube.

CRITICAL STEP The downstream fabrication protocol uses 100 nmol of PLGA–PEG–DNA; thus, aliquoting 500 μL equates to a theoretical 100 nmol of PLGA–PEG–DNA (assuming 200 μM was the target PLGA–PEG–mal reaction concentration). Premixing the different PLGA–PEG–DNA sequences before drying ensures more precise control over the mixture ratio, whereas later the volumes may be difficult to control due to solvent evaporation.

17. Dry the PLGA–PEG–DNA aliquots in a vacuum centrifuge at 70 °C for 2–3 h.

18. Once dried, store at −20 °C.

PAUSE POINT Dried PLGA–PEG–DNAs are stable for over a year. PLGA–PEG–DNA can be stable if dissolved in organic solvent, although any aqueous solutions should be avoided as this will lead to hydrolysis of either the PLGA ester linkages or the thiol–mal bond.

19. Urea– polyacrylamide gel electrophoresis (PAGE) is used to verify PLGA–PEG–DNA conjugation (Fig. 2b,c).

20. Prepare ~20 μL of a 0.2 μM solution of PLGA–PEG–DNA (diluted in 1× TE) and dilute to 0.1 μM using 20 μL of 2× urea–PAGE loading buffer.

21. Similarly, make a 0.1 μM dilution of pure DNA (in loading buffer) used for the reactions.

22. Heat the sample for 3 min at 70 °C.

23. During heating, prepare a urea–PAGE gel by loading a vertical gel chamber with 1× TBE buffer and pre-running the gel for 10 min at 120 V. Use a syringe or pipette to clean the melted gels in each lane using TBE buffer within the chamber.

24. Load 1 pmol (~10 μL) of 0.1 μM sample in triplicate alongside 1 pmol of control pure DNA lanes. Run the gel for 1.5 h at 120 V.

25. Prepare a 25 mL of 1× Sybr Gold (10,000× dilution) in 1× TBE.

26. Dispense into a wide disposable glass dish, cover the dish with the lid and protect from light.

27. After the gel has finished running, release the gel from the cast and transfer to the 1× Sybr Gold solution.

28. Place onto an orbital shaker at room temperature for 5–10 min protected from light.

29. Rinse the stained gel with 1× TBE and transfer into a new glass dish containing buffer to prevent gel dehydration.

30. Image the gel using a gel-doc reader or laser scanner.

31. Import the gel image into ImageJ. After adjusting brightness and contrast, perform gel densitometry analysis as described by the ImageJ operational manual (see ‘Software’)[70].

32. Use the intensity of the top PLGA–PEG–DNA band and the lower, unreacted DNA to calculate the efficiency of the reaction using the equation below and record to track batch variation.

$$(\text{Intensity PLGA}-\text{PEG}-\text{DNA})/(\text{Intensity PLGA}-\text{PEG}-\text{DNA}+\text{Intensity DNA}).$$

CRITICAL STEP Disulfide bonds can form between the thiol–DNA and can appear in the gel above the unreacted thiol-DNA (Fig. 2b). We typically do not include the disulfide band intensity since it is negligible relative to the main unreacted thiol–DNA band.

TROUBLESHOOTING

PLGA particle fabrication

TIMING 6 h—CRITICAL This procedure describes the fabrication of 2 μm particles bearing a maximally dense surface DNA scaffold at 1:1, R:G DNA sequence ratios (for sequence information, see Table 1), where R and G are different DNA sequences. This procedure assumes that 100 nmol of PLGA–PEG–DNA was dried in Step 18 with a 1:1 mixture of DNA G and R sequences (PLGA–PEG–G and PLGA–PEG–R, respectively). 100 nmol of PLGA–PEG–DNA generates ~100 OD_{550} in 400 μL volume (40 OD_{550} in 1 mL) or ~2×10^9 particles. For fabricating particles of other target diameters, refer to Table 3 for modifying reagent amounts within this section and to the ‘Anticipated results’ section for representative morphologies and size distributions (Fig. 2g,h).

33. Weigh 50 mg of unmodified PLGA 50:50 (38–54 kDa, PLGA) into a 15 mL tube (fabrication tube).

34. Use a glass pipette to add 400 μL of EtOAc into the tube.

    CRITICAL STEP Keep EtOAc-containing tubes open for as little time as possible to minimize evaporation—this will reduce the size variability between batches. Do not hold tubes near the liquid as this may contribute to heating.

35. Wrap the tube with parafilm and place vertically on a shaker table overnight to dissolve.

36. The next day, place stock tubes of EtOAc, water and fabrication tubes on ice to reduce evaporation when opened.

37. Resuspend the 1:1 R:G PLGA–PEG–DNA tube from Step 18 with 100 μL of water and 100 μL of EtOAc. Reuse this pipette tip whenever transferring PLGA–PEG–DNA for a given sequence ratio (switch if using a different sequence ratio).

38. Place the PLGA–PEG–DNA tube into the bath sonicator for 10 min or until fully resuspended.

39. Transfer the PLGA–PEG–DNA into the 15 mL fabrication tube in 100 μL increments to reduce material loss inside the pipette tip.

40. To wash the PLGA–PEG–DNA tube, add 300 μL of water and 100 μL of 5× particle fabrication buffer (see ‘Reagent setup’).

41. Using the saved PLGA–PEG–DNA pipette tip, transfer this solution into the fabrication tube. If the pipette tip gets clogged, briefly pipette the EtOAc fraction within the fabrication tube to dissolve the clog.

42. Sonicate the fabrication tube and vortex until mixed. Place the fabrication tube on ice.

43. Place a magnetic stir plate with a 250 mL beaker and a stir magnet into a fume hood. This will be needed after probe sonication after Step 50.

44. Prepare a 50 mL conical tube partially filled with ice to act as a secondary container for the fabrication tube during probe sonication. Set up a vortexer, 0.2% (wt/vol in water) PVA and separate ice container near the probe sonicator.

45. For the sonication setup, clean the sonication microtip probe using 70% (vol/vol in water) ethanol and allow to dry.

CRITICAL STEP Ensure that the sonication program is set to the recommended settings (Box 1). Sonication will need to pause halfway through, so if your sonicator does not allow for this function then adjust the number of cycles accordingly.

Vortex the reaction tube and place into the 50 mL secondary ice container.

46. Position the sonication probe into the fabrication tube solution, avoiding the tube walls.

47. Initiate the sonication program, moving the microtip throughout the solution to ensure a more homogeneous sonication. After two cycles, pause sonication and vortex the reaction tube before finishing the remaining cycles.

48. Immediately after sonication add 9 mL of 0.2% (wt/vol) PVA into the fabrication tube, invert to mix, then vortex.

49. Dispense the contents of fabrication tube into the 250 mL beaker from Step 43 and turn on the magnetic stirrer for ~2.5 h without any heating.

CRITICAL STEP This step will evaporate the EtOAc residue. For larger volumes, use a rotary evaporator.

TROUBLESHOOTING

50. After 2.5 h, place a 40 μm filter onto a 50 mL conical tube and pour the particle solution through the filter. Use a micropipette to transfer any remaining solution.

51. Centrifuge the particle tubes at 225*g* for 10 min.

CRITICAL STEP If nanoparticles were fabricated, then after Step 52 the supernatant will contain the nanoparticles while any large particle contaminants will be contained within the pellet. If larger microparticles (>2 μm) were fabricated, then proceed as written without protocol modification.

52. Discard the supernatant and resuspend in 2 mL of TE containing 0.1% (vol/vol) Tween 20 (TE–Tween) using a micropipette.

CRITICAL STEP If nanoparticles were fabricated, then collect the supernatant and discard any visible pellet after Step 52. For all subsequent nanoparticle centrifugation steps in this protocol, spin at 16,000*g* for 10 min.

53. Distribute the 2 mL into smaller microcentrifuge tubes and centrifuge at 6,000*g* for 5 min.

54. Resuspend each tube in 200 μL of TE–Tween.

55. Spin again at 6,000*g* for 5 min, resuspending again in 200 μL TE–Tween. During the final resuspension, combine all tubes into a single tube with a total volume of ~400 μL TE–Tween.

56. Prepare a small sample for Nanodrop quantification. Since the stock concentration is large, use a larger dilution volume to allow for sufficiently large pipetting volumes from the stock solution (~0.5–1 μL). Assuming a successful fabrication yield of ~100 OD_{550} in 400 μL, use the dilution example below to generate a dilution of ~0.5 OD_{550}:

A. Generalized dilution equation used: $C_1 \times V_1 = C_2 \times V_2$

B. (100 OD_{550} stock) × (X μL stock sampled) = (0.5 OD_{550} target concentration) × (100 μL total dilution volume); X μL stock sampled = 0.5 μL

C. (Total dilution volume) – (X μL stock sampled) = (volume of TE–Tween to dilute stock sample); volume of TE–Tween to dilute stock sample = 99.5 μL

D. Dilution factor = (total dilution volume)/(volume of stock sampled); dilution factor = 200

CRITICAL STEP Nanodrop particle absorbance is linear between 0.2 and 1.0 at OD_{550}, so the estimated dilution fold would need to be adjusted accordingly. The estimate of 0.5 used above is an appropriate initial target as some amount of error will probably maintain the measured range between 0.2 and 1.0.

CRITICAL STEP Microparticles settle quickly, creating a concentration gradient and a particle pellet over time. Whenever handling microparticles, ensure the tubes are sufficiently resuspended.

57. After using an appropriate buffer (TE–Tween) to blank the Nanodrop, measure the OD_{550} of the diluted sample and solve for the stock concentration via the equation below and using the dilution factor calculated in Step 57. If the measured OD_{550} is below 0.2, then remake the dilution using a lower dilution factor.

A. (Stock OD_{550}) = (dilution factor) × (measured OD_{550} of diluted sample)

58. Using the equation from Step 57, set aside a small sample of diluted particles to generate ~20 μL at 5–10 OD_{550}. Save this sample for imaging and size quantification later.

    CRITICAL STEP For nanoparticles, refer to Step 99 for the necessary sample amount and the dilution concentration for Zetasizer measurements.

59. To each ~400 μL tube of particles, add 100 μL of 5% (wt/vol) PVA and mix.

60. In a secondary container, prepare a small volume of liquid nitrogen.

61. Flash freeze the tubes by submerging in liquid nitrogen below the cap level using a tube holder (e.g., long forceps).

62. Place the frozen tubes into a lyophilization chamber for 24 h with the tube caps open.

    PAUSE POINT Lyophilized particles can be stored for up to 2 years at −20 °C. Particles stored after 2 years should be reassessed for DNA-scaffold density (see ‘Particle surface DNA loading analysis’)

Particle surface DNA loading analysis

TIMING 4 h—CRITICAL This protocol describes the quantification of particle scaffold DNA density and relative ratio of DNA sequences via the detection of hybridized, fluorescently labeled cDNA (5′ end label) using a plate spectrophotometer. The procedure assumes particles are taken from lyophilized stock. The total particle amount required for fluorescent detection varies depending on the particle size since each formulation has a different nM/OD_{550} loading capacity. Thus, the fluorescence detection limit of the spectrophotometer should be used to predict the amount of particles needed to adapt this method for other particle sizes.

63. Remove the lyophilized particles from Step 63 onto a disposable weigh boat.

64. Use a razor blade to cut a small fraction of the particle for OD_{550} measurement. Target a concentration of 20 OD_{550} in 100 μL and readjust later after OD_{550} quantification is made.

    CRITICAL STEP 20 OD_{550} in 100 μL was chosen to ensure that the particle signal will be above the signal detection limit for our spectrophotometer. Additionally, if users are not careful during pipetting steps there could be substantial particle loss, which is mitigated by increasing the initial particle quantity.

65. Resuspend the particle sample in 500 μL of water for 5 min.

66. Centrifuge the particles at 6,000*g* for 5 min.

    CRITICAL STEP Since the particles were lyophilized in a nonvolatile buffer, the buffer salts are still contained in the pellet. Water should be used to resuspend to prevent high concentrations of buffer salts.

67. Remove the supernatant and wash with 500 μL of TE–Tween.

68. Repeat spinning and washing one more time with the final resuspension in 100 μL of TE–Tween.

69. Make a sample dilution in a separate tube.

70. Measure the diluted sample OD_{550}. Use the OD_{550} to calculate the stock tube concentration.

CRITICAL STEP If there is less than 20 OD_{550} in 100 μL, repeat Steps 64–67 to resuspend a newly cut portion of the particle as described previously and add to the existing particle volume after sufficient wash steps described in this step. Repeat Steps 70–71.

71. Calculate the hybridization component volumes according to Table 4, assuming a particle concentration target of 20 OD_{550} in 100 μL total hybridization volume.

CRITICAL STEP The total loading capacity of cDNA of high-scaffold density 2 μm particles approaches 75–150 nM of cDNA per OD_{550} depending on batch-to-batch variation[20]. For the 1:1 R:G particle here, both compR and compG DNAs will maximally load between 37.5 and 75 nM/OD_{550}, respectively. cDNA should be loaded at three times the maximal theoretical loading capacity (~225 nM/OD_{550} for each cDNA on the 1:1 particle) to ensure surface saturation regardless of particle batch variability.

CRITICAL STEP 200 nm particle loading approaches 1,000–2,000 nM of cDNA per OD_{550}, whereas the 8 μm particle loading approaches 10–20 nM of cDNA per OD_{550}, depending on the batch variation. The loading capacity should be determined for different particle sizes before experimental use. This should be adjusted in Table 4 for calculating hybridization reaction conditions depending on the particle size used.

CRITICAL STEP The hybridization buffer will constitute half of the total volume. The remaining half will be used for particle volume and cDNA. If 100 μL has not been reached, calculate the volume for TE–Tween to fill the remainder. The total hybridization volume may exceed the target volume depending on the concentration of reagents, so extra TE–Tween may not be required (seen as a negative or zero value for the extra TE–Tween calculation).

72. Transfer a quantity of particles into a microcentrifuge tube such that, once diluted, it will result in 20 OD_{550} in 100 μL (hybridization tube). If the volume of particles needed in Step 72 exceeds 50 μL, then centrifuge particles and remove supernatant until 50 μL of volume remains.

73. To the hybridization tube, add 50 μL 2× hybridization buffer, cDNAs and extra TE–Tween (if needed).

74. Mix the solution using a micropipette followed by bath sonication for 15 s to ensure particle dispersion.

75. Incubate particles on a shaker for 30 min at 37 °C.

CRITICAL STEP Particle hybridization is achieved in less than 2 min, although to ensure surface saturation we hybridized for 30 min. During this time, settling occurs at high particle concentrations, which is more apparent when using larger-diameter microparticles. If this is substantial, vortex the particles halfway through their incubation period.

76. During particle incubation, generate fluorescent-cDNA standard curves in a black-walled microwell plate.

- **A.** For each fluorescent cDNA:
  - **i.** Start with 200 µL of a 2 µM DNA concentration in 9% (vol/vol) DMSO in PBS (PBS–DMSO).
  - **ii.** Remove 100 µL to perform twofold serial dilutions until reaching the limit of detection for the plate spectrophotometer, leaving 100 µL per well.
  - **iii.** Separately, make blank wells containing 100 µL PBS–DMSO for background subtraction.
  - **iv.** Cover the well-plate top and set aside to protect from light.

    CRITICAL STEP Particles will be loaded onto the plate in PBS–DMSO, so the ladder should be made in the same buffer.

77. After hybridization, add 400 µL of TE–Tween and centrifuge at 6,000*g* for 5 min at 4 °C.

78. Remove supernatant and wash twice more.

79. Use 120 µL TE–Tween for the final resuspension.

CRITICAL STEP After particles have been hybridized, all centrifugation steps should occur at 4 °C to minimize dehybridization of loaded cargos.

CRITICAL STEP It is important to remove the majority of the supernatant to prevent background signal. It is additionally important to not disturb the pellet during any steps, as this will reduce the total signal detected during later steps.

80. Add 50 µL of hybridized particles (particle replicate tubes) into two separate centrifuge tubes—these will be used for repeated measures.

81. With the remaining 20 µL volume, dilute a small volume for OD_{550} calculation to determine the concentration in the particle replicate tubes. This value will be needed to calculate the final DNA nM/OD_{550}.

82. Centrifuge replicate particle tubes at 6,000*g* for 5 min and remove 45 µL of supernatant from each.

83. Add 45 µL of DMSO to each particle tube to dissolve particles.

CRITICAL STEP 5 µL of wash buffer should be remaining after supernatant removal to reduce particle loss. If previous wash steps were not thorough, the 5 µL of remaining supernatant could include background DNA signal. The 45 µL

of removed supernatant can be saved and measured to determine the background fluorescence contribution.

84. For replicate measurements, add 90 μL of PBS into the microwell plate from Step 77 and 10 μL of dissolved particles.

85. Resuspend all wells thoroughly and do not generate bubbles.

86. Read the fluorescence of the microplate on a microplate spectrophotometer in top-down mode with settings in accordance with the respective fluorophores used.

CRITICAL STEP Filters should be carefully selected to minimize signal crossover between fluorophores. Other settings, such as channel voltage, should be optimized for each machine.

87. For fluorescence analysis, average the blank PBS–DMSO wells and subtract from all wells. Create a linear best-fit curve for the fluorescent ladder lanes.

CRITICAL STEP Since the ladder fluorescent signal could be widely different than the measured particle signal, ensure that the ladder range used for generating the best-fit curve are within one-to-two dilution steps away from the measured particle signal to increase accuracy.

88. Calculate the fluorophore concentration of each well using the best-fit curve above. Correct for sample dilution by dividing each well fluorescence concentration by 1/10 of the OD_{550} value determined in Step 82 to determine stock nM/OD_{550}.

CRITICAL STEP Particles were diluted tenfold in Step 85. This factor needs to be corrected for the OD_{550} in the plate.

89. Average the nM/OD_{550} values from each well and report as the mean ± s.e.m. Calculate the surface ratio between R:G signals using the equation below. The ratio of cDNAs is reflective of the ratio of the scaffold DNAs:

1. Ratio of R-nM/OD_{550} (R) to G-nM/OD_{550} (G)
    - If R > G then the ratio of R to G is (R/G):1
    - If R < G, then the ratio of R to G is 1:(G/R)

TROUBLESHOOTING

Particle size quantification

TIMING 2 h—CRITICAL Microparticle size distributions are assessed using confocal microscopy imaging (option A). While brightfield requires less material preparation, confocal imaging of fluorescent particles produces defined silhouettes and reduces off-target quantification of debris; thus, confocal imaging is recommended for accurate size quantification. The selected magnification should be used to provide a sufficient field-of-view to capture a large number of particles, while still maintaining visualization of small-diameter particles. Since nanoparticle fabrication may be of interest, we suggest the use of

dynamic light scattering instruments such as Zetasizer (option B). Since Zetasizer does not rely on fluorescence measurements, unlike the confocal microscopy method, nanoparticles do not need to be hybridized with fluorescent cDNA and can be analyzed immediately after Step 56.

Option A (microparticle size quantification using confocal microscopy):

90. Particles must first be hybridized using saturating levels of fluorescent cDNA as described in Steps 72–76 and 78–80. A small amount of particles are needed for imaging (~5–10 OD_{550} in 30 μL), so adjust starting particle amount to minimize particle waste.

91. Pipette 10 μL of diluted, fluorescent particle (target ~5–10 OD_{550}) onto a clear microscope slide and overlay a coverslip.

92. Seal the coverslip corners with clear nail polish.

93. After the corners have partially dried and flattened, seal the sides of the slips by connecting each corner with nail polish. This will prevent sample drying and allow for slide inversion on the microscope if needed.

94. Visualize particles under confocal microscopy (Fig. 2g).

95. Adjust laser power and exposure settings for the relevant laser line, being careful to avoid photobleaching. Height focus should be set using the fluorescence channel.

96. Acquire at least five representative images.

97. Analyze images using ImageJ to determine particle diameters. Size distribution curves can be generated in software such as Graphpad (Fig. 2h).

Option B (nanoparticle size quantification using Zetasizer):

1. Prepare a 1 mL dilution of nanoparticles using 0.1 μm filtered deionized water, targeting 0.01 OD_{550}.

2. Dispense an appropriate volume into a disposable cuvette and perform size analysis using the Zetasizer and the manufacturer’s instructions. Intensity-weighted size distributions and other variation metrics, such as the average diameter or polydispersity index (PDI), can be exported and visualized within software such as GraphPad (Fig. 2h).

TROUBLESHOOTING

Antibody conjugation with complementary DNA

TIMING 1 d—CRITICAL This procedure describes the conjugation of antibodies with amine-labeled cDNA using an NHS–PEG–mal linker at a 2 mg antibody scale. This protocol does not change whether the DNA is labeled, but for most applications we recommend a dyeless DNA. If a dye-labeled DNA is used, special attention should be placed to the charge of the dye; we have found that positively charged dyes may have increased association with the antibody and thus leads to purification difficulties. Ab–DNA can be labeled for

quantification purposes after purification if required (see ‘Preparation of antibodies for surface loading quantification’).

98. Calculate the volume needed for 2 mg of antibody and prepare a Glen size-exclusion column that is appropriately sized for the antibody volume, as per the manufacturer’s instructions. Buffer exchange washes should be 10 mM EDTA in 1× PBS, Ca^{2+}/Mg^{2+} free (PBS–EDTA).

CRITICAL STEP Ensure that the buffer does not contain any amine-groups (e.g., Tris) as this will compete to react with NHS reagent used later.

99. Buffer exchange the antibody into PBS–EDTA per Glen column manufacturer’s instructions and collect into a new tube (reaction tube).

100. Measure the antibody A_{280} using a Nanodrop with an appropriate dilution. Place the antibody at 4 °C. The protein concentration can be calculated using the following equations:

- (A_{280} × dilution)/1.33 = (mg/mL antibody)
- ((mg/mL antibody) × 1,000)/155 = nmol antibody, where 155 is the antibody molecular weight (kDa).

101. Calculate volume of amine-cDNA needed for 4× molar excess relative to antibody in Step 103. Move this volume into a new tube (DNA reaction tube). The following equation can be used:

- nmol DNA needed = 4 × (nmol antibody)
- mL of DNA needed = (nmol DNA needed)/(μM DNA stock)

CRITICAL STEP Here, a subsaturating amount of DNA—as determined using SDS–PAGE immediately after DNA conjugation without purification—was used to prioritize Ab–DNA purity over conjugation efficiency (Fig. 3b,c). Higher amounts of DNA could be used to improve the Ab–DNA yield as long as the removal of unreacted DNA is confirmed. Importantly, the ratio of DNA to biomolecule should be optimized for every new biomolecule and linker.

102. Calculate the mg of NHS–PEG–mal for 20× molar excess relative to DNA from Step 104. Dissolve linker in a small volume of DMSO, with at least 30 μL per 0.8 mg of linker.

103. Add 20× molar-excess-dissolved linker to the DNA tube and incubate for 1 h at 37 °C. If the reaction DMSO volume exceeds 5% (vol/vol), add HEPES (100 mM, pH 7.2) until 5% DMSO is reached.

104. When the DNA–PEG–mal reaction from the previous step is nearly complete, dilute TCEP to 5 mM in PBS–EDTA.

105. Calculate the volume of 5 mM TCEP needed for 4.5× molar excess relative to antibody amount determined in Step 103.

106. Add this TCEP volume into the antibody tube and incubate for 1 h at 37 °C.

107. Afterwards place the antibody at 4 °C.

CRITICAL STEP A lower molar excess can be used but may require longer incubation; longer timing or increased molar excess can result in different reduction cleavage products.

108. Precipitate the DNA as described in Step 5. During the precipitation, a second Glen column should be equilibrated to PBS–EDTA. The final volume after DNA precipitation will be 200 μL, so prepare an appropriately sized Glen column.

109. After 30 min at −20 °C, centrifuge the DNA reaction tube at 18,000*g* for 10 min at 4 °C.

110. Remove the supernatant and resuspend in 200 μL PBS–EDTA.

111. Use the Glen column to buffer exchange to PBS–EDTA to remove any excess unreacted linker from the DNA–PEG–mal.

112. Determine the DNA–PEG–mal concentration from the Nanodrop A_{260} and Beer's Law. Reference Table 1 for the relevant extinction coefficients.

113. Add 4× molar excess of DNA–PEG–mal into the antibody tube and incubate for 1 h at 37 °C. Afterwards, place the antibody reaction tube at 4 °C overnight.

Ab–DNA purification

TIMING 1–2 d—CRITICAL The following steps are required for removal of free, unreacted DNA–PEG–mal from the Ab–DNA conjugate, which can compete for surface hybridization.

114. Use a ring-stand clamp to suspend a resin gravity column over a liquid waste container Assemble the column by placing the column filter at the bottom end nearest the exit port and capping the bottom. Vortex a bottle of Protein G resin beads and add 1.5 mL of the bead suspension followed by a sufficient volume of 1× Protein G binding buffer (binding buffer, diluted in water) to fill the column.

CRITICAL STEP 1.5 mL of suspension results in ~0.75 mL column volume (CV) of resin after liquid drainage. Varied resin amounts can be used depending on the amount of protein being purified.

115. Remove the column cap and allow for the buffer to drain. When ~3/4 of the column height remains, cap the bottom, and wait 20 min for the resin to settle.

116. Place a second column filter into the column and push until above the binding resin.

CRITICAL STEP Do not trap bubbles beneath the filters as this can slow the elution of the column.

117. Add 5 CVs (~3.75 mL) of binding buffer and allow to drain.

118. Remove the waste container under the column and replace with a 15 mL capture conical tube.

119. Remove the capture tube and replace with another 15 mL conical tube.

120. Add the Ab–DNA from the first capture tube.

121. Repeat twice more by loading the flow through to ensure maximum column binding.

122. Discard the last flow through.

123. Place a waste container underneath the filter column and wash with 10 CVs of binding buffer.

124. While the column is washing, label ~13 1.5 mL tubes: 5 for the acidic elutions, 3 for the neutral and 5 for the basic.

125. Add 55 μL of 10× acidic elution neutralization buffer into each acidic elution tube and 55 μL 10× of basic elution neutralization buffer into each basic elution tube.

CRITICAL STEP If different elution volumes are captured per tube, the volume of neutralization buffer should be adjusted to achieve a final 1× concentration.

126. Add 3 CV (2.5 mL) of acidic elution buffer into the column and begin capturing 500 μL of flow through into each acidic capture tube.

127. Mix each tube afterwards to ensure the neutralization buffer has mixed into the flow through.

128. After all acidic buffer has passed, add 3 CV of binding buffer and capture a third of the volume into each of the neutral tubes.

129. After all the binding buffer has eluted, add 3 CV of basic elution buffer and capture 500 μL of flow through into each basic capture tube.

130. Mix each tube afterwards to ensure the neutralization buffer has mixed into the flow through.

131. Place a waste container underneath the column and add 5–10 CV of binding buffer.

132. After draining, cap the bottom and add binding buffer to cover just above the top resin.

133. Label and store at 4 °C if subsequent purifications are needed.

134. Quantify the A_{260} and A_{280} of each elution tube using a Nanodrop (Fig. 3d).

135. Dispose all tubes where the A_{280} indicates minimal protein recovery (<5–10% of the original theoretical protein amount) and also dispose when the A_{280}/A_{260} ratio is less than 0.9.

CRITICAL STEP This step is the most critical for improving the purity of the final Ab–DNA. The A_{280}/A_{260} ratio can slightly vary, although the tubes that primarily contain the unbound DNAs should have a ratio much less than 1.0.

136. Dialyze the Ab–DNA with 1× PBS using a 50 mL dialysis column (10K MWCO) and place onto an orbital shaker at 4 °C as per the manufacturer's instructions.

137. Swap the 1× PBS after 2 h and 4 h cumulative time.

138. After the final swap, dialyze overnight.

139. The next day, collect the Ab–DNA from the dialysis column and store at 4 °C.

CRITICAL STEP This step removes the glycine and other buffer components that may inhibit downstream quantifications and purifications. The glycine must be removed if additional Fc-affinity column purifications are needed, otherwise the antibody cannot bind to the resin.

140. Use a microBCA kit to determine the protein concentration (in mg/mL) within the Ab–DNA conjugate according to the manufacturer's recommendations.

141. The DNA concentration within the Ab–DNA is required for hybridization calculations, but this requires additional steps to calculate since both the antibody and the DNA independently contribute to both A_{260} and A_{280}. Refer to Box 2 to solve for the DNA concentration within the Ab–DNA.

CRITICAL STEP If a dye-labeled Ab–DNA was used, then the $A_{260\ (\text{DNA component of Ab–DNA})}$ can be estimated on a plate spectrophotometer using a standard fluorescent curve of known cDNA–dye concentrations and comparing the fluorescence of a known dilution of Ab–DNA.

142. Use urea–PAGE to confirm that free DNA has been removed from the Ab–DNA conjugate (Fig. 3e).

143. Prepare dilutions of Ab–DNA and pure DNA, run the gel and analyze according to Steps 19–32.

CRITICAL STEP If a dye-less DNA was used for conjugation, urea–PAGE must be performed to later stain the DNA with Sybr Gold, which is not compatible with SDS–PAGE gels. If a dye-labeled DNA was used, then SDS–PAGE gel is recommended as the antibody bands are more clearly defined.

TROUBLESHOOTING

144. Calculate the Ab–DNA purity with the equation below. If the sample is not pure (e.g., purity <0.95), then the purification Steps 120–145—using the column saved from Step 136—must be repeated before proceeding:

- Ab–DNA purity = (intensity of Ab–DNA band)/((intensity of Ab–DNA band) + (intensity of DNA band))

145. Prepare a sufficiently sized Glen column using 0.1 M TEAA (pH 7.0) as the exchange buffer.

146. Buffer exchange the Ab–DNA into the TEAA and aliquot into separate tubes for lyophilization.

147. Label the estimated protein and DNA amount in each tube to calculate the new concentrations when later resuspending.

CRITICAL STEP TEAA is a volatile buffer and thus does not leave salts after lyophilization, which could damage the proteins at high concentrations.

148. Freeze the Ab–DNA using liquid nitrogen as described in Steps 61–63 and lyophilize overnight.

149. The next day, resuspend an Ab–DNA aliquot in 0.1 μm-filtered PBS so that the concentration of the antibody is at least 6.5 μM, using the concentrations determined in Steps 143–144 to determine the new concentrations after resuspension. Store remaining aliquots at −20 °C.

CRITICAL STEP Higher resuspension concentrations (>1 mg/mL) are important for the stability of proteins and to have more reasonable volumes to work with during particle hybridization. To note, resuspending the Ab–DNA at too high concentrations could result in protein aggregation[71,72]. Thus, concentrations between 1 and 10 mg/mL are recommended, which is comparable to the concentrations of the purchased antibody stocks used within this protocol.

PAUSE POINT Lyophilized proteins are stable at −20 °C for over 2 years. The antibody can remain stable at 4 °C for over a year. The shelf-life of other proteins should be assessed and monitored.

150. (Optional) Sodium azide (0.05%) can be added to a desired concentration to limit microbial growth once resuspended and stored at 4 °C.

Preparation of antibodies for surface loading quantification

TIMING 2 h—CRITICAL Flow cytometry can be immediately used to verify the ratio between protein species on particle surfaces if the conjugated cDNA was labeled with a fluorescent dye. If unlabeled cDNA was used for conjugation, NHS-dye labeling of the antibody is first required. Below we describe the labeling of αCD28–compR. The procedure is identical for labeling αCD3-compG but with a different fluorophore. To reduce nonspecific interactions between the Ab–DNA and the particle, we recommend using negatively charged dyes for Ab–DNA labeling.

151. For later quantification, record the A_{260} and A_{280} of the Ab–DNA. Calculate the ratios R1 = A_{280}/(μM antibody) and R2 = A_{260}/(μM DNA), where the respective antibody and DNA concentrations are known from Step 152 after resuspension from lyophilized stock.

152. Resuspend NHS–Alexafluor-488 (AF488) to 2 mM in DMSO.

153. Aliquot ~50 μg (0.32 nmol) of purified αCD28–compR (~100 μL at 0.5 mg/mL).

154. Add 8× molar excess of 2 mM AF488 into the antibody aliquot and react for 1 h at 37 °C.

155. Record the final reaction volume.

CRITICAL STEP Do not exceed 5% (vol/vol) DMSO to reduce protein denaturing. To prevent this, either make a more concentrated stock of NHS–dye or dilute with HEPES (100 mM, pH 7.2).

156. Prepare an appropriately sized Zeba spin desalting column according to the manufacturer's instructions, replacing the buffer with 1× TE. Load the αCD28–compR–AF488 onto the column and spin as recommended.

157. Measure the A_{260}, A_{280} and A_{488} to solve for the antibody, DNA and AF488 concentrations using the below equations:

- μM antibody = A_{280}/R1, where R1 is from Step 154. Similarly, μM DNA = A_{260}/R2, where R2 is from Step 154
- AF488 μM = A_{488}/(extinction coefficient AF488)
- Number of AF488–dye per antibody = (μM AF488)/(μM antibody)

CRITICAL STEP Depending on the fluorophore intensity and dilution, the absorbance may be greater than 1.0. If so, redo this step using a higher dilution. The number of AF488–dyes per antibody should be above 1.0 and can be used to indicate successful conjugation.

Particle surface loading of antibody

TIMING 2 h—CRITICAL The following describes the loading and quantification of αCD28–compR–AF488 and αCD3–compG–AF647 DNA conjugates onto microparticles presenting a 1:1 R:G DNA scaffold. These steps assume NHS–dye-labeled antibodies were prepared previously, although the procedure is identical for any fluorescently tagged antibody (e.g., using dye-labeled DNA during Ab–DNA synthesis). If this procedure is performed under sterile conditions and using sterile materials, these particles are applicable for use in vitro and in vivo settings (see Critical Step following Step 169).

158. Assuming particles are lyophilized in −20 °C storage, prepare a small quantity of particles to allow for 1 OD_{550} in a final volume of 100 μL TE–Tween by following Steps 64–69 (adjusting for the desired OD_{550}).

159. Assuming that antibodies have been prepared and labeled using NHS–dye, calculate the necessary volume of αCD28–compR–AF488 and αCD3–compG–AF647 to reach a final concentration of 30 nM each in 100 μL (total hybridization volume).

CRITICAL STEP The antibody loading capacity on 2 μm particles is ~20 nM/OD_{550}, which was previously determined using plate spectrophotometry as described in 'Particle surface DNA loading analysis'[20]. The maximum loading capacity of other biomolecules should be determined for every new biomolecule type and particle size to ensure an appropriate excess is given during surface hybridization. Given that the scaffold ratio is 1:1, each antibody will maximally load ~10 nM/OD_{550}. Since loading is at 3× excess of the theoretical limit, each antibody is hybridized at 30 nM/OD_{550} for a combined antibody concentration of 60 nM/OD_{550}.

CRITICAL STEP Particle loading should occur between 1 and 10 OD_{550}. The total reaction volume is flexible, although the total reagent use should be considered. If done under sterile conditions, particles can be used for biological applications using a small sample for flow analysis.

160. Refer to Table 4 for calculating the hybridization volumes, adjusting for the target OD_{550}, antibody loading capacities and antibody concentrations.

161. Spin the 100 µL of 1 OD_{550} particles for 5 min at 6,000*g* and remove supernatant until the calculated particle volume needed from Step 163 is reached.

162. To a 1.5 mL tube, add 50 µL of 2× hybridization buffer, the Ab–DNAs and extra TE–Tween using the values in Step 163.

163. Resuspend the particles and add into this reaction tube.

164. Use the micropipette to mix and sonicate briefly (~5–10 s). Incubate for 30 min at 37 °C.

165. Wash particles twice according to Steps 78–79 at 4 °C.

166. After the last wash, resuspend in 500 µL TE–Tween.

CRITICAL STEP For adapting to sterile use, particles should be resuspended using sterile PBS rather than TE–Tween. Additional PBS washes could be used to ensure the removal of Tween 20 detergent or other hybridization components. However, particle loss may increase without the use of a detergent; thus, OD_{550} should be verified before particle dosing. While in vitro use is described later, particles for in vivo use should be concentrated using centrifugation to a desired volume suitable for localized or systemic injections as previously demonstrated[20].

Quantification of antibody loading onto particles using flow cytometry

TIMING 4–6 h—CRITICAL This procedure quantifies the microparticle surface loading of αCD28–compR–AF488 and αCD3–compG–AF647 using flow cytometry. Blank and single-antibody loaded particles are made using the method described in Steps 163–169 and are used for compensation controls and downstream calculations. Use single DNA-sequence-scaffolded particles (R or G only) for single-color controls to saturate the surface with their respective antibody species. The plate spectrophotometer used in the previous section ‘Particle surface DNA loading analysis’ can be used as an alternative quantification tool, although this uses prohibitively more material compared with flow cytometry.

167. Perform flow cytometric analysis on particles from Step 169. Reference Box 3 for performing surface-loading analysis using software such as FlowJo. We have included representative flow cytometry fluorescence histograms and calculated surface loadings for particles hybridized using a variety of loading methods as described in the section ‘Experimental design’ (Fig. 4). Example data from Steps 161–169 that used the 1:1 R:G surface scaffold and particle surface-saturating amount of Ab–DNA is provided (Fig. 4h).

TROUBLESHOOTING

T cell enrichment from leukapheresis products

TIMING 2 h—CRITICAL This procedure describes the isolation of either $CD4^+$ or $CD8^+$ T cells from leukapheresis blood product using commercial negative selection beads.

168. In a sterilized BSC, isolate $CD4^+$ or $CD8^+$ T cells from leukapheresis blood using the EasySep Enrichment kit per the manufacturer's instructions. Wash steps should be performed using sterile-filtered PBS–FBS wash buffer (see 'Reagent Setup'). When required, cells should be centrifuged at 300*g* for 5 min at 4 °C.

169. After cells have been enriched, spin down the cells at 300*g* for 5 min at 4 °C. Calculate the volume to resuspend cells between 10 and 50×10^6 cells/mL. Remove the supernatant and resuspend in sterile freezing medium to the desired concentration. Aliquot 1 mL of cells into each liquid nitrogen compatible freezing vial and place into a polyethylene CoolCell. Immediately transfer the CoolCell into the −80 °C freezer overnight. Transfer freezing vials to liquid nitrogen storage the following day.

PAUSE POINT T cells can be stored in liquid nitrogen for over a year and thawed when needed.

T cell expansion using ICEp

TIMING ~11 d—CRITICAL This procedure describes $CD4^+$ T cell culturing using ICEps, which is identical for $CD8^+$ T cells. Cells will be expanded in a 96-well (flat-bottom) culture plate throughout, although they can be transferred to larger well-plate volumes as long as the appropriate cell concentrations are maintained. ICEps should be prepared sterilely with αCD3 and αCD28 1 d before T cell activation as described in the previous section, 'Particle surface loading of antibody'. The quantity of particles required should be determined before T cell activation to reduce material waste. Complete T cell media (media) should contain 100 U/mL hIL-2.

170. Centrifuge αCD3- and αCD28-loaded ICEps at 6,000*g* for 5 min at 4 °C.

171. In a BSC, carefully remove supernatant and resuspend to 1 OD_{550} (~20×10^6 particles/mL) in media. OD_{550} can be measured to verify desired particle concentration.

CRITICAL STEP We will seed 25,000 T cells per 96-well plate, so 1.25 μL of particles (at 1 OD_{550}) will eventually be added to each well for 1× particle to cell excess. Additional particle amounts can be added, although the total well volume should stay consistent between conditions.

172. Warm media in a 37 °C water bath.

173. Aliquot 9 mL of warmed media into a 15 mL tube.

174. Remove a $CD4^+$ enriched T cell vial from liquid nitrogen storage and thaw in the water bath. Just before fully thawing, move the vial into the BSC.

175. Gently pipette to resuspend the cell pellet and transfer the volume into the 9 mL of warmed media to dilute the DMSO.

176. Spin cells at 300*g* for 5 min at 4 °C.

177. Remove the supernatant and resuspend cells in 10 mL of media and count the cells. Depending on the number of T cell conditions, dilute an appropriate volume of cells in media to ~0.278 × 10^6 cells/mL.

178. After mixing, pipette 90 μL of cells per well in a 96-well plate.

179. Thoroughly mix the ICEps without generating bubbles and add an appropriate volume to each well (1.25 μL of 1 OD_{550} for 1× particle-to-cell). Occasionally resuspend stock ICEps to prevent particle settling.

180. Add additional media for a total well volume of ~100 μL. The cells are now approximately at 0.25 × 10^6 cells/mL.

181. Using a multichannel pipette, gently mix all wells to thoroughly distribute ICEps and cells. Transfer the seeded culture plate into a sterile incubator set to 37 °C and 5% CO_2.

182. After 24 h (day 1) visualize the plate under a bright-field microscope to observe cell clustering and look for any signs of contamination.

183. After 48 h (day 2), double the well volume using prewarmed media (~100 μL) by dispensing around the well perimeter, attempting not to disturb the cell clusters. Cells are typically not ready to be split at this day due to a freezing-related growth delay.

184. On day 4, resuspend the T cell wells and take a small sample for counting.

185. Calculate the volume containing 25,000 cells and reseed this volume into an unused well. Add media for a total well volume of 100 μL.

186. Track the cell expansion fold between well splitting and repeat every 2 d until growth slows or a predetermined end point has been reached (Fig. 5a,b).

CRITICAL STEP At this concentration, a 2 d splitting procedure lets cells expand upwards of 10–15 fold without filling the entire well. Other plating conditions requires different schedules.

187. At the experiment endpoint, stain and fix cells for flow cytometry analysis (Fig. 5c–e).

TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 5.

Timing

- Steps 1–32, PLGA–PEG–DNA conjugate synthesis: 2 d

- Steps 33–63, PLGA particle fabrication: 6 h (not including overnight polymer dissolving)
- Steps 64–90, particle surface DNA loading analysis: 4 h
- Steps 91–100, particle size quantification: 2 h
- Steps 101–116, antibody conjugation with complementary DNA: 1 d
- Steps 117–153, Ab–DNA purification: 1–2 d (depending on number of purifications)
- Steps 154–160, preparation of antibodies for surface loading quantification: 2 h
- Steps 161–169, particle surface loading of antibody: 2 h
- Step 170, quantification of antibody loading onto particles using flow cytometry: 4–6 h (including analysis timing)
- Steps 171–172, T cell enrichment from leukapheresis products: 2 h
- Steps 173–190, T cell expansion using ICEp: ~11 d (including flow cytometry analysis)

Anticipated results

Fabrication of PLGA particles with dense DNA scaffolds

A critical step in determining the particle DNA-scaffold density is the synthesis of polymer–DNA amphiphiles. As the sole surfactant for the emulsion-based fabrication protocol, the surface presentation of the DNA domain is driven by hydrophobic–hydrophilic interactions[56,73] (Fig. 1(i)). Polymer–DNA amphiphiles are generated from the conjugation of PLGA(10k)–PEG(5k)–mal (PLGA–PEG–mal, Akina #AI053) with thiol–DNA–17mer via the Michael addition reaction in DMF/TE (vol/vol, 90:10) solvent, which can then be directly used for the emulsion protocol without prior purification (Fig. 1(i) and Fig. 2a). We found that the input amount of the PLGA–PEG–mal determines the particle size in the downstream emulsion protocol, so we maintained a constant 100 nmol of polymer reactant for each fabrication procedure targeting a particle diameter of 2 μm. Thus, the input molar excess of thiol–DNA relative to polymer—which directly correlates with the conjugation efficiency of PLGA–PEG–DNA—determines the DNA-scaffold density on the yielded particle product (Fig. 2b–f). Notably, we found that there was a conjugation efficiency variation associated with different lots of PLGA–PEG–mal made by Akina Inc. (Fig. 2c,e) and the quality of thiol–DNA (Fig. 2d), of which the latter became less of an issue when we sourced the thiol–DNA from IDT Inc. instead of in-house synthesis as previously reported[20]. The 1:1 PLGA–PEG–mal to thiol–DNA reaction ratio was chosen since the conjugation efficiency for the high-quality PLGA–PEG–mal lot approached saturation as identified by urea–PAGE (see PLGA Lot-1 in Fig. 2b,e). Poor-quality PLGA–PEG–mal may require high amounts of thiol–DNA to reach saturation, which is not economically viable and contributes to a lower DNA-scaffold density (see PLGA Lot-2 in Fig. 2b,e,f). Here, urea–PAGE provided an effective and essential tool for the quality control of PLGA–PEG–DNA, which should be routinely performed. As mentioned, the input amount of PLGA–PEG–mal

relative to unmodified PLGA during emulsion determines the size profile; therefore, it can be adjusted to obtain particles with varied intended size (Table 3 and Fig. 2g,h). This protocol produces ~200 nm diameter nanoparticles with a PDI range of less than 0.200, as determined by the built-in Zetasizer software, which indicates highly uniform particles (Fig. 2h, Size A)[74]. Further, these particles maintain similar mean diameters across batches, indicating low batch variance. For micron-scale particles, large size distributions have been reported using bulk probe-sonication protocols, which was also observed here (Fig. 2h, Size B and C)[55]. While a given batch may be polydisperse, batch-to-batch variation was minimal, as indicated by the similar diameter and s.d. ranges compared with their averaged value across batches. Therefore, across size scales, this protocol generates DNA-scaffolded particles with minimal batch-to-batch variation.

Ab–DNA conjugation and purification

To chemically modify antibodies with minimal activity loss, a selective reduction protocol using a precise molar excess of TCEP (4.5×) is used to generate free thiols for cDNA attachment (Fig. 3a,b). Therefore, it is important to accurately measure the antibody concentration to determine the TCEP dose. During antibody handling, Ca^{2+}/Mg^{2+}-free PBS buffer supplemented with 10 mM EDTA is used to keep the thiol groups from oxidizing and reforming disulfide linkages[75]. SDS–PAGE serves as a handy tool to check the extent of antibody reduction and fractionation after Ab–DNA conjugation (Fig. 3b). Linker-attached cDNA with mal functionalization (DNA–PEG–mal) is given in excess to ensure a high yield of DNA–Ab conjugates. However, the excess amount of unreacted DNA needs to be removed to avoid competition in the downstream surface hybridization step. Hence, we titrated the DNA molar excess to the antibody and found that a range of 4–6× is the minimal excess for the highest conjugation efficiency, as determined by the saturating trend for the conjugation reaction (Fig. 3c). Fc affinity-based chromatography was found to be the only method that effectively removed unreacted DNA. To ensure a high recovery of the costly antibodies and a complete removal of excess DNA, we provide guidelines to determine the appropriate elution fractions to collect from the purification (Fig. 3d) and to check for residual free-DNA after purification (Fig. 3e) (see ‘Ab-DNA purification’).

Particle surface functionalization and quantification

The ratiometric and density control of one or more functionalities on particle surfaces are achieved through cargo-directed (Fig. 4a–e) and scaffold-directed (Fig. 4f–h) strategies. For the former method, one or multiple functionalities (cDNA–protein cargos) are hybridized onto the surfaces with a total input amount below the predetermined loading capacity of the cargo (Fig. 4a–e). The density and relative ratio of different cargos are adjusted by the input mixture ratio before surface hybridization (Fig. 4d,e). For the latter method, one or multiple functionalities are hybridized onto particles with different densities of DNA scaffolds (with one or more sequences), and the loading input of the cargos are controlled at 3× molar excess to the predetermined loading capacity of each cargo (Fig. 4f–h). Flow cytometry enables the precise ratiometric quantification of surface-decorated cargos resulting from either method. While the cargo-directed method does not require unique particle scaffold formulations, it can have limitations in precision when cargos with different chemical properties (e.g., size and charge) are co-loaded[20]. Currently, we focus on

using this method to characterize the relative densities and ratios of biomolecules, although the absolute numbers could be further quantified by establishing standard titrations in the future.

Exemplified human T cell activation ex vivo

DNA-scaffolded PLGA microparticles (2 μm) were coated with cDNA-conjugated agonistic antibodies αCD3 and αCD28 at various ratios to provide stimulatory and co-stimulatory signals for human T cell activation and ex vivo expansion, which is a key step for T cell manufacturing[29]. As reported previously, the ratiometric control of αCD3 to αCD28 had an impact on T cell expansion fold (Fig. 5a), and here we found that the particle to cell excess also affected cell expansion[20] (Fig. 5b). Additionally, we expect that the size of the particles and the stability of the polymer may also matter for cell activation, so these chemiophysical parameters—in addition to details of material–cell interactions—would need to be systematically investigated among multiple T cell donors when adopting this type of material for T cell manufacturing[24,76,77]. Other than cell quantity, cell quality that is directly associated with the therapeutic efficacy after infusion into patients should also be evaluated. Here, we exemplified a flow cytometry-based immune profiling to evaluate cell differentiation (memory and effector fates, Fig. 5c,d) and exhaustion (co-expression of inhibitory receptors, Fig. 5c,e). While cell phenotyping provides important metrics relating to cell quality, the functionality of manufactured cells should also be evaluated in vivo in related animal models[78].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Z. Gartner and B. Moser for relevant DNA synthesis, S. Douglas and K. Shen for gel imaging, W. Lim for sonication equipment, the University of California, San Francisco (UCSF) Nikon Imaging center for confocal microscopy, and V. Nguyen and the UCSF Flow Cytometry Core (RRID: SCR_018206) for their flow cytometry expertise and equipment use—funded in part by the Diabetes Research Center via the National Institutes of Health (NIH) grant P30 DK063720. P.H. was supported by the UCSF Medical Scientist Training Program training grant T32GM141323 via the National Institute of General Medical Sciences (NIGMS). X.H. acknowledges the start-up fund provided by the School of Biomedical Engineering, Science, and Health Systems at Drexel University. This work was also partially funded by the UCSF Diabetes Center Pilot and Feasibility award funded in part by NIH grant P30 DK063720 (P.H., T.D. and Q.T.), NIH grant 1U54CA244438 (T.D.), the Northern California JDRF Center of Excellence 5-COE-2019-860-S-B (Q.T.) and the JDRF grant 2-SRA-2022-1221-S-B (T.D.). We thank E. Ronin and P. Ho for helpful discussion and trainings. We also thank E. Hansen for help with protocol validation and assistance.

Data availability

Any raw data that supports the plots within this paper are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

References

1. Mitchell MJ et al. Engineering precision nanoparticles for drug delivery. Nat. Rev. Drug Discov 20, 101–124 (2021). [PubMed: 33277608]

2. Blanco E, Shen H & Ferrari M Principles of nanoparticle design for overcoming biological barriers to drug delivery. Nat. Biotechnol 33, 941–951 (2015). [PubMed: 26348965]
3. Dellacherie MO, Seo BR & Mooney DJ Macroscale biomaterials strategies for local immunomodulation. Nat. Rev. Mater 4, 379–397 (2019).
4. Wang H & Mooney DJ Biomaterial-assisted targeted modulation of immune cells in cancer treatment. Nat. Mater 17, 761–772 (2018). [PubMed: 30104668]
5. Waldman AD, Fritz JM & Lenardo MJ A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat. Rev. Immunol 20, 651–668 (2020). [PubMed: 32433532]
6. Tan S, Li D & Zhu X Cancer immunotherapy: pros, cons and beyond. Biomed. Pharmacother 124, 109821 (2020). [PubMed: 31962285]
7. Sterner RC & Sterner RM CAR-T cell therapy: current limitations and potential strategies. Blood Cancer J. 11, 1–11 (2021). [PubMed: 33414374]
8. Romano M, Fanelli G, Albany CJ, Giganti G & Lombardi G Past, present, and future of regulatory T cell therapy in transplantation and autoimmunity. Front. Immunol 10, 43 (2019). [PubMed: 30804926]
9. Ferreira LMR, Muller YD, Bluestone JA & Tang Q Next-generation regulatory T cell therapy. Nat. Rev. Drug Discov 18, 749–769 (2019). [PubMed: 31541224]
10. Herold KC et al. An anti-CD3 antibody, teplizumab, in relatives at risk for type 1 diabetes. N. Engl. J. Med 381, 603–613 (2019). [PubMed: 31180194]
11. Balcerek J et al. Polyclonal regulatory T cell manufacturing under cGMP: a decade of experience. Front. Immunol 12, 744763 (2021). [PubMed: 34867967]
12. Dong S et al. The effect of low-dose IL-2 and Treg adoptive cell therapy in patients with type 1 diabetes. JCI Insight 6, e147474 (2021). [PubMed: 34324441]
13. Lin MJ et al. Cancer vaccines: the next immunotherapy frontier. Nat. Cancer 3, 911–926 (2022). [PubMed: 35999309]
14. Larson RC & Maus MV Recent advances and discoveries in the mechanisms and functions of CAR T cells. Nat. Rev. Cancer 21, 145–161 (2021). [PubMed: 33483715]
15. Tang L et al. Enhancing T cell therapy through TCR-signaling-responsive nanoparticle drug delivery. Nat. Biotechnol 36, 707–716 (2018). [PubMed: 29985479]
16. Bonati L & Tang L Cytokine engineering for targeted cancer immunotherapy. Curr. Opin. Chem. Biol 62, 43–52 (2021). [PubMed: 33684633]
17. Hwang J-R, Byeon Y, Kim D & Park S-G Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. Exp. Mol. Med 52, 750–761 (2020). [PubMed: 32439954]
18. Veerman RE, Güçlüler Akpinar G, Eldh M & Gabrielsson S Immune cell-derived extracellular vesicles—functions and therapeutic applications. Trends Mol. Med 25, 382–394 (2019). [PubMed: 30853173]
19. Chang JT, Wherry EJ & Goldrath AW Molecular regulation of effector and memory T cell differentiation. Nat. Immunol 15, 1104–1115 (2014). [PubMed: 25396352]
20. Huang X et al. DNA scaffolds enable efficient and tunable functionalization of biomaterials for immune cell modulation. Nat. Nanotechnol 16, 214–223 (2021). [PubMed: 33318641]
21. Majedi FS et al. Augmentation of T-cell activation by oscillatory forces and engineered antigen-presenting cells. Nano Lett. 19, 6945–6954 (2019). [PubMed: 31478664]
22. Cheung AS, Zhang DKY, Koshy ST & Mooney DJ Scaffolds that mimic antigen-presenting cells enable ex vivo expansion of primary T cells. Nat. Biotechnol 36, 160–169 (2018). [PubMed: 29334370]
23. Rhodes KR, Meyer RA, Wang J, Tzeng SY & Green JJ Biomimetic tolerogenic artificial antigen presenting cells for regulatory T cell induction. Acta Biomater. 112, 136–148 (2020). [PubMed: 32522714]
24. Kim JV, Latouche J-B, Rivière I & Sadelain M The ABCs of artificial antigen presentation. Nat. Biotechnol 22, 403–410 (2004). [PubMed: 15060556]

25. Boozer C, Ladd J, Chen S & Jiang S DNA-directed protein immobilization for simultaneous detection of multiple analytes by surface plasmon resonance biosensor. Anal. Chem 78, 1515–1519 (2006). [PubMed: 16503602]
26. Liu Y & Yu J Oriented immobilization of proteins on solid supports for use in biosensors and biochips: a review. Microchim. Acta 183, 1–19 (2016).
27. Bilal M, Asgher M, Cheng H, Yan Y & Iqbal HMN Multi-point enzyme immobilization, surface chemistry, and novel platforms: a paradigm shift in biocatalyst design. Crit. Rev. Biotechnol 39, 202–219 (2019). [PubMed: 30394121]
28. Smith MR, Tolbert SV & Wen F Protein-scaffold directed nanoscale assembly of T cell ligands: Artificial antigen presentation with defined valency, density, and ratio. ACS Synth. Biol 7, 1629–1639 (2018). [PubMed: 29733631]
29. Wang X & Rivière I Clinical manufacturing of CAR T cells: foundation of a promising therapy. Mol. Ther. Oncolytics 3, 16015 (2016). [PubMed: 27347557]
30. Arcangeli S et al. CAR T cell manufacturing from naive/stem memory T lymphocytes enhances antitumor responses while curtailing cytokine release syndrome. J. Clin. Invest 132, e150807 (2022). [PubMed: 35503659]
31. López-Cantillo G, Urueña C, Camacho BA & Ramírez-Segura C CAR-T cell performance: how to improve their persistence? Front. Immunol 13, 878209 (2022). [PubMed: 35572525]
32. Arcangeli S et al. Next-generation manufacturing protocols enriching TSCM CAR T cells can overcome disease-specific T cell defects in cancer patients. Front. Immunol 11, 1217 (2020). [PubMed: 32636841]
33. Elmowafy EM, Tiboni M & Soliman ME Biocompatibility, biodegradation and biomedical applications of poly(lactic acid)/poly(lactic-co-glycolic acid) micro and nanoparticles. J. Pharm. Investig 49, 347–380 (2019).
34. Roybal KT et al. Precision tumor recognition by T cells with combinatorial antigen-sensing circuits. Cell 164, 770–779 (2016). [PubMed: 26830879]
35. Maynard SA, Winter CW, Cunnane EM & Stevens MM Advancing cell-instructive biomaterials through increased understanding of cell receptor spacing and material surface functionalization. Regen. Eng. Transl. Med 7, 533–547 (2021).
36. Zhong JX, Raghavan P & Desai TA Harnessing biomaterials for immunomodulatory-driven tissue engineering. Regen. Eng. Transl. Med 10.1007/s40883-022-00279-6 (2022).
37. Mertgen A-S et al. Multifunctional biomaterials: combining material modification strategies for engineering of cell-contacting surfaces. ACS Appl. Mater. Interfaces 12, 21342–21367 (2020). [PubMed: 32286789]
38. Bao G, Mitragotri S & Tong S Multifunctional nanoparticles for drug delivery and molecular imaging. Annu. Rev. Biomed. Eng 15, 253–282 (2013). [PubMed: 23642243]
39. Saminathan A, Zajac M, Anees P & Krishnan Y Organelle-level precision with next-generation targeting technologies. Nat. Rev. Mater 7, 355–371 (2022).
40. Lagreca E et al. Recent advances in the formulation of PLGA microparticles for controlled drug delivery. Prog. Biomater 9, 153–174 (2020). [PubMed: 33058072]
41. Yu W, Liu R, Zhou Y & Gao H Size-tunable strategies for a tumor targeted drug delivery system. ACS Cent. Sci 6, 100–116 (2020). [PubMed: 32123729]
42. Pradal J et al. Effect of particle size on the biodistribution of nano- and microparticles following intra-articular injection in mice. Int. J. Pharm 498, 119–129 (2016). [PubMed: 26685724]
43. Adakkattil R, Thakur K & Rai V Reactivity and selectivity principles in native protein bioconjugation. Chem. Rec 21, 1941–1956 (2021). [PubMed: 34184826]
44. Trads JB, Tørring T & Gothelf KV Site-selective conjugation of native proteins with DNA. Acc. Chem. Res 50, 1367–1374 (2017). [PubMed: 28485577]
45. Saha B, Songe P, Evers TH & Prins MWJ The influence of covalent immobilization conditions on antibody accessibility on nanoparticles. Analyst 142, 4247–4256 (2017). [PubMed: 29068008]
46. Zamecnik CR, Lowe MM, Patterson DM, Rosenblum MD & Desai TA Injectable polymeric cytokine-binding nanowires are effective tissue-specific immunomodulators. ACS Nano 11, 11433–11440 (2017). [PubMed: 29124929]

47. Makaraviciute A, Jackson CD, Millner PA & Ramanaviciene A Considerations in producing preferentially reduced half-antibody fragments. J. Immunol. Methods 429, 50–56 (2016). [PubMed: 26779832]
48. Sapsford KE et al. Functionalizing nanoparticles with biological molecules: developing chemistries that facilitate nanotechnology. Chem. Rev 113, 1904–2074 (2013). [PubMed: 23432378]
49. Khandare J & Minko T Polymer–drug conjugates: progress in polymeric prodrugs. Prog. Polym. Sci 31, 359–397 (2006).
50. Martínez-Jothar L et al. Insights into maleimide-thiol conjugation chemistry: conditions for efficient surface functionalization of nanoparticles for receptor targeting. J. Control. Release 282, 101–109 (2018). [PubMed: 29526739]
51. Chiodi E, Marn AM, Geib MT & Ünlü MS The role of surface chemistry in the efficacy of protein and DNA microarrays for label-free detection: an overview. Polymers 13, 1026 (2021). [PubMed: 33810267]
52. Pei X et al. Putting precision and elegance in enzyme immobilisation with bio-orthogonal chemistry. Chem. Soc. Rev 51, 7281–7304 (2022). [PubMed: 35920313]
53. Wasserberg D, Cabanas-Danés J, Subramaniam V, Huskens J & Jonkheijm P Orthogonal supramolecular protein assembly on patterned bifunctional surfaces. Chem. Commun 54, 1615–1618 (2018).
54. Meder F, Kaur S, Treccani L & Rezwan K Controlling mixed-protein adsorption layers on colloidal alumina particles by tailoring carboxyl and hydroxyl surface group densities. Langmuir 29, 12502–12510 (2013). [PubMed: 23875793]
55. Wongrakpanich A, Khunkitchai N, Achayawat Y & Suksiriworapong J Ketorolac-loaded PLGA-/PLA-based microparticles stabilized by hyaluronic acid: effects of formulation composition and emulsification technique on particle characteristics and drug release behaviors. Polymers 15, 266 (2023). [PubMed: 36679147]
56. Makadia HK & Siegel SJ Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. Polymers 3, 1377–1397 (2011). [PubMed: 22577513]
57. Vivek K, Harivardhan Reddy L & Murthy RSR Comparative study of some biodegradable polymers on the entrapment efficiency and release behavior of etoposide from microspheres. Pharm. Dev. Technol 12, 79–88 (2007). [PubMed: 17484147]
58. Ghasemiyeh P & Mohammadi-Samani S Polymers blending as release modulating tool in drug delivery. Front. Mater 8, 752813 (2021).
59. Fu J et al. DNA-scaffolded proximity assembly and confinement of multienzyme reactions. Top. Curr. Chem 378, 38 (2020).
60. Wiener J, Kokotek D, Rosowski S, Lickert H & Meier M Preparation of single- and double-oligonucleotide antibody conjugates and their application for protein analytics. Sci. Rep 10, 1457 (2020). [PubMed: 31996713]
61. van der Sleen LM & Tych KM Bioconjugation strategies for connecting proteins to DNA-linkers for single-molecule force-based experiments. Nanomaterials 11, 2424 (2021). [PubMed: 34578744]
62. von Witting E, Hober S & Kanje S Affinity-based methods for site-specific conjugation of antibodies. Bioconjugate Chem. 32, 1515–1524 (2021).
63. Manning MC, Chou DK, Murphy BM, Payne RW & Katayama DS Stability of protein pharmaceuticals: an update. Pharm. Res 27, 544–575 (2010). [PubMed: 20143256]
64. Frokjaer S & Otzen DE Protein drug stability: a formulation challenge. Nat. Rev. Drug Discov 4, 298–306 (2005). [PubMed: 15803194]
65. Wang W Protein aggregation and its inhibition in biopharmaceutics. Int. J. Pharm 289, 1–30 (2005). [PubMed: 15652195]
66. Themeli M, Rivière I & Sadelain M New cell sources for T cell engineering and adoptive immunotherapy. Cell Stem Cell 16, 357–366 (2015). [PubMed: 25842976]
67. Stock S, Schmitt M & Sellner L Optimizing manufacturing protocols of chimeric antigen receptor T cells for improved anticancer immunotherapy. Int. J. Mol. Sci 20, 6223 (2019). [PubMed: 31835562]

68. Eskandari SK et al. Regulatory T cells engineered with TCR signaling–responsive IL-2 nanogels suppress alloimmunity in sites of antigen encounter. Sci. Transl. Med 12, eaaw4744 (2020). [PubMed: 33177180]

69. Ghaffari S et al. Optimizing interleukin-2 concentration, seeding density and bead-to-cell ratio of T-cell expansion for adoptive immunotherapy. BMC Immunol. 22, 43 (2021). [PubMed: 34217218]

70. Schneider CA, Rasband WS & Eliceiri KW NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675 (2012). [PubMed: 22930834]

71. Wang Y et al. Phase transitions in human IgG solutions. J. Chem. Phys 139, 121904 (2013). [PubMed: 24089716]

72. Wang SS, Yan YS & Ho K US FDA-approved therapeutic antibodies with high-concentration formulation: summaries and perspectives. Antib. Ther 4, 262–272 (2021). [PubMed: 34909579]

73. Vincent MP, Navidzadeh JO, Bobbala S & Scott EA Leveraging self-assembled nanobiomaterials for improved cancer immunotherapy. Cancer Cell 40, 255–276 (2022). [PubMed: 35148814]

74. Danaei M et al. Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceutics 10, 57 (2018). [PubMed: 29783687]

75. Fass D & Thorpe C Chemistry and enzymology of disulfide cross-linking in proteins. Chem. Rev 118, 1169–1198 (2018). [PubMed: 28699750]

76. Delcassian D, Sattler S & Dunlop IE T cell immunoengineering with advanced biomaterials. Integr. Biol 9, 211–222 (2017).

77. Wang C, Sun W, Ye Y, Bomba HN & Gu Z Bioengineering of artificial antigen presenting cells and lymphoid organs. Theranostics 7, 3504–3516 (2017). [PubMed: 28912891]

78. Si X, Xiao L, Brown CE & Wang D Preclinical evaluation of CAR T cell function: in vitro and in vivo models. Int. J. Mol. Sci 23, 3154 (2022). [PubMed: 35328572]

Key references

Huang X et al. Nat. Nanotechnol 16, 214–223 (2021): 10.1038/s41565-020-00813-z [PubMed: 33318641]

Box 1

Sonication settings for particle fabrication

The following sonication settings were chosen for the S-4000 probe sonicator (Qsonica). The settings should be adjusted for other sonication systems and yielded particles should be quality checked to match the characteristics described within this protocol.

1. Total energy: 230–250 J
2. Amplitude: 30
3. Pulse sequence timing: 5 s on, 10 s off
4. Total sonication time: 25 s (5 total pulse sequences)

Box 2

Equations for determining the DNA concentration within Ab–DNA solution

Perform the following steps for determining the specific concentration contributions from antibody and DNA within the Ab–DNA conjugate solution:

1. Measure the A_{260} and A_{280} for dilutions of (1) purified Ab–DNA and (2) pure antibody (pure Ab).
2. Record the dilution factor, D, used to measure the Ab–DNA A_{260} and A_{280}.
3. (Equation 1) $A_{280\ (\text{Ab Component of Ab–DNA})} \times D/1.33$ = antibody mg/mL.
4. Solve for $A_{280\ (\text{Ab Component of Ab–DNA})}$ where the antibody mg/mL was calculated from microBCA in Step 143.
5. (Equation 2) $A_{280\ (\text{pure Ab})}/A260\ (\text{pure Ab}) = G$. Solve for G.
6. (Equation 3) $A_{280\ (\text{Ab component of Ab–DNA})}/A_{260\ (\text{Ab component of Ab–DNA})} = G$.
7. Solve Equation 3 for $A_{260\ (\text{Ab component of Ab–DNA})}$, where $A_{280\ (\text{Ab component of Ab–DNA})}$ is solved in line iv and G is solved in line v.
8. (Equation 4) $A_{260(\text{Ab–DNA})} = A_{260(\text{DNA component of Ab–DNA})} + A_{260(\text{Ab component of Ab–DNA})}$
9. Solve Equation 4 for $A_{260\ (\text{DNA component of Ab–DNA})}$ using the $A_{260\ (\text{Ab–DNA})}$ measured on a Nanodrop and the $A_{260\ (\text{Ab component of Ab–DNA})}$ solved in line vii.
10. Solve for the DNA concentration in the stock solution with $A_{260\ (\text{DNA component of Ab–DNA})} \times D$/(extinction coefficient of DNA used for conjugation) = DNA μM.

Box 3

Quantification of particle surface loading using flow cytometry

Flow cytometry should be performed including single-color and blank controls. The mean fluorescent intensities (MFIs) can be used to calculate the antibody surface occupancy from the below equations. Optional normalization to the particle DNA loading can be performed for more convenient comparisons of surface ratios between particles batches when comparing particles with varied total surface protein density. This method is useful for comparing surface DNA ratios but cannot be used to compare DNA densities between batches.

1. Surface occupancy of antibody (% αCD3)
   - % αCD3 = ($MFI_{\alpha CD3} - M_{FIblank}$ particle)/ ($MFI_{\text{single-color-control }\alpha CD3} - MFI_{\text{blank particle}}$), where the MFI is the signal coming from the respective channel as the αCD3 antibody dye. Repeat for % αCD28 using relevant values
2. Ratio of αCD3:αCD28
   - If % αCD3 > % αCD28, then the ratio of αCD3:αCD28 is (% αCD3/% αCD28):1
   - If % αCD3 < % αCD28, then the ratio of αCD3:αCD28 is 1:(% αCD28/% αCD3)
3. Optional: normalization of surface occupancy
   - $\%\ \alpha CD3_{norm}$ = (% αCD3)/(% αCD3 + % αCD28), repeat similar calculation for normalizing % αCD28 using relevant values

Key points

- The protocol describes the fabrication of DNA scaffolds, the bioconjugation of biomolecules with complementary DNAs, conjugate assembly onto the DNA scaffolds and their immunomodulatory effect on primary human T cells in culture.
- Steric hindrance typically limits the use of orthogonal chemistry and covalent surface attachment strategies, whereas this DNA hybridization-based approach maintains control over the loading of each biomolecule species.

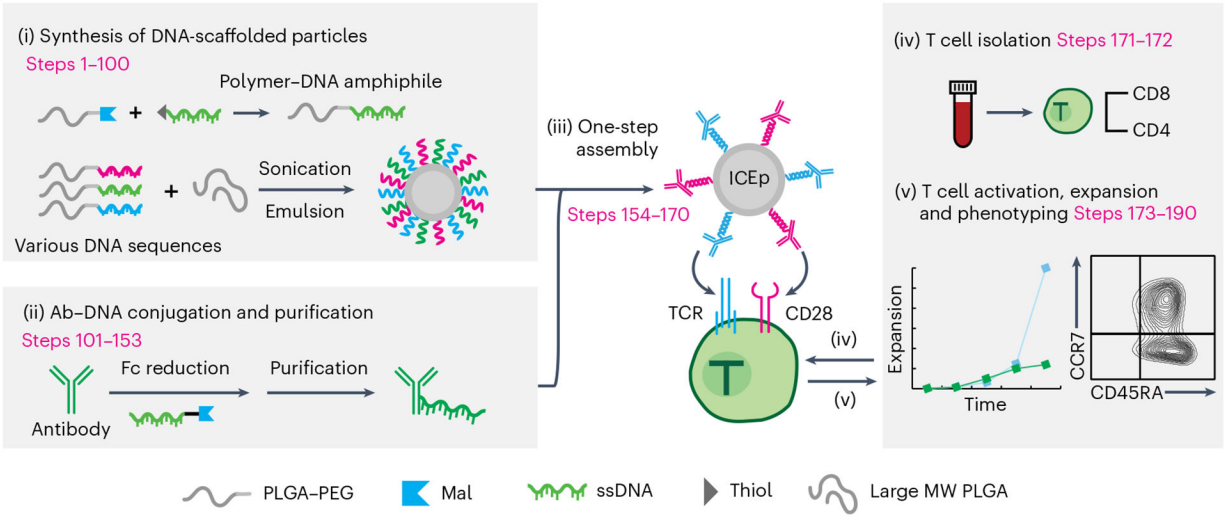

Fig. 1 |. Schematic of the fabrication protocol for precision ICEps.

The precise functionalization of immunomodulatory signals on synthetic material surfaces is enabled by attaching DNA handles on both components and associating them via DNA hybridization. This protocol involves (i) synthesizing particles with dense surface DNA scaffolds (with one or multiple sequences) through emulsion-based fabrication using polymer–DNA amphiphiles as surfactants, (ii) conjugating the cDNA to the immunomodulatory biomolecules with minimal bioactivity loss and complete removal of free DNA, and (iii) loading cDNA–biomolecule conjugates on particle surfaces through one-step hybridization. Here, ICEps are exemplified for their use in human T cell ex vivo expansion, which is highlighted with essential details in (iv) T cell isolation and (v) cell culture and activation that can impact phenotypic outcome of cell products. TCR, T cell receptor.

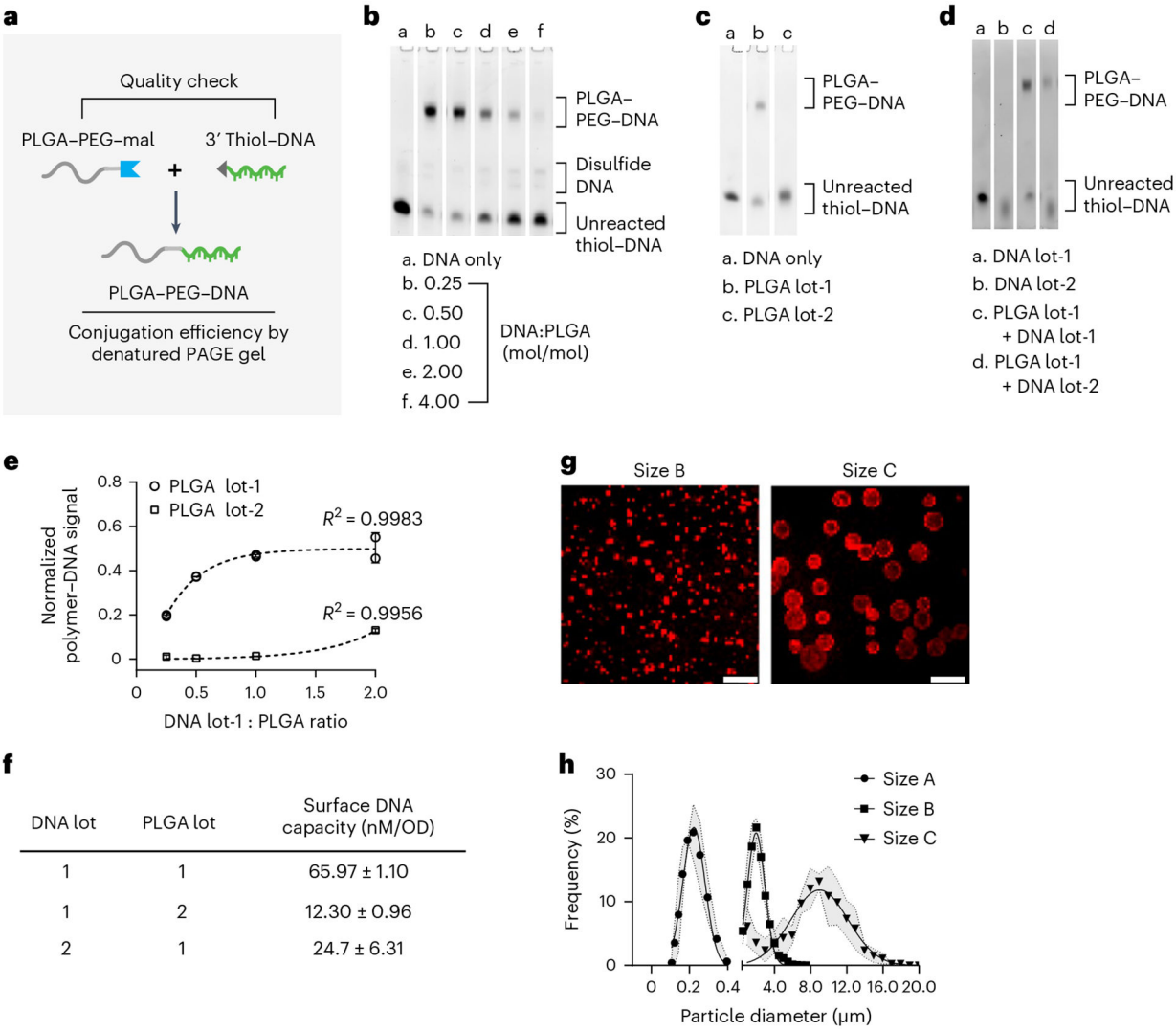

Fig. 2 |. Quality control of PLGA particles with dense DNA scaffolds.

a, A schematic of the synthesis of polymer–DNA amphiphiles and their quality check via gel electrophoresis—an essential step in achieving a high-DNA-scaffold density upon particle fabrication. **b**–**d**, Urea–PAGE of PLGA–PEG–DNA conjugates from the synthesis reactions using varying molar ratios of thiol–DNA to PLGA–PEG–mal (**b**), different lots of PLGA–PEG–mal (**c**) and different lots of thiol–DNA (**d**). The total DNA input into each lane was controlled at 1 pmol. **e**, Normalized PLGA–PEG–DNA amount to the total DNA amount in each lane of gel images in **b** and **c** using densitometry analysis in ImageJ. PLGA Lot-1 and Lot-2 were fit using exponential plateau (R^2 = 0.9983, root mean square error (RMSE) 0.0057) and exponential growth (R^2 = 0.9956, RMSE 0.0041) models, respectively. **f**, Surface-loading capacity of fluorescently labeled cDNA on microparticles (2 μm diameter) fabricated using PLGA–PEG–mal from different lots of PLGA–PEG–mal and thiol–DNA in **c** and **d**. Data are mean ± s.e.m of n = 3 technical replicates. **g**, Representative confocal microscope images (40× magnification) of particles fabricated using different protocols that yield different sizes and hybridized with Cy3-labeled cDNA. Scale bar, 20

μm. **h**, Size distribution of particles measured using Zetasizer (size A: mean diameter 220.42 nm, mean diameter range 201.54–234.35 nm, PDI range 0.120–0.176, $n = 3$ independent samples) or shown in g using ImageJ analysis (size B: mean diameter 1.90 μm, mean diameter range 1.74–2.10 μm, s.d. average 1.01 μm, s.d. range 0.99–1.01, $n = 3$ independent samples; size C: mean diameter 7.78 μm, mean diameter range 6.57–8.61 μm, s.d. average 3.75 μm, s.d. range 3.40–3.89 μm, $n = 4$ independent samples). Size frequencies were fit with gaussian distribution curves ((adjusted R^2, RMSE): size A (0.8323, 3.343); size B (0.9462, 1.720); size C (0.6897, 2.543)) and the shaded regions represent error envelopes of ±1 s.d. for each discrete frequency bin.

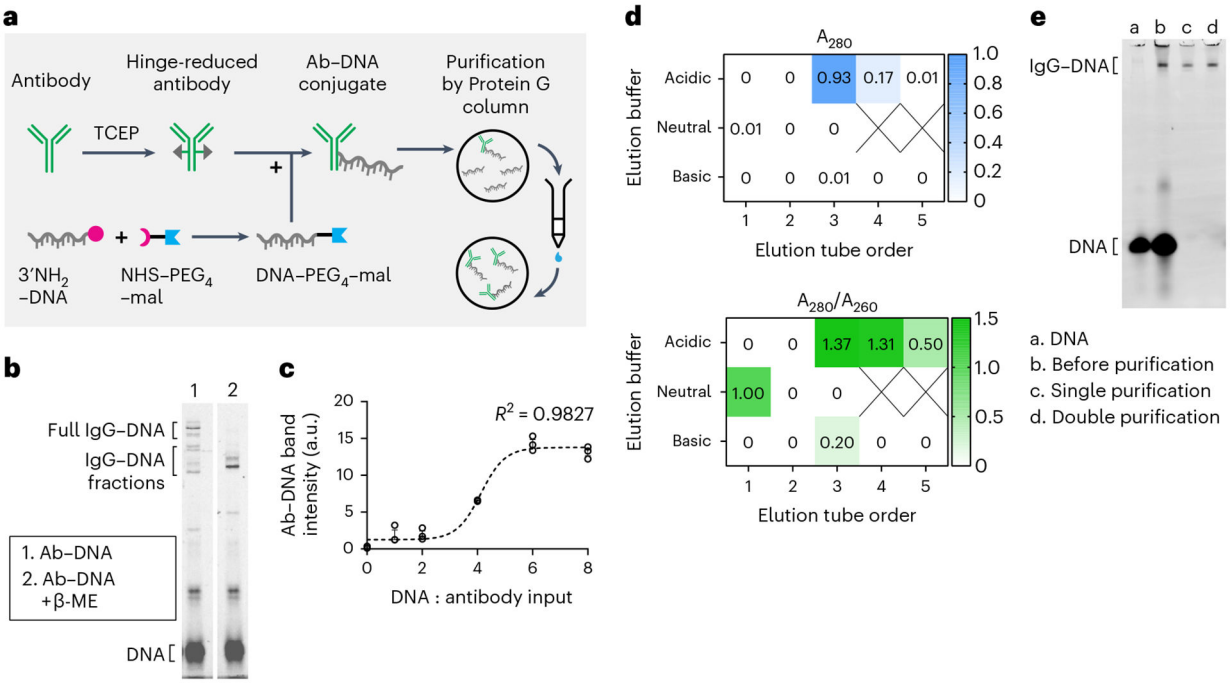

Fig. 3 |. Protocol and quality checkpoints of Ab–DNA conjugation and purification.

a, A schematic of Ab–DNA conjugation through selective reduction of antibody hinge-region disulfides and Fc affinity-based chromatography for purification to remove excess, unreacted DNA. **b**, SDS–PAGE of Ab–DNA conjugates from selective reduction by TCEP treatment at 4.5× molar excess (lane 1) versus full reduction by β-mercaptoethanol (β-ME) treatment (lane 2). **c**, Densitometric analysis of Ab–DNA bands from urea–PAGE of reactions with varying ratios of DNA to antibody input. Data were fit using a sigmoidal dose–response curve (R^2 = 0.9827, RSME 0.8078). Data represent mean ± s.e.m of n = 3 technical replicates. **d**, Example heat maps depicting the A_{280} or A_{280}/A_{260} ratios of Ab–DNA conjugates eluted from Fc affinity-based chromatography columns. **e**, Urea–PAGE of Ab–DNA conjugates with and without purification.

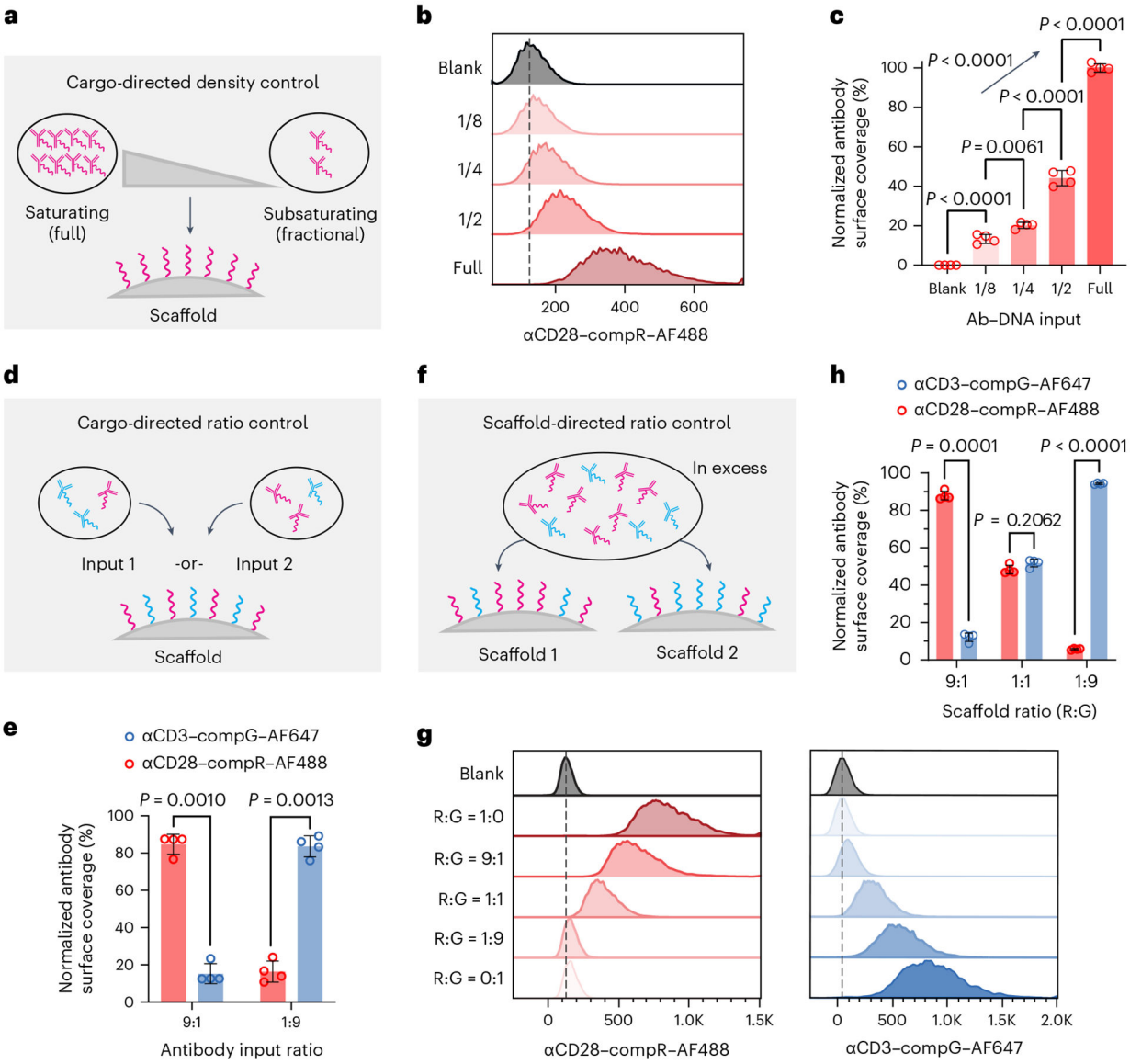

Fig. 4 |. Density and ratiometric control of cargos co-loaded onto particle surfaces.

a, A schematic of titrating the input amount of cDNA cargo to control the surface density on particles. **b**,**c**, Flow cytometry histograms (**b**) and normalized mean fluorescence intensities (MFIs) (**c**) of particles hybridized with varying input amounts of Ab–DNA (αCD28–compR–AF488, full: 20 nM/OD_{550}, 1/2: 10 nM/OD_{550}, 1/4: 5 nM/OD_{550}, 1/8: 2.5 nM/OD_{550}). A linear trend was determined using a one-way ANOVA ($F_{1,15}$ = 3,944, P < 0.0001) and inter-Ab–DNA input P values were determined by one-way ANOVA ($F_{4,15}$ = 1,151, P < 0.0001) followed by Tukey's post hoc test. **d**, A schematic of the ratiometric control of surface cargos by the input ratio of Ab–DNA cargos at the hybridization-based assembly step. **e**, Flow cytometry-based quantification of particles (R:G of 1:1) that are hybridized with different ratios of Ab–DNA (αCD28–compR–AF488 and αCD3–compG–AF647) with a constant total amount of 20 nM/OD. Data are normalized MFI to the maximal loading capacity from particles with only one sequence of scaffold (R:G of 1:0 or R:G of 0:1).

P values were determined by multiple two-tailed paired *t*-tests. **f**, A schematic of the ratiometric control of surface cargos by the DNA-scaffold ratio of different sequences. **g**, Flow cytometry histograms of particles fabricated with varying ratios of DNA scaffolds (R:G of 9:1, R:G of 1:1, R:G of 1:9) and hybridized with equal input amount of cargos (αCD28–compR–AF488 and αCD3–compG–AF647) in excess. **h**, Normalized MFI of histograms in g to the maximal loading capacity from particles with only one sequence of scaffold (R:G of 1:0 or R:G of 0:1). *P* values were determined by multiple two-tailed paired *t*-tests. Data in **c**,**e** and **h** represent mean ± s.d. of $n = 4$ experimental replicates from two independent experiments.

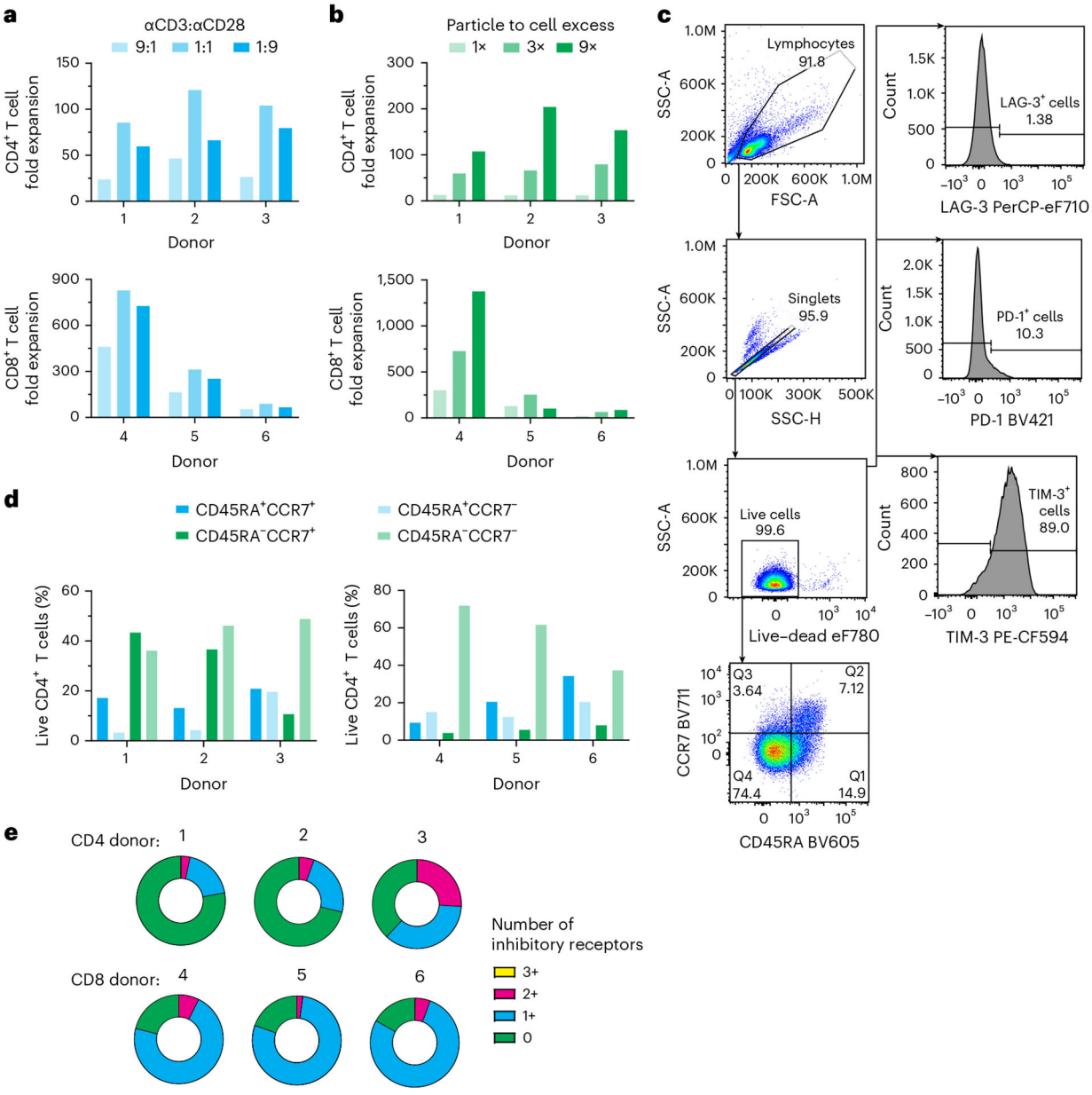

Fig. 5 |. ICEp activation of human T cells and their phenotypic characterization.

a, Expansion fold of T cells ($CD4^+$ and $CD8^+$) at day 11 from the activation by ICEp with varying ratios of αCD3 to αCD28 on particle surfaces and 3× excess of particles to cells. **b**, Expansion fold of T cells ($CD4^+$ and $CD8^+$) at day 11 from the activation by ICEp (αCD3–compR:αCD28–compG, 1:9) with varying particle-to-cell excess. **c**, Representative flow cytometry gating strategy for T cell phenotyping. SSC, side scatter; FSC, forward scatter; -A, area; -H, height. **d**, Populations of naive and stem cell-like memory ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$) and effector memory ($CD45RA^-CCR7^-$ and $CD45RA^+CCR7^-$) cells in expanded cells ($CD4^+$ and $CD8^+$) at day 11. Cells were activated using ICEp (αCD3–compR:αCD28–compG, 1:9) with 3× excess of particles to cells. **e**, Populations of cells with co-expression of inhibitory receptors (LAG-3,

PD-1 and TIM-3) at day 11. Cells were activated using ICEp (αCD3–compR:αCD28–compG, 1:9) with 3× excess of particles to cells. Data in **a**,**b**,**d** and **e** represent $n = 6$ independent donors ($n = 3$ for $CD4^+$ and $n = 3$ for $CD8^+$ T cell experiments).

Table 1 |

DNA sequences used for polymer and protein bioconjugation

Name	Sequence (5’ to 3’)	3’ Modification	Extinction coefficient ($M^{-1} \times cm^{-1}$)
R	AGTGGGAGCGCGTGATG	Thiol (C3 S-S)	173,700
G	GTTCATCTGCACCACCG	Thiol (C3 S-S)	148,100
B	GCCTTTACGATGTCCTT	Thiol (C3 S-S)	144,400
compR[a]	C[*]A[*]T[*]C[*]ACGCGCTCCCACT[*]A[*]A[*]T[*]T[*]	NH_2 (Amino C7)	188,400
compG[a]	C[*]G[*]G[*]TGGTGCAGATGAACTT[*]C[*]A[*]G[*]	NH_2 (Amino C7)	215,000
compB[a]	A[*]A[*]G[*]GACATCGTAAAGGCA[*]T[*]T[*]T[*]	NH_2 (Amino C7)	216,300

[*] internal phosphorothioate bond.

[a] cDNAs can be optionally labeled with fluorophore at the 5′-end for quantification purposes.

Table 2 |

PLGA–PEG–DNA conjugation reaction template

Predetermined	
a. PLGA–PEG–mal : DNA ratio	1
b. Target PLGA–PEG–mal concentration (μM)	200
c. PLGA–PEG–mal molecular number (Da)	22,941
d. Total DNA (μM)	See Step 8
e. Volume of DNA (μL)	See Step 8
Calculated	
f. Total reaction volume (μL)	(e × d × a)/b
g. Triethylamine volume (μL)	f/100
h. Extra TE buffer volume (μL)	(f/10) − e
i. PLGA–PEG–mal needed (mg)	$(a \times d \times e \times c)/(1 \times 10^9)$
j. 30 mg/mL PLGA–PEG–mal DMF volume (μL)	(1,000 × i)/30
k. Extra DMF volume (μL)	0.9 × f − j − g

Table 3 |

Fabrication conditions for achieving varied particle diameters

Particle diameter (μm)	PLGA-PEG-DNA (nmol)	Unmodified PLGA (MW 38,000–54,000) (mg)	$V_{organic}$ (μL)	$V_{aqueous}$ (μL)
0.2 (Size A)	200	5	500	1,000 (50μL 5% PVA)
2 (Size B)	100	50	500	500 (no PVA)
8 (Size C)	20	50	500	500 (no PVA)

Table 4 |

Particle surface hybridization of complementary DNA

Predetermined	
a. Target hybridization volume (μL)	100
b. Target particle OD_{550} (in target hybridization volume)	10–20
c. Stock particle OD_{550}	See Step 71
d. Concentration of cDNA (μM)	500
e. 2× DNA hybridization buffer volume (μL)	50
f. Loading capacity of cDNA (nM/OD_{550})[a]	150
Calculated	
g. Particle volume needed (μL)[b]	(a × b)/c
h. Total hybridization capacity of cDNA (μM)[c]	(b × f)/1,000
i. cDNA volume (3× capacity excess; μL)	(3 × h × a)/d
j. Extra TE-Tween volume (μL)	a − e − g − i, or 0

[a]When loading antibody onto 2 μm particles, the loading capacity is 20 nM/OD_{550} (ref. 20). These values must be determined for each biomolecule species and particle size. 200 nm diameter nanoparticle cDNA loading capacity is between 1,000 and 2,000 nM/OD_{550} and the 8 μm particles load between 12 and 20 nM/OD_{550}, depending on batch-to-batch variation. Antibody loading capacity has not been determined for the nanoparticles or 8 μm particles.

[b]If this volume exceeds 50 μL, centrifuge this particle volume as previously described and remove supernatant until 50 μL remains. Adjust the calculation for Extra TE–Tween volume accordingly.

[c]This value represents the total loading capacity of all represented cDNAs. If ratiometric particles are used, this value should be distributed between each sequence based on the particle scaffold DNA surface ratio.

Table 5 |

Troubleshooting table

Step	Problem	Possible reason	Solution
32	Poor PLGA–PEG–DNA conjugation or conjugate band intensity weak compared with unreacted DNA band	Improperly purified PLGA–PEG–mal contains mal-bearing precursors used during vendor polymer fabrication	Purify PLGA–PEG–mal via phase precipitation. If not solved, contact vendor
		Large amount of disulfide–DNA formed, evident from the band between the free DNA and PLGA–PEG–DNA bands	Verify accurate EDTA concentration and reduce DNA concentration used after precipitation and resuspension
		Improper moisture control can lead to mal hydrolysis in PLGA–PEG–mal	Allow the polymer container from freezer to warm to room temperature before opening to reduce condensation. Backfill with desiccated, inert gas before closing. Store in freezer desiccation chamber
50	Large aggregates seen after sonication	Improper mixture of conjugation reaction components	Ensure that the unmodified PLGA is thoroughly dissolved before adding addition reaction components. Vortex the reaction tube before sonication and after two sonication cycles
90	Low amount of DNA loading or off-target surface DNA ratios	PLGA–PEG–DNA sequence batches with varying conjugation efficiencies	PLGA–PEG–DNA conjugation efficiency should be tracked. If one batch failed or had low conjugation efficiency, the reaction should have been redone and the poor conjugate should not have been used to fabricate particles
		Polymer concentration too high after diluting DMSO-degraded particles, leading to reaggregation	After diluting the DMSO-degraded particles, do not exceed 1–2 OD_{550} per 100 μL. Minimize wait time before plate-reader analysis
		Improper particle handling during spin-down steps or dilutions	During supernatant removal steps, ensure that particles are not accidently removed. Ensure thorough mixing before any dilutions or aliquoting
		Particle scaffold fidelity is impaired due to particle age or mishandling	During lyophilized particle resuspension, select the proper solution to not increase salt concentration. Monitor particle DNA loading over time, as the mal–thiol bond can hydrolyze, leading to a less dense scaffold
		Improper ratio-mixture of PLGA–PEG–DNAs before particle fabrication	When drying polymers at a fixed ratio, ensure that the volumes mixed are accurate and no liquid is stuck inside the pipette tip
100	Particle sizes are highly variable between batches	Incorrect amount of unmodified PLGA	Ensure the unmodified PLGA amount is within ±1% weight target between batches
		Improper mixture of particle fabrication components	Ensure that all components are thoroughly mixed before emulsification steps. Pay extra attention to the pipetting volumes for viscous or volatile components
		Evaporation of EtOAc during mixing	Cool all liquid reagents on ice before mixing and avoid heat transfer from hands by holding tubes away from bottom. Reduce time that volatile tubes are opened
146	Low Ab–DNA gel band intensity	Poor conjugation efficiency	Titrate DNA amount to find optimal concentration; if no conjugation is observed, verify quality of the cDNA and/or use fresh TCEP. Ensure quality of the NHS–PEG–mal linker and keep in proper storage conditions
		Incorrect staining or gel imaging procedure	Verify staining reagent is compatible with selected gel type. Ensure the correct pmol of biomolecule was loaded in the lanes. Make sure that the gel-doc voltage and filter channel is appropriate for the dye used
170	Low antibody signal on particle surface	Poor or incorrect dye labeling	Increase the dye-to-antibody ratio and verify the reaction calculations are correct. If still low signal, verify the NHS–dye concentration or repeat labeling reaction

Step	Problem	Possible reason	Solution
		DNA impurities leading to competition for hybridization	Ensure that the Ab–DNA purity is above 95% for removing unreacted DNA in Step 147; perform additional column purifications and increase stringency on A_{280}/A_{260} cutoffs for elution collection
190	Poor T cell expansion	Donor variation	Evaluate multiple donors as some may just have poor expansion at baseline. Compare with a gold-standard expansion reagent, such as Dynabeads, to ensure the biomaterial is not at fault
		Low antibody activity due to improper handling	Verify antibody structural integrity using SDS–PAGE. Perform cell-staining studies using stock Ab–DNA and comparing binding with unmodified controls (flow cytometry). Perform new conjugation with newly purchased antibody if the stock unmodified antibody quality is suspected
		Incorrect number of particles given	It is critical to not lose particles during wash steps. Before adding to culture, remeasure the stock particle OD_{550} to ensure the correct volume of particles are added