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Corresponding Author:	Jordan Green, Ph.D. Johns Hopkins University Baltimore, MD UNITED STATES
Corresponding Author's Institution:	Johns Hopkins University
Corresponding Author E-Mail:	green@jhu.edu
Order of Authors:	Elana Ben-Akiva
	Kelly Rhodes
	Randall Meyer
	Jordan Green, Ph.D.
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TITLE:

Fabrication of Anisotropic Polymeric Artificial Antigen Presenting Cells for Cd8+ T Cell Activation

AUTHORS:

Elana Ben-Akiva[1]*, Kelly R Rhodes[1]*, Randall A Meyer[1], Jordan J Green[2]

[1]Biomedical Engineering, Translational Tissue Engineering Center, Institute for Nanobiotechnology, Johns Hopkins University School of Medicine, Baltimore, USA
[2]Biomedical Engineering, Translational Tissue Engineering Center, Institute for Nanobiotechnology, Ophthalmology, Oncology, Neurosurgery, Materials Science and Engineering, Chemical and Biomolecular Engineering, and the Bloomberg-Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, USA

* These authors contributed equally

CORRESPONDING AUTHOR:
Jordan J. Green (green@jhu.edu)

EMAIL ADDRESSES OF CO-AUTHORS:
Elana Ben-Akiva (ebenaki1@jhmi.edu)
Kelly R Rhodes (krhodes7@jhmi.edu)
Randall A Meyer (rmeyer16@jhmi.edu)

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SUMMARY:
Here, we present a protocol to quickly and reproducibly generate biologically inspired, biodegradable articifical antigen presenting cells (aAPC) with tunable size, shape, and surface protein presentation for T cell expansion *ex vivo* or *in vivo*.

ABSTRACT:
Artificial antigen presenting cells (aAPC) are a promising platform for immune modulation due to their potent ability to stimulate T cells. Acellular substrates offer key advantages over cell-based aAPC, including precise control of signal presentation parameters and physical properties of the aAPC surface to modulate its interactions with T cells. aAPC constructed from anisotropic particles, particularly ellipsoidal particles, have been shown to be more effective than their spherical counterparts at stimulating T cells due to increased binding and larger surface area available for T cell contact, as well as reduced nonspecific uptake and enhanced pharmacokinetic properties. Despite increased interest in anisotropic particles, even widely accepted methods of generating anisotropic particles such as thin-film stretching can be challenging to implement and

use reproducibly.

To this end, we describe a protocol for the rapid, standardized fabrication of biodegradable anisotropic particle-based aAPC with tunable size, shape, and signal presentation for T cell expansion *ex vivo* or *in vivo*, along with methods to characterize their size, morphology, and surface protein content, and to assess their functionality. This approach to fabricating anisotropic aAPC is scalable and reproducible, making it ideal for generating aAPC for "off-the-shelf" immunotherapies.

INTRODUCTION:

Artificial antigen presenting cells (aAPC) have shown promise as immunomodulatory agents because they can generate a robust antigen-specific T cell response. Essential to these platforms are their ability to efficiently present crucial signals for T cell activation. Acellular aAPC are an attractive alternative to cell-based aAPC because they are easier and less costly to fabricate, face fewer challenges during scale-up and translation, and alleviate risks associated with cell-based therapies. Acellular aAPC also allow for a high degree of control over signal presentation parameters and physical properties of the surface that will interface with T cells[1].

aAPC must recapitulate a minimum of two signals essential for T cell activation. Signal 1 provides antigen recognition and occurs when the T cell receptor (TCR) recognizes and engages with an MHC class I or II bearing its cognate antigen, culminating in signaling through the TCR complex. To bypass the antigen specificity requirement, aAPC systems often bear an agonistic monoclonal antibody against the CD3 receptor, which nonspecifically stimulates the TCR complex. Recombinant forms of MHC, particularly MHC multimers, have also been used on the surface of aAPC to provide antigen specificity[2,3]. Signal 2 is a costimulatory signal that directs T cell activity. To provide the costimulation necessary for T cell activation, the CD28 receptor is generally stimulated with an agonistic antibody presented on the aAPC surface, although other costimulatory receptors such as 4-1BB have been successfully targeted[4]. Signal 1 and 2 proteins are typically immobilized on the surface of rigid particles to synthesize aAPC. Historically, aAPC have been fabricated from a variety of materials, including polystyrene[4,5] and iron dextran[6]. Newer systems utilize biodegradable polymers like poly(lactic-co-glycolic acid) (PLGA) to generate aAPC that can be easily coupled to signal proteins, are suitable for direct administration *in vivo*, and can facilitate the sustained release of encapsulated cytokines or soluble factors to augment T cell activation[7,8].

In addition to the presence of necessary signal proteins, receptor engagement over a sufficiently large surface area during the aAPC/T cell interaction is essential for T cell activation. Thus, physical parameters of the aAPC such as size and shape drastically alter their available contact area and affect their ability to stimulate T cells. Micron-sized aAPC have been shown to be more effective at stimulating T cells than their nanoscale counterparts[9,10]. However, nano-aAPC can have superior biodistribution and better drainage to the lymph nodes that may enhance their performance *in vivo* over micro-aAPC[11]. Shape is another variable of interest in particle-based aAPC systems. Anisotropic aAPC have recently been shown to be more effective than isotropic particles at stimulating T cells, mainly due to enhanced interaction with target cells

coupled with reduced non-specific cell uptake. Cells preferentially bind to the long axis of ellipsoidal particles, and the larger radius of curvature and flatter surface allow for more contact between the aAPC and T cell[12]. The long axis of ellipsoidal particles also discourages phagocytosis, resulting in increased circulation time compared to spherical particles following *in vivo* administration[12,13]. Because of these advantages, ellipsoidal particles mediate greater expansion of antigen-specific T cells *in vitro* and *in vivo* compared to spherical particles, an effect observed at both the micro and nanoscales[12,13]. There are various strategies to fabricate anisotropic particles, but thin-film stretching is a simple, widely accepted method used to generate a range of diverse particle shapes[14]. Following synthesis, particles are cast into films and stretched in one or two dimensions at a temperature above the glass transition temperature of the particle material. The film is then dissolved to retrieve the particles. Despite growing interest in anisotropic particles, current approaches for fabricating particle-based aAPC are mostly limited to isotropic systems, and methods of altering particle shape can be difficult to implement, incompatible with certain aAPC synthesis strategies, and lack precision and reproducibility[15]. Our thin-film stretching technique can be performed manually or in an automated fashion to rapidly generate anisotropic particles synthesized from a variety of biodegradable polymers, stretched to a desired aspect ratio in one or two dimensions[15].

Based on our previous work, we developed a biodegradable particle-based approach combined with scalable thin-film stretching technology to rapidly generate aAPC with tunable size and shape in a standardized fashion for T cell expansion *ex vivo* or *in vivo*. Our protein conjugation strategy can be used to couple any protein(s) of interest to carboxyl groups on the particle surface at a desired density, giving this aAPC system a high degree of flexibility. We also describe methods to characterize the size, morphology, and surface protein content of aAPC, and to evaluate their functionality *in vitro*. This protocol can be easily adapted to expand immune cells *ex vivo* or *in vivo* for a variety of immunotherapeutic applications.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Johns Hopkins University.

1. Fabrication of Spherical PLGA Particles of Tunable Size

1.1. Preparation of materials for particle synthesis

1.1.1. Prepare 5% w/w polyvinyl alcohol (PVA) solution.

1.1.1.1. Add 500 mL of deionized (DI) water to an Erlenmeyer flask with a magnetic stir bar and place on hot plate stirrer at 500 rpm and monitor temperature with thermometer. Cover flask with tinfoil to prevent evaporation.

1.1.1.2. When water temperature reaches approximately 70 °C, add 25 g total of PVA in small batches over time, waiting for PVA to dissolve before adding more.

1.1.1.3. Once all PVA is dissolved (typically 30-60 min), let solution cool and sterile filter. Store at 4 °C for future use.

1.1.2. Prepare film casting solution of 10% w/w PVA and 2% w/w glycerol.

1.1.2.1. Add 500 mL of DI water to an Erlenmeyer flask with a magnetic stir bar. Add 8 mL of glycerol at room temperature and mix by trituration.

1.1.2.2. Place flask on hot plate stirrer at 500 rpm and monitor temperature with thermometer. Cover flask with tinfoil to prevent evaporation.

1.1.2.3. When solution temperature reaches approximately 70 °C, add 50 g total of PVA in small batches over time, waiting for PVA to dissolve before adding more.

1.1.2.4. Once all PVA is dissolved (typically 60 min), let solution cool and sterile filter using a bottle-top vacuum filter system with a pore size of 0.22 µm. Store at room temperature for future use.

1.1.3. Prepare a 50 mL 1% w/w PVA solution. Add 40 mL of DI water and 10 mL of 5% PVA solution (made in 1.1.1) to a 100-150 mL beaker.

1.1.4. Prepare a 100 mL 0.5% w/w PVA solution. Add 90 mL of DI water and 10 mL of 5% PVA solution to a 150-250 mL beaker.

1.1.5. Add a magnetic stir bar to the 0.5% PVA solution and place in a chemical hood on a stir plate at room temperature at 500 rpm.

1.2. Microparticle synthesis

1.2.1. Weigh out 100 mg of poly(lactic-co-glycolic acid) (PLGA) into a scintillation vial and dissolve in 5 mL of dichloromethane (DCM). Vortex to dissolve the PLGA.

1.2.2. Place homogenizer in the 50 mL 1% PVA solution so that the homogenizer is as close to the bottom of the beaker as possible without touching it. Turn on homogenizer and adjust to desired speed—3200 rpm for 5 µm diameter particles, 5000 rpm for 3 µm, 15,000 rpm for 1 µm (increasing homogenization speed decreases particle size). Once at the desired speed, add the PLGA solution to the beaker and homogenize for 1 minute.

1.2.3. After homogenization, pour the 1% PVA, PLGA microparticle solution into the 100 mL 0.5% PVA solution on a stir plate and stir for at least 4 hours for solvent evaporation in a chemical hood.

1.2.4. Wash the particles 3 times in DI water.

1.2.4.1. Pour the particle solution into 50 mL conical tubes and centrifuge at 3000 x g for 5 minutes. Pour out supernatant and add approximately 20 mL of DI water.

1.2.4.2. Resuspend particles by vortexing. Once resuspended, fill up conical tubes to 50 mL with DI water.

1.2.4.3. Wash again the same way two more times.

1.3. Nanoparticle synthesis

1.3.1. Weigh out 200 mg of PLGA into a scintillation vial and dissolve in 5 mL of DCM. Vortex to dissolve the PLGA.

1.3.2. Place a beaker with 50 mL 1% PVA solution into container filled with ice. Place the sonicator probe in the beaker as close to the bottom as possible without touching. Begin sonication and immediately add PLGA solution into the beaker. Sonicate with a power of 12 W for 2 minutes to generate nanoparticles with an approximate diameter of 200 nm.

1.3.3. After sonication, pour the 1% PVA, PLGA nanoparticle solution into a 100 mL 0.5% PVA solution on a stir plate and stir for at least 4 hours for solvent evaporation in a chemical hood.

1.3.4. Pour the particle solution into 50 mL conical tubes and centrifuge at 3000 x g for 5 minutes to remove microparticles. Remove supernatant and pour into high speed centrifuge tubes.

1.3.5. Wash the particles 3 times with DI water. Centrifuge at 40,000 x g for 15 minutes. Pour out supernatant and resuspend in DI water by vortexing. Wash again the same way two more times.

2. Fabrication of Polymeric Particles of Tunable Shape

2.1. After washing PLGA particles three times, resuspend the particles in approximately 1 mL of DI water. Add the film casting solution to particles for final particle concentration of 2.5 mg/mL particles.

2.2. Pipette the particle suspension in 10 mL aliquots into 75 x 50 mm rectangular Petri dishes for one-dimensional stretching or in 15 mL aliquots into 100 x 100 mm square Petri dishes for two-dimensional stretching. Remove bubbles by either pipette or pushing bubbles to the side and let films dry overnight in a chemical hood.

2.3. Once films have dried, remove films from plastic dishes with tweezers and cut off edges of films with scissors. Save edges in a 50 mL conical tube to be used as spherical particles.

2.3.1. Load a film onto the automated thin film stretching device by mounting a film onto aluminum blocks. For 1D stretching, mount film onto aluminum blocks of one axis of the stretcher

(**Figure 2**) by placing two short edges of film in between two pieces of neoprene rubber. Using an Allen wrench, screw the metal grips on top of rubber to hold the film in place. For 2D stretching, mount all four edges onto four aluminum blocks to stretch on both axes (**Figure 2**).

2.3.2. Measure and record the length of film in between aluminum blocks on one axis for 1D stretching or both axes for 2D stretching. Calculate the distance required to stretch the film in one or two directions based on desired fold-stretch.

2.3.3. Place the film-loaded stretching device in an oven at 90 °C and bring the film to temperature over 10 minutes. Place a large beaker in the oven with a small amount of water.

2.3.4. Stretch film.

2.3.5. When stretching is completed, remove the stretching device from oven and let the film cool to room temperature for 20 minutes.

2.3.6. For 1D stretching, cut the film out of the stretching device at the edges. For 2D stretching, cut out and save the center square of film that is uniformly stretched on both axes. Place films in conical tubes with 2 films per tube and discard the rest of the film.

2.3.7. Add approximately 25 mL of DI water to each conical tube and vortex until the films are dissolved.

2.3.8. Once the films are dissolved, wash particles 3 times with DI water.

2.3.8.1. For microparticles, fill up conical tubes to 50 mL and centrifuge at 3000 x g for 5 minutes, pour out supernatant, add approximately 20 mL of DI water, and vortex to resuspend particles.

2.3.8.2. For nanoparticles, transfer particles to high speed centrifuge tubes and centrifuge at 40,000 x g for 15 minutes, pour out supernatant, add approximately 20 mL of DI water, and vortex to resuspend particles.

2.3.9. After the third wash, resuspend particles in approximately 1 mL of DI water. Record the weight of the microcentrifuge tube and add particles to the tube. Freeze particles in a -80 °C freezer for 1 hour or flash freeze in liquid nitrogen. Once frozen, lyophilize particles overnight.

2.3.10. Once lyophilized, weigh particles in the microcentrifuge tube and subtract recorded weight of the empty tube to determine the weight of lyophilized particles.

3. Surface Protein Conjugation to Create Artificial Antigen Presenting Cells

3.1. Prepare 2-(N-Morpholino)ethanesulfonic acid (MES) buffer. Make a 0.1 M solution of MES in water and adjust the pH to 6.0 by titration with 1M sodium hydroxide (NaOH).

3.2. Resuspend lyophilized PLGA/PBAE micro/nanoparticles at 20 μg/mL in MES buffer by vortexing. Fill a polypropylene microcentrifuge tube with 900 μL of MES buffer and add 100 μL of the particle solution to the tube.

3.3. Prepare EDC/NHS solution. Dissolve 40 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 48 mg N-hydroxysulfoxuccinimide (NHS) in 1 mL MES buffer.

3.4. Add 100 μL EDC/NHS solution to the particles and vortex to mix.

3.5. Incubate the tube on an inverter at room temperature for 30 minutes.

3.6. Spin down microparticles at 5,000 x g for 5 minutes, or nanoparticles at 17,000 x g for 5 minutes. Discard the supernatant and resuspend in 1 mL PBS by vortexing (microparticles) or sonicating at 2-3 W for 5 seconds (nanoparticles).

3.7. For microparticles, add 8 μg of the desired signal 1 protein, and 10 μg anti-mouse CD28 (clone 37.51) to the particles. For nanoparticles, add 16 μg signal 1 and 20 μg anti-mouse CD28. Add PBS to bring the volume in the tube to 1.1 mL.

3.8. Incubate the tube on an inverter at 4 °C overnight.

3.9. The next day, wash the particles. Spin down microparticles at 5,000 x g for 5 minutes, or nanoparticles at 17,000 x g for 5 minutes. Discard the supernatant, and resuspend particles in 1 mL of sterile PBS by vortexing (microparticles) or sonication at 2-3 W for 5 seconds (nanoparticles). Repeat twice.

3.10. Resuspend the particles at the desired concentration in culture medium for immediate use. For long-term storage, resuspend particles at 10 mg/mL in a 100 mM sucrose solution. Freeze, lyophilize, and store the particles at -80 °C.

4. Characterization and Evaluation of aAPC

4.1. Characterization of aAPC size and shape (**Figure 3**)

4.1.1. Characterize microparticle size and shape by imaging particles using scanning electron microscopy (SEM). For SEM imaging, spread lyophilized particles onto carbon tape adhered to an aluminum tack and sputter coat with gold-palladium.

4.1.2. Analyze size and aspect ratio of particles using image analysis software.

4.1.3. To determine particle size, set scale using scale bar and measure particle diameter. Repeat for approximately 100 particles to determine the average particle diameter and generate a histogram of particle sizes.

4.1.4. To determine aspect ratio, measure the distance across the long axis and the short axis of particles and divide long axis by short axis. Repeat for approximately 50 particles of each shape to determine the average particle aspect ratio for each shape and generate histograms.

4.1.5. Characterize nanoparticle size and shape by imaging particles using transmission electron microscopy (TEM). Analyze size and aspect ratio of nanoparticles using image analysis software as described in 5.1.2. Alternatively, measure spherical nanoparticle size using dynamic light scattering (DLS) or nanoparticle tracking analysis (NTA).

4.2. Protein Conjugation Efficiency

4.2.1. Prepare aAPC according to Methods 1-3, but in Step 3.7 use fluorescently labeled signal 1 protein and anti-mouse CD28. After conjugation and washing, resuspend the aAPC in 1 mL PBS for a final concentration of 2 mg/mL aAPC.

4.2.2. Prepare protein standards in a black polystyrene 96-well microplate. Add 5 μg of fluorescently labeled signal 1 protein to PBS in the first well of the plate and make 10 1:2 dilutions in PBS across the row of the plate. Leave the last well blank. Repeat this step to generate another set of standards with fluorescently labeled anti-CD28.

4.2.3. Pipette 100 μL of the aAPC solution into replicate wells of the black polystyrene microplate in triplicate.

4.2.4. Read the fluorescence on a fluorescence plate reader at the appropriate wavelengths. Use the fluorescence readings from the protein standards to generate standard curves for each fluorescent antibody. Using the standard curve, calculate the concentrations of signal 1 and anti-CD28 in each sample well, and then calculate the amount of surface protein and conjugation efficiency (**Figure 4A, 5A**).

4.3. Evaluation of aAPC for *in vitro* stimulation of CD8+ T cells

4.3.1. Prepare B' media (RPMI medium supplemented with L glutamine, 10% FBS, 1% Non-essential amino acid solution, 1% Sodium pyruvate, 1% MEM Vitamin solution, 92 μM β-mercaptoethanol, 10 ng/mL ciprofloxacin, and 30 U/mL IL-2.

4.3.2. Sacrifice a Black 6 mouse via carbon dioxide exposure.

4.3.3. Harvest the spleen from the mouse according to a previously established protocol[16]. Collect the spleen in a 50 mL conical tube with 10- 15 mL PBS. Using a pestle, mash the spleen through a 70 μm cell strainer into a 50 mL conical tube. During mashing, wash the strainer with 40 mL PBS.

4.3.4. Spin the splenocytes at 300 x g for 5 minutes. Pour off the supernatant and resuspend the splenocytes in 4 mL of Ack Lysing Buffer to lyse the red blood cells. Allow the tube to sit undisturbed for 1 minute, then add PBS to bring the volume in the tube to 20 mL.

4.3.5. Spin the cells at 300 x g for 5 minutes. Pour off the supernatant and resuspend the cells in 1 mL of PBS. Count the cells using a hemocytometer.

4.3.6. Spin the cells at 300 x g for 5 minutes and resuspend in the desired volume of cell separation buffer. Isolate CD8+ T cells from the single cell suspension using a CD8+ negative selection T cell isolation kit, following the manufacturer's protocol.

4.3.7. Following magnetic separation, spin CD8+ T cells at 300 x g for five minutes and remove the cell separation buffer. Resuspend cells in 1 mL of PBS and count the cells. Label cells with carboxyfluorescein succinyl ester (CFSE) according to the manufacturer's protocol.

4.3.8. Incubate 8,333 CD8+ T cells and 0.0833 mg (or desired dose) of aAPC in 150 μL B' media in each well of a 96 well U-bottom tissue culture-treated plate.

4.3.9. Incubate at 37 °C for 7 days. After 3-4 days, refresh the culture medium by adding 75 μL fresh medium to each well.

4.3.10. After 3 days of incubation, analyze CFSE labelled cells on a flow cytometer to assess proliferation. Each peak on the flow cytometry CFSE histogram represents a generation of cells due to the CFSE dilution with each successive cell division.

4.3.11. After 7 days, use a hemocytometer to count the number of cells in each well. Prior to counting, stain dead cells with a Trypan Blue solution. Exclude dead cells from final cell counts. Normalize the final cell concentration to the initial concentration to calculate the fold-expansion (**Figures 4 and 5**).

REPRESENTATIVE RESULTS:

A schematic for the automated 2D thin film stretching device is given in **Figure 1**. A schematic and description for a 1D thin film stretching device is given in Ho et al.[17] The stretcher is constructed from aluminum parts using standard milling and machining techniques. Similar to the 1D stretcher, the 2D stretcher consists of metallic grips and guide rails. Bidirectional lead screws are used to translate linear to rotational motion. The lead screws are the attached via mechanical taps to identical stepper motors with sufficient torque. The 8 stepper motor control wires can be soldered onto 8-pin heat-resistant amphenol connectors for easy attachment to the control console in an oven for thin film stretching. Polytetrafluoroethylene (PTFE) coated wire of sufficient length must be used to connect the stepper motors to the drivers in the control console. The recommended computer control scheme is given in **Figure 1A**. The two motors must be connected through heat resistant wiring to 2 independent drivers. The two drivers must then be connected to a microcontroller to interface with a computer. The drivers

should be connected to the X-Axis and Y-Axis outputs on the microcontroller. The drivers and microcontroller both require an external power supply. Prior to connecting the power supply to these three components it is recommended that a 4 A fuse be inserted in between each of the powered connections to protect the components from current overload. Finally, the microcontroller can be linked via a Parallel Port Input to a computer using a DB25 Male to Male cable. The electronics used to control the stepper motors are heat sensitive and therefore must be placed outside of any heat source (such as an oven) used during operation to heat the thin films to sufficient temperature to enable stretching. Although the recommended motors are heat resistant up to the temperatures specified in this protocol for stretching particles, the motors and drivers will build up additional heat while they are attached to the main power supply. Therefore, it is recommended that the device only be turned on during the period of actual film stretching to minimize potential heat build-up.

PLGA nano- and microparticles were synthesized using the single emulsion techniques described in this protocol and imaged using TEM (**Figure 3A**) and SEM (**Figure 3B**), respectively. Spherical nanoparticles had a diameter of 237.3 ± 4.0 nm as measured by DLS and 224 nm as measured by NTA (**Figure 3C)**. Microparticles were synthesized by homogenization at 5000 rpm to generate spherical particles with an average diameter of 3 ± 1 μm (**Figure 3D**). The particles were stretched using the automated film stretching device at 90 °C in one dimension to generate prolate ellipsoidal nano- and microparticles and stretched at 70 °C in two dimensions to generate oblate ellipsoidal particles. The aspect ratios of the microparticles of all three shapes were analyzed by measuring the long axis and short axis distance of particles and dividing the two. Spherical microparticles had an aspect ratio of 1.05 ± 0.04, while 1D stretched prolate ellipsoidal particles had a larger aspect ratio of 3.6 ± 0.8 (**Figure 3E**). 2D stretched oblate ellipsoidal particles had an aspect ratio of 1.2 ± 0.2, roughly maintaining an aspect ratio of one.

EDC/NHS reaction chemistry was used to conjugate a fluorescently labelled peptide-loaded MHC IgG dimer and anti-CD28 antibody to the surface of stretched and spherical PLGA particles. Conjugation efficiency results demonstrate similar amounts of protein on the surface of spherical and ellipsoidal micro-aAPC (**Figure 4A**) and nano-aAPC (**Figure 5A**) and demonstrate that protein coupling during aAPC synthesis occurs in a concentration-dependent manner. To evaluate the effect of shape on aAPC functionality, spherical and prolate ellipsoidal aAPC conjugated with gp100-loaded MHC IgG dimer and anti-CD28 were used to stimulate PMEL transgenic CD8+ T cells. T cells were labelled with CFSE and evaluated by flow cytometry after 3 days to assess proliferation (**Figure 4B, 5B**). Prolate ellipsoidal aAPC were found to induce higher levels of T cell proliferation at sub-saturating doses than spherical aAPC, with the best separation achieved at a 0.01 mg dose. After 7 days, the T cells were manually counted. Prolate ellipsoidal aAPC more effectively stimulated T cells compared to their spherical counterparts at the microscale (**Figure 4C**) and nanoscale (**Figure 5C**), and dose-dependent T cell expansion was observed.

Figure Legends:

Figure 1: Schematic representation of automated thin film stretcher. (A) Schematic of control console for thin film stretcher. (B) Schematic of mechanical hardware for thin film stretcher. (Left) Overhead view of mechanical hardware. (Right) Cross-section of gripping mechanism for thin films.

Figure 2: Photographs of assembled automated thin film stretcher to stretch polymeric particles into anisotropic shapes. The 2D thin film stretching device is composed of two axes with aluminum mounts that grip the film. The two axes contain lead screws in opposing directions so that they move apart from each other. To automate the stretching procedure, a USB linked microcontroller is connected to two stepper motor drivers that relay signals to unipolar stepper motors through a thermal cable.

Figure 3: Size and aspect ratio analysis of spherical and ellipsoidal PLGA particles. (A) TEM and (B) SEM images of spherical, 1D stretched prolate ellipsoidal, and 2D stretched oblate ellipsoidal PLGA (A) nanoparticles and (B) microparticles. (C) Spherical nanoparticles were sized by NTA and determined to be 224 nm in diameter. SEM images of PLGA microparticles were analyzed for (D) size distribution of spherical particles and (E) aspect ratios of all particle shapes. (C) Reproduced and adapted with permission from *Small*[13], Copyright Wiley-VCH 2015.

Figure 4: Characterization and functional assessment of spherical and prolate ellipsoidal micro-aAPC. (A) Conjugation efficiency of fluorescently-labelled peptide-loaded MHC IgG dimer and anti-CD28 antibody to the surface of spherical and prolate ellipsoidal microparticles. (B) CD8+ T cells were labelled with CFSE and incubated with spherical and 1D-stretched micro-aAPC at 0.01, 0.1, and 1 mg doses, or non-cognate controls. After 3 days, cells were evaluated by flow cytometry to assess proliferation. (C) T cells were also evaluated after 7 days by manual counting. Cell counts were normalized to the initial count to calculate fold-expansion. For comparison between prolate ellipsoidal and spherical fold expansion, * = $\boldsymbol{p} < 0.05$, ** = $\boldsymbol{p} < 0.01$, and *** = $\boldsymbol{p} < 0.001$. Error bars represent standard error of the mean (SEM) for 3 replicates. Reproduced and adapted with permission from *Biomaterials*[12], Copyright Elsevier 2014.

Figure 5: Characterization and functional assessment of spherical and prolate ellipsoidal nano-aAPC. (A) Conjugation efficiency of fluorescently-labelled peptide-loaded MHC IgG dimer and anti-CD28 antibody to the surface of spherical and prolate ellipsoidal nanoparticles. (B) CD8+ T cells were labelled with CFSE and incubated with spherical and prolate ellipsoidal nano-aAPC of varying fold-stretch at 0.01, 0.1, and 1 mg doses. After 3 days, cells were evaluated by flow cytometry to assess proliferation. (C) T cells incubated with prolate ellipsoidal particles of varying fold stretch (ranging from 1.5 to 3.5) were also evaluated after 7 days by manual counting. Cell counts were normalized to an untreated condition to calculate fold-expansion. * = $\boldsymbol{p} < 0.05$, ** = $\boldsymbol{p} < 0.01$, and *** = $\boldsymbol{p} < 0.001$ compared to spherical. Error bars represent standard error of the mean (SEM) for 3 replicates. Reproduced and adapted with permission from *Small*[13], Copyright Wiley-VCH 2015.

DISCUSSION:

This protocol details a versatile method for the precise generation of anisotropic polymeric particles. The thin film stretching technique described here is scalable, highly reproducible and inexpensive. Alternative techniques for generating anisotropic particles suffer from many limitations, including high cost, low throughput, and limited particle size. The thin film stretching approach is also advantageous because the particles are modified to be anisotropic after synthesis, and, as a result, is compatible with a wide range of particle sizes and synthesis techniques. **Figure 1** details the setup of the automated two-dimensional stretching device. This device can also be used without the electronic components by manually turning the screws until the film has reached the desired degree of stretching. However, we have found that the automated process is much more consistent and rapid than manual operation[15]. Various techniques have been developed to synthesize anisotropic particles, such as microfluidic approaches[17-19], layer-by-layer coating[21], and other bottom-up synthesis approaches[21,22]. However, these approaches do not enable strong control over particle geometry and are not as versatile in terms of shapes that can be generated and particle materials that can be used. A popular top-down method for fabricating nonspherical particles is Particle Replication in Non-Wetting Templates (PRINT)[24]. Although PRINT enables precise control over particle shape, it requires expensive machinery and is not as accessible and simple to implement as the thin film stretching method.

The single emulsion technique can be used to fabricate PLGA particles of various sizes, ranging from the nano to microscale[12,13]. By varying homogenization speed or sonication amplitude, microparticle and nanoparticle size, respectively, can be modulated. Once spherical particles have been generated, the thin film stretching method described here can be used to deform the particles into various shapes[15]. In this protocol, we describe the generation of prolate and oblate ellipsoidal particles by stretching in one or two dimensions, respectively. Spherical particles are cast into a thin plastic film, which is heated above the glass transition temperature of PLGA and stretched in one or two dimensions to deform the particles. The aspect ratio of the particles is highly controllable. By tuning the degree of film stretch, the aspect ratio of the particles can be modulated, and we have found that measured particle aspect ratio is highly correlated with the predicted value[12,13]. Various other shapes can be generated by modifying the temperature during stretching or the degree of stretching. For example, biconcave discoidal particles resembling the shape of red blood cells can be generated by stretching microparticles 1.5-fold in two dimensions at 90 °C.[15]. This film stretching technique has also been used to transform spherical polystyrene particles into many anisotropic shapes, including worms, barrels, and rectangular discs[21]. The film stretching device can be used by manually controlling the screws or the device can be automated as shown in **Figure 1** to make the process more efficient and consistent[15]. This simple technique reliably produces anisotropic particles that retain their shape under physiologic conditions[24]. Furthermore, this method has been applied to other polymeric materials, in addition to PLGA, such as polycaprolactone (PCL) and hybrid particles made of PLGA and poly(beta-amino ester) (PBAE).

This protocol also describes how PLGA particles of varied size and shape can be conjugated with the surface proteins required for CD8+ T cell activation to act as aAPC. Proteins can be covalently conjugated to anisotropic and spherical PLGA micro- and nanoparticles by EDC/NHS mediated

coupling of primary amines on proteins to carboxyl groups on the particle surface. The efficiency of protein conjugation can be measured by coupling fluorescently labeled protein to the surface of particles as described in this protocol, and we have found that this technique couples protein to particles at 15-20% efficiency[12,13]. Prolate ellipsoidal micro- and nanoparticle aAPC are more effective than their spherical counterparts at activating CD8+ T cell proliferation and expansion *in vitro*[12,13]. Ellipsoidal aAPC have enhanced binding to and interaction with T cells due to their larger surface area for contact[12]. Anisotropic particles also have superior properties over spherical particles *in vivo* due to their enhanced biodistribution and resistance to phagocytosis[13]. This platform is highly modular and has the potential to be adapted to many other drug delivery applications. Using this procedure, polymeric particles of tunable shape and size can be generated and the particle surface can be conjugated with any protein of interest.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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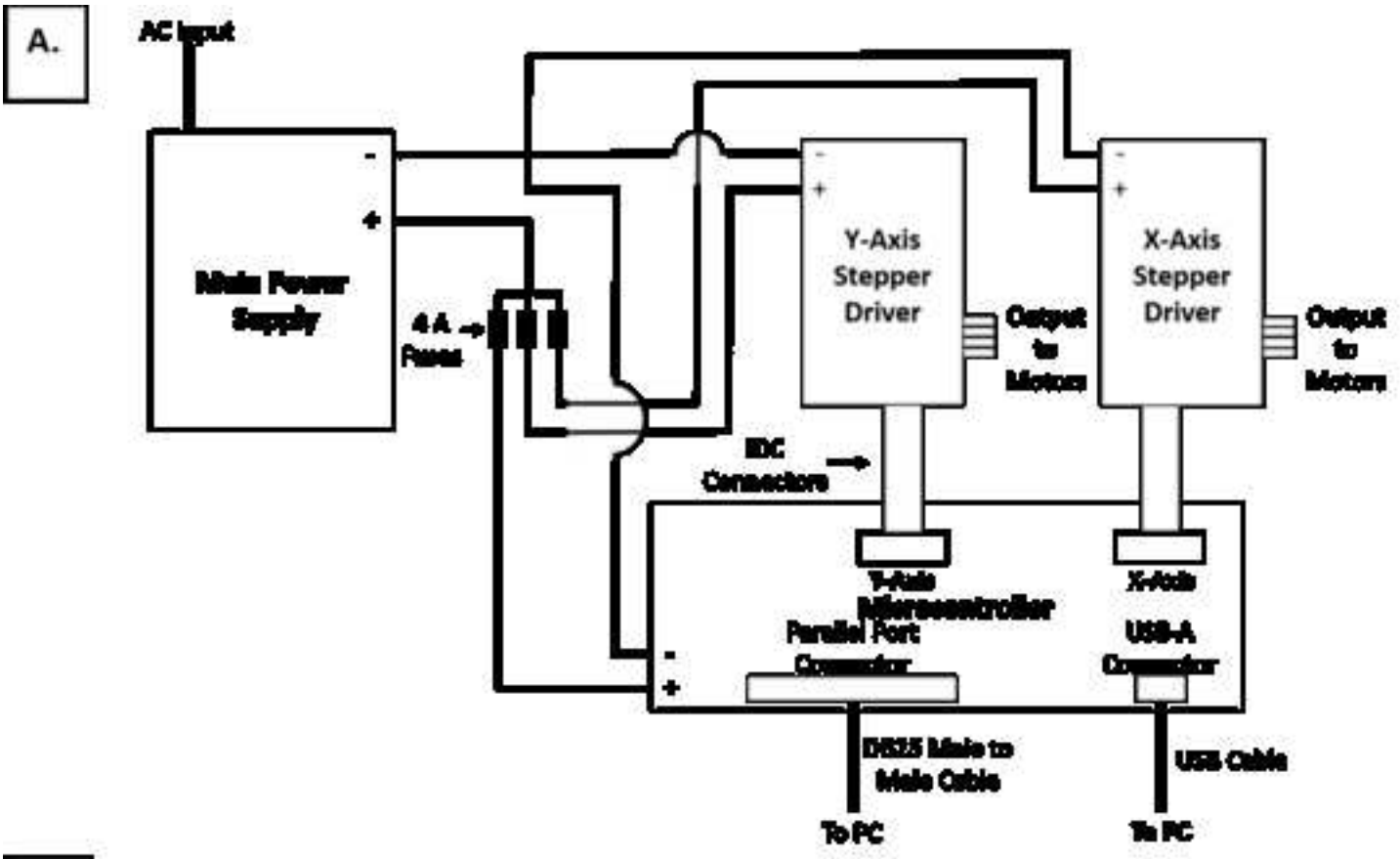

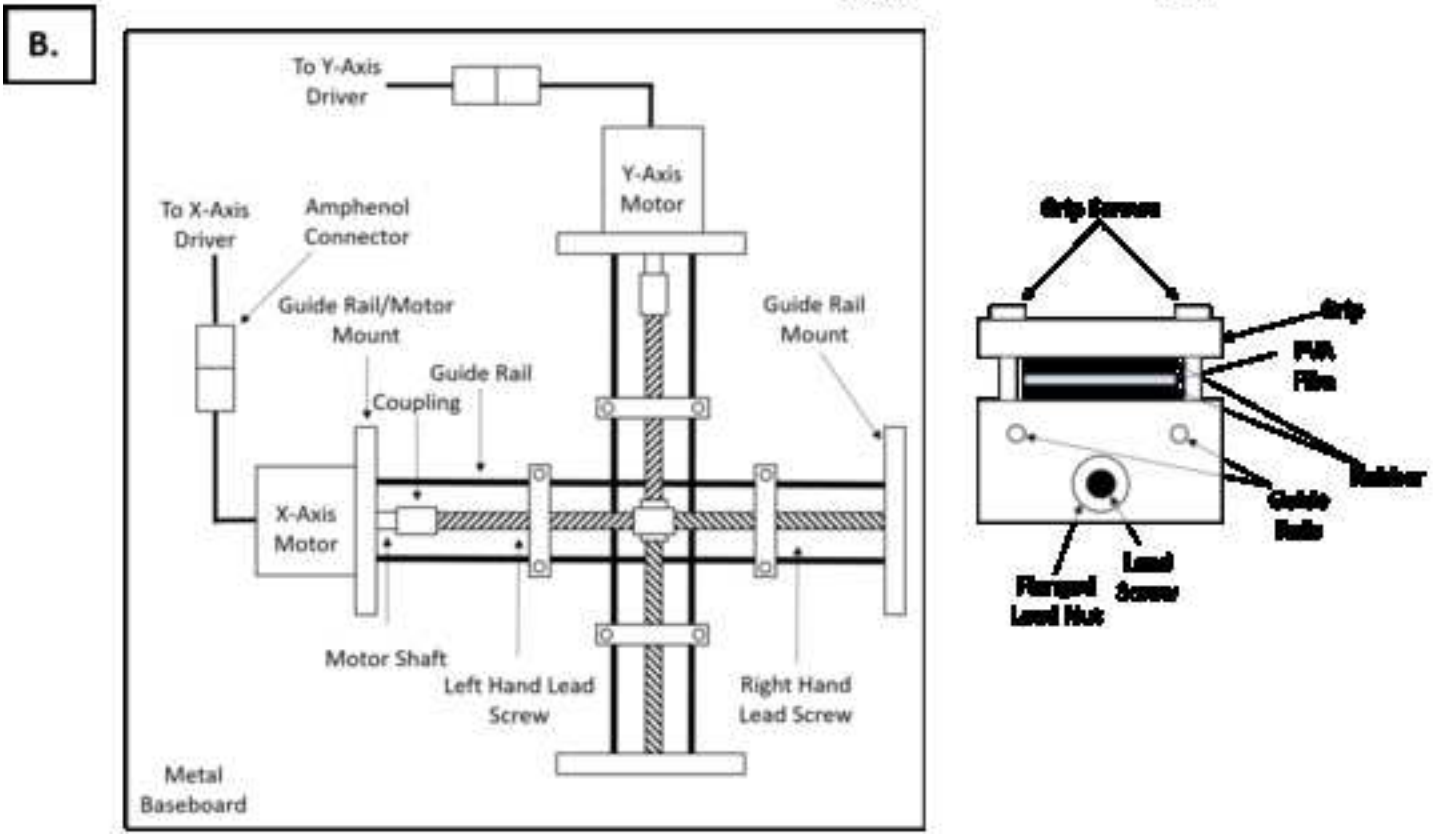

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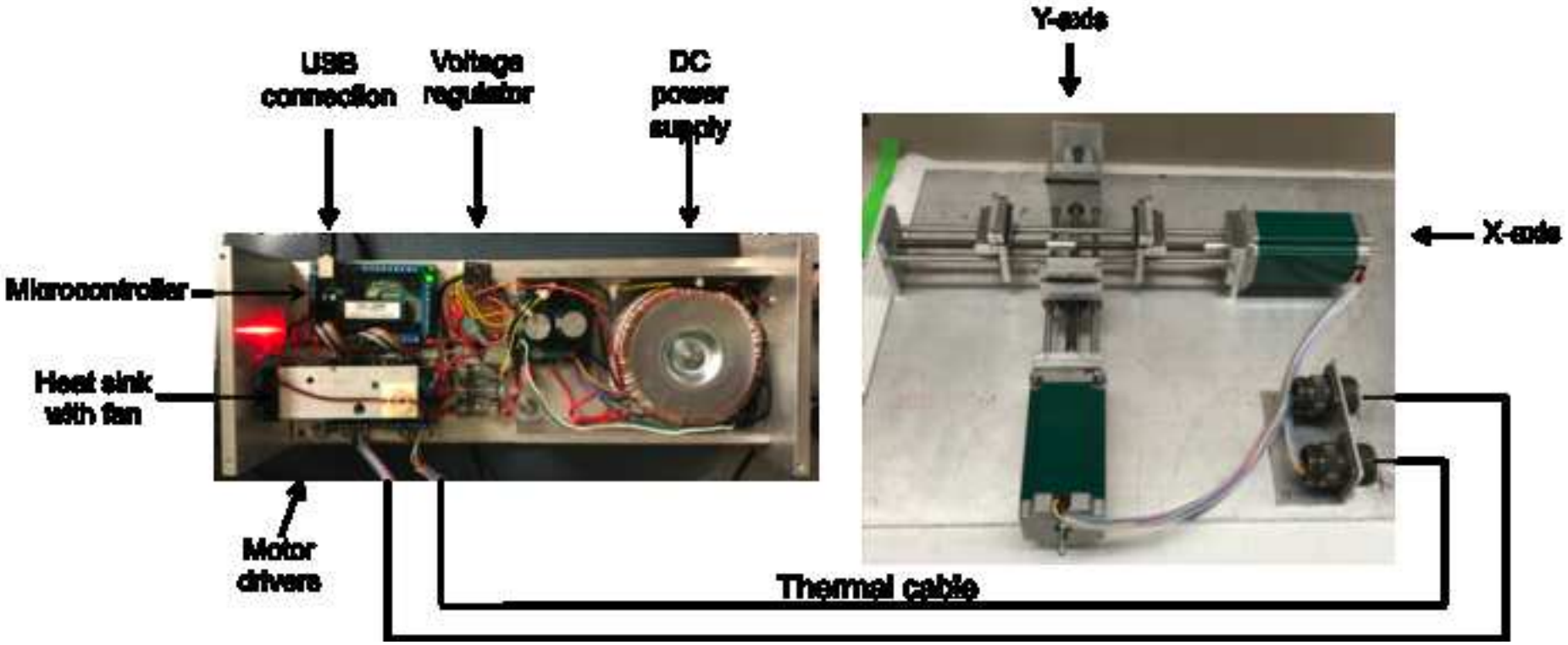

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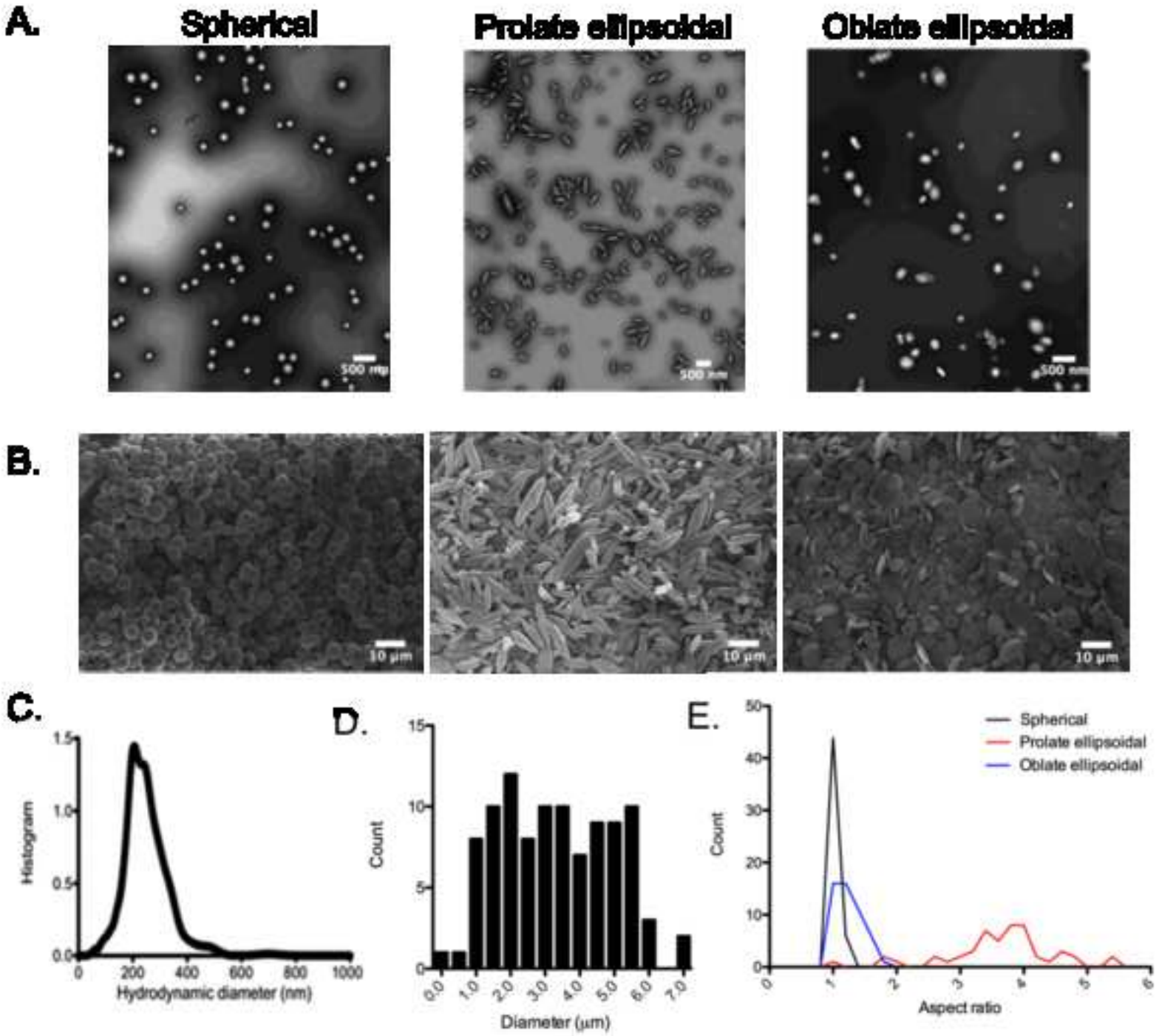

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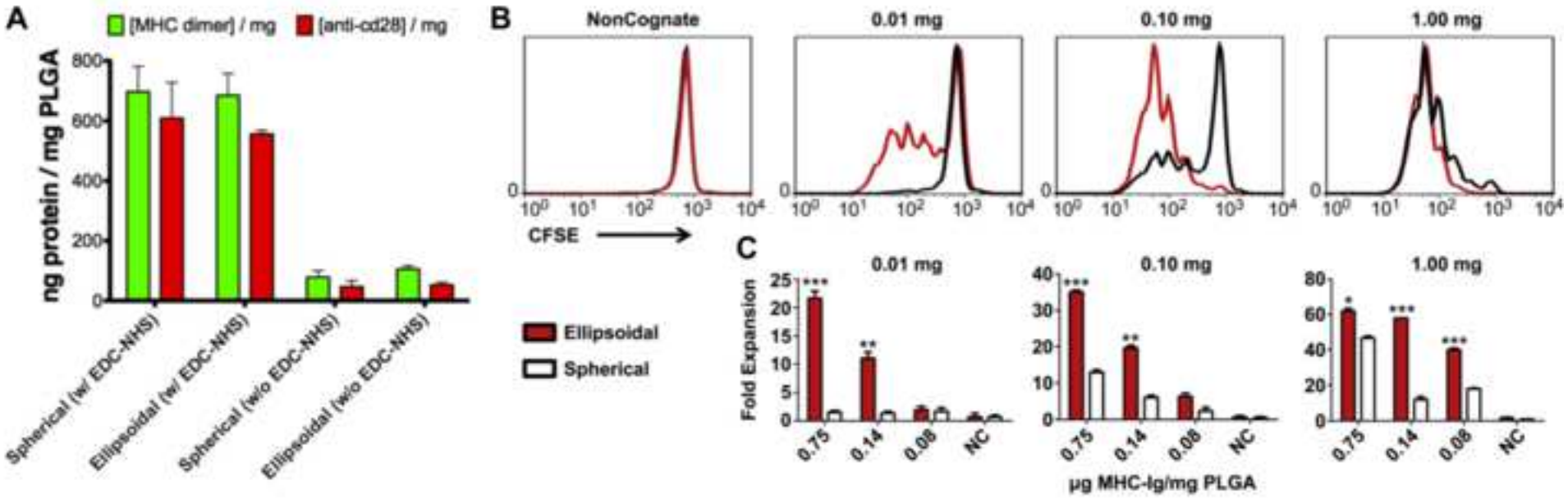

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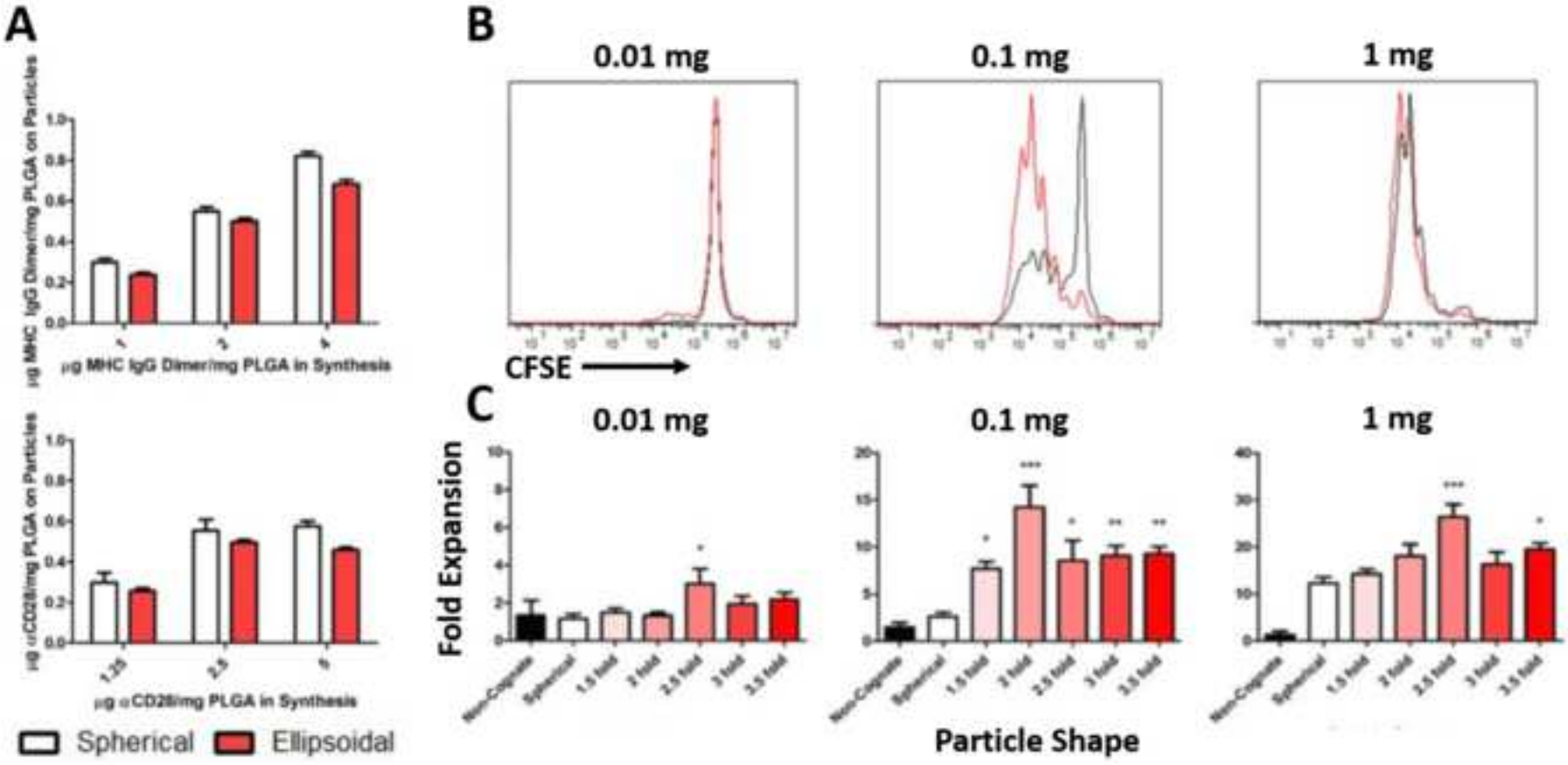

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Poly(vinyl alcohol), MW 25000, 88% hydrolyzed	Polysciences, Inc.	02975-500
Glycerol	Sigma-Aldrich	G9012
Digital Thermometer	Fluke	N/A
Immersion Temperature Probe	Fluke	N/A
Digital Hotplate & Stirrer	Benchmark Scientific	H3760-HS
Multipoint stirrer	Thermo Fisher Scientific	50093538
Resomer RG 504 H, Poly(D,L-lactide-co-glycolide)	Sigma-Aldrich	719900
Dichloromethane	Sigma-Aldrich	D65100
Homogenizer	IKA	0003725001
Sonicator	Sonics & Materials, Inc.	N/A
Sonicator sound abating enclosure	Sonics & Materials, Inc.	N/A
Sonicator probe	Sonics & Materials, Inc.	N/A
Sonicator microtip	Sonics & Materials, Inc.	N/A
High speed centrifuge	Beckman Coulter	N/A
High speed centrifuge rotor	Beckman Coulter	369691
High speed polycarbonate centrifuge tubes	Thermo Fisher Scientific	3118-0050
Rectangular disposable petri dish	VWR International	25384-322
Square disposable petri dish	VWR International	10799-140
LEAF Purified anti-mouse CD3ε Antibody	Biolegend	100314
InVivoMab anti-mouse CD28, clone 37.51	Bio X Cell	BE0015-1
N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride	Sigma-Aldrich	E6383
N-Hydroxysulfosuccinimide sodium salt	Sigma-Aldrich	56485
MES	Sigma-Aldrich	M3671
Alexa Fluor 488 anti-mouse CD3 Antibody	Biolegend	100212
APC anti-mouse CD28 Antibody	Biolegend	102109
Corning 96 Well Solid Polystyrene Microplate	Sigma-Aldrich	CLS3915
Protein LoBind Tubes, 1.5 mL	Eppendorf	22431081
RPMI 1640 Medium (+ L-Glutamine)	ThermoFisher Scientific	11875093

Fetal Bovine Serum	Sigma-Aldrich	F4135
Ciprofloxacin	Sigma-Aldrich	17850
2-Mercaptoethanol	Sigma-Aldrich	M6250
Recombinant Human IL-2 (carrier-free)	Biolegend	589102
Sodium Pyruvate (100 mM)	ThermoFisher Scientific	11360070
MEM Non-Essential Amino Acids Solution (100X)	ThermoFisher Scientific	11140050
MEM Vitamin Solution (100X)	ThermoFisher Scientific	11120052
CD8a+ T Cell Isolation Kit, mouse	Miltenyi Biotech	130-104-075
CellTrace CFSE Cell Proliferation Kit	ThermoFisher Scientific	C34554
LS Columns	Miltenyi Biotech	130-042-401
MidiMACS Separator	Miltenyi Biotech	130-042-302
MACS Multistand	Miltenyi Biotech	130-042-303
Flow Cytometer	Accuri C6	
Synergy 2 Multi-Detection Microplate Reader	BioTek	
autoMACS Running Buffer	Miltenyi BIotech	130-091-221
Cell Strainer	ThermoFisher Scientific	22363548
ACK Lysing Buffer	ThermoFisher Scientific	A1049201
C57BL/6J (Black 6) Mouse	The Jackson Laboratory	000664
U-Bottom Tissue Culture Plates	VWR	353227
40 V DC Power Supply	Probotix	LPSK-4010
PTFE Coated Wire	Mouser	602-5858-100-01
Stepper Motor Driver	Probotix	MondoStep5.6
IDC Connector Kit	Probotix	IDCM-10-12
Microcontroller	Probotix	PBX-RF
4A Fuses	Radio Shack	2701026
DB25 Male to Male Cable	Probotix	DB25-6
USB-A to USB-B Cable	Staples	2094915
8-Pin Amphenol Connectors Male and Female	Mouser	654-97-3100A-20-7P and 654-97-3106A20-7S
Stepper Motor	Probotix	HT23-420-8
Right Hand Lead Screw	Roton	60722

Left Hand Lead Screw	Roton	60723
Screws	McMaster Carr	92196A151
Neoprene Rubber	McMaster Carr	8698K51
Right Handed Flanged Lead Nut	Roton	91962
Left Handed Flanged Lead Nut	Roton	91963
Linux Control Computer	Probotix	LCNC-PC
Corning bottle-top vacuum filter system	Sigma-Aldrich	CLS431097
Trypan Blue Solution, 0.4 %	ThermoFisher Scientific	15250061

Comments/Description

Model name: Fluke 52 II
Model name: Fluke 80PK 22

Model number: VC 505
Part number: 630-0427
Part number: 630-0220
Part number: 630-0423
Model number: J-20XP (discontinued), alternative model: J-26XP
Model number: JA-17
50 mL, screw cap
75 x 50 x 10 mm
100 mm x 100 mm

flat bottom, black polystyrene

Heat Inactivated, sterile-filtered

Sterile, 70 µm nylon mesh

Male, at least 7 weeks old

Sterile, 96-well tissue culture treated polystyrene plates

This is for a 100 ft. spool but an equivalent wire will work

Equivalent fuses will work as well

Equivalent cable will work as well

Any computer with matching specification and Linux operating system will work

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Jordan J. Green, Ph.D.
The Johns Hopkins University School of Medicine
Departments of Biomedical Engineering and Materials Science & Engineering
400 N. Broadway, Smith Building Room 5017
Baltimore, MD 21231
410-614-9113 / FAX 443-287-6298 / green@jhu.edu

July 7, 2018

Dear Dr. Nandita Singh:

Enclosed please find our revised manuscript entitled "Fabrication of anisotropic polymeric artificial antigen presenting cells for CD8+ T cell activation" submitted for consideration as an article in *JoVE*. We thank the editors for their helpful comments to improve the manuscript. This submission addresses the final editorial comments which are each described point by point below:

1. 4.3.2: Please specify how to sacrifice the mouse, and include a reference (at least) for spleen harvesting.

Reference has been added.

2. Figure 4: There is still no Figure 4C; it looks like panel C is labeled as panel B, and panel B is unlabeled.

Figure 4 has been updated. 4(C) shows the results after T cells were evaluated after 7 days by manual counting.

3. Figure 5: Please explain what '1.5 fold', '2 fold', etc. mean (x-axis for panel C)

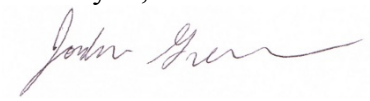
The Figure 5 caption has been revised. 5(C) shows results from T cells incubated with prolate ellipsoidal particles of varying fold stretch (ranging from 1.5 to 3.5).

4. Figures 4/5: Please explain what the error bars and stars signify.

The captions have been updated: * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ from statistical comparisons. Error bars represent standard error of the mean (SEM) for 3 replicates.

We believe this work will make an important contribution to *JoVE* and that it is of broad interest to its readership. Please let me know if there is any other information that we can provide.

Thank you,



Jordan J. Green, Ph.D.
Professor of Biomedical Engineering
The Johns Hopkins University School of Medicine

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TITLE:

Fabrication of anisotropic polymeric artificial antigen presenting cells for CD8+ T cell activation

AUTHORS:

Ben-Akiva, Elana*
Biomedical Engineering, Translational Tissue Engineering Center, Institute for Nanobiotechnology
Johns Hopkins University School of Medicine
Baltimore, USA
ebenaki1@jhmi.edu

Rhodes, Kelly R*
Biomedical Engineering, Translational Tissue Engineering Center, Institute for Nanobiotechnology
Johns Hopkins University School of Medicine
Baltimore, USA
krhodes7@jhmi.edu

Meyer, Randall A
Biomedical Engineering, Translational Tissue Engineering Center, Institute for Nanobiotechnology
Johns Hopkins University School of Medicine
Baltimore, USA
rmeyer16@jhmi.edu

Green, Jordan J
Biomedical Engineering, Translational Tissue Engineering Center, Institute for Nanobiotechnology, Ophthalmology, Oncology, Neurosurgery, Materials Science and Engineering, Chemical and Biomolecular Engineering, and the Bloomberg~Kimmel Institute for Cancer Immunotherapy
Johns Hopkins University School of Medicine
Baltimore, USA
green@jhu.edu

* These authors contributed equally

CORRESPONDING AUTHOR: Jordan J. Green, green@jhu.edu

KEYWORDS: aAPC, immunoengineering, polymer, anisotropic, particle, shape

SHORT ABSTRACT:

Here, we present a protocol to quickly and reproducibly generate biologically inspired, biodegradable articifical antigen presenting cells (aAPC) with tunable size, shape, and surface protein presentation for T cell expansion *ex vivo* or *in vivo*.

LONG ABSTRACT:

Artificial antigen presenting cells (aAPC) are a promising platform for immune modulation due to their potent ability to stimulate T cells. Acellular substrates offer key advantages over cell-based aAPC, including precise control of signal presentation parameters and physical properties of the aAPC surface to modulate its interactions with T cells. aAPC constructed from anisotropic particles, particularly ellipsoidal particles, have been shown to be more effective than their spherical counterparts at stimulating T cells due to increased binding and larger surface area available for T cell contact, as well as reduced nonspecific uptake and enhanced pharmacokinetic properties. Despite increased interest in anisotropic particles, even widely accepted methods of generating anisotropic particles such as thin-film stretching can be challenging to implement and use reproducibly.

To this end, we describe a protocol for the rapid, standardized fabrication of biodegradable anisotropic particle-based aAPC with tunable size, shape, and signal presentation for T cell expansion ex vivo or in vivo, along with methods to characterize their size, morphology, and surface protein content, and to assess their functionality. This approach to fabricating anisotropic aAPC is scalable and reproducible, making it ideal for generating aAPC for "off-the-shelf" immunotherapies.

INTRODUCTION:

Artificial antigen presenting cells (aAPC) have shown promise as immunomodulatory agents because they can generate a robust antigen-specific T cell response. Essential to these platforms are their ability to efficiently present crucial signals for T cell activation. Acellular aAPC are an attractive alternative to cell-based aAPC because they are easier and less costly to fabricate, face fewer challenges during scale-up and translation, and alleviate risks associated with cell-based therapies. Acellular aAPC also allow for a high degree of control over signal presentation parameters and physical properties of the surface that will interface with T cells. [1]

aAPC must recapitulate a minimum of two signals essential for T cell activation. Signal 1 provides antigen recognition and occurs when the T cell receptor (TCR) recognizes and engages with an MHC class I or II bearing its cognate antigen, culminating in signaling through the TCR complex. To bypass the antigen specificity requirement, aAPC systems often bear an agonistic monoclonal antibody against the CD3 receptor, which nonspecifically stimulates the TCR complex. Recombinant forms of MHC, particularly MHC multimers, have also been used on the surface of aAPC to provide antigen specificity. [2,3] Signal 2 is a costimulatory signal that directs T cell activity. To provide the costimulation necessary for T cell activation, the CD28 receptor is generally stimulated with an agonistic antibody presented on the aAPC surface, although other costimulatory receptors such as 4-1BB have been successfully targeted. [4] Signal 1 and 2 proteins

are typically immobilized on the surface of rigid particles to synthesize aAPC. Historically, aAPC have been fabricated from a variety of materials including polystyrene [4,5] and iron dextran. [6] Newer systems utilize biodegradable polymers like poly(lactic-co-glycolic acid) (PLGA) to generate aAPC that can be easily coupled to signal proteins, are suitable for direct administration *in vivo*, and can facilitate the sustained release of encapsulated cytokines or soluble factors to augment T cell activation. [7,8]

In addition to the presence of necessary signal proteins, receptor engagement over a sufficiently large surface area during the aAPC/T cell interaction is essential for T cell activation. Thus, physical parameters of the aAPC such as size and shape drastically alter their available contact area and affect their ability to stimulate T cells. Micron-sized aAPC have been shown to be more effective at stimulating T cells than their nanoscale counterparts. [9,10] However, nano-aAPC can have superior biodistribution and better drainage to the lymph nodes that may enhance their performance *in vivo* over micro-aAPC. [11] Shape is another variable of interest in particle-based aAPC systems. Anisotropic aAPC have recently been shown to be more effective than isotropic particles at stimulating T cells, mainly due to enhanced interaction with target cells coupled with reduced non-specific cell uptake. Cells preferentially bind to the long axis of ellipsoidal particles, and the larger radius of curvature and flatter surface allow for more contact between the aAPC and T cell. [12] The long axis of ellipsoidal particles also discourages phagocytosis, resulting in increased circulation time compared to spherical particles following *in vivo* administration.[12,13] Because of these advantages, ellipsoidal particles mediate greater expansion of antigen-specific T cells *in vitro* and *in vivo* compared to spherical particles, an effect observed at both the micro and nanoscales. [12,13] There are various strategies to fabricate anisotropic particles, but thin-film stretching is a simple, widely accepted method used to generate a range of diverse particle shapes.[14] Following synthesis, particles are cast into films and stretched in one or two dimensions at a temperature above the glass transition temperature of the particle material. The film is then dissolved to retrieve the particles. Despite growing interest in anisotropic particles, current approaches for fabricating particle-based aAPC are mostly limited to isotropic systems, and methods of altering particle shape can be difficult to implement, incompatible with certain aAPC synthesis strategies, and lack precision and reproducibility.[15] Our thin-film stretching technique can be performed manually or in an automated fashion to rapidly generate anisotropic particles synthesized from a variety of biodegradable polymers, stretched to a desired aspect ratio in one or two dimensions. [15]

Based on our previous work, we developed a biodegradable particle-based approach combined with scalable thin-film stretching technology to rapidly generate aAPC with tunable size and shape in a standardized fashion for T cell expansion *ex vivo* or *in vivo*. Our protein conjugation strategy can be used to couple any protein(s) of interest to carboxyl groups on the particle surface at a desired density, giving this aAPC system a high degree of flexibility. We also describe methods to characterize the size, morphology, and surface protein content of aAPC, and to evaluate their functionality *in vitro*. This protocol can be easily adapted to expand immune cells *ex vivo* or *in vivo* for a variety of immunotherapeutic applications.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Johns Hopkins University.

1. Fabrication of spherical PLGA particles of tunable size

1.1. Preparation of materials for particle synthesis

1.1.1. Prepare 5% w/w polyvinyl alcohol (PVA) solution. Add 500 mL of deionized (DI) water to an Erlenmeyer flask with a magnetic stir bar and place on hot plate stirrer at 500 rpm and monitor temperature with thermometer. Cover flask with tinfoil to prevent evaporation. When water temperature reaches approximately 70 °C, add 25 g total of PVA in small batches over time, waiting for PVA to dissolve before adding more. Once all PVA is dissolved (typically 30-60 min), let solution cool and sterile filter. Store at 4 °C for future use.

1.1.2. Prepare film casting solution of 10% w/w PVA and 2% w/w glycerol. Add 500 mL of DI water to an Erlenmeyer flask with a magnetic stir bar. Add 8 mL of glycerol at room temperature and mix by trituration. Place flask on hot plate stirrer at 500 rpm and monitor temperature with thermometer. Cover flask with tinfoil to prevent evaporation. When solution temperature reaches approximately 70 °C, add 50 g total of PVA in small batches over time, waiting for PVA to dissolve before adding more. Once all PVA is dissolved (typically 60 min), let solution cool and sterile filter using a bottle-top vacuum filter system with a pore size of 0.22 µm. Store at room temperature for future use.

1.1.3. Prepare a 50 mL 1% w/w PVA solution. Add 40 mL of DI water and 10 mL of 5% PVA solution (made in 1.1.1) to a 100-150 mL beaker.

1.1.4. Prepare a 100 mL 0.5% w/w PVA solution. Add 90 mL of DI water and 10 mL of 5% PVA solution to a 150-250 mL beaker.

1.1.5. Add a magnetic stir bar to the 0.5% PVA solution and place in a chemical hood on a stir plate at room temperature at 500 rpm.

1.2. Microparticle synthesis

1.2.1. Weigh out 100 mg of poly(lactic-co-glycolic acid) (PLGA) into a scintillation vial and dissolve in 5 mL of dichloromethane (DCM). Vortex to dissolve the PLGA.

1.2.2. Place homogenizer in the 50 mL 1% PVA solution so that the homogenizer is as close to the bottom of the beaker as possible without touching it. Turn on homogenizer and adjust to desired speed—3200 rpm for 5 µm diameter particles, 5000 rpm for 3 µm, 15,000 rpm for 1 µm

(increasing homogenization speed decreases particle size). Once at the desired speed, add the PLGA solution to the beaker and homogenize for 1 minute.

1.2.3. After homogenization, pour the 1% PVA, PLGA microparticle solution into the 100 mL 0.5% PVA solution on a stir plate and stir for at least 4 hours for solvent evaporation in a chemical hood.

1.2.4. Wash the particles 3 times in DI water. Pour the particle solution into 50 mL conical tubes and centrifuge at 3000 x g for 5 minutes. Pour out supernatant and add approximately 20 mL of DI water. Resuspend particles by vortexing. Once resuspended, fill up conical tubes to 50 mL with DI water. Wash again the same way two more times.

1.3. Nanoparticle synthesis

1.3.1. Weigh out 200 mg of PLGA into a scintillation vial and dissolve in 5 mL of DCM. Vortex to dissolve the PLGA.

1.3.2. Place a beaker with 50 mL 1% PVA solution into container filled with ice. Place sonicator probe in beaker as close to bottom of beaker as possible without touching. Begin sonication and immediately add PLGA solution into beaker. Sonicate with a power of 12 W for 2 minutes to generate nanoparticles with an approximate diameter of 200 nm.

1.3.3. After sonication, pour the 1% PVA, PLGA nanoparticle solution into a 100 mL 0.5% PVA solution on a stir plate and stir for at least 4 hours for solvent evaporation in a chemical hood.

1.3.4. Pour the particle solution into 50 mL conical tubes and centrifuge at 3000 x g for 5 minutes to remove microparticles. Remove supernatant and pour into high speed centrifuge tubes. Wash the particles 3 times with DI water. Centrifuge at 40,000 x g for 15 minutes. Pour out supernatant, and resuspend in DI water by vortexing. Wash again the same way two more times.

2. Fabrication of polymeric particles of tunable shape

2.1. After washing PLGA particles three times, resuspend the particles in approximately 1 mL of DI water. Add the film casting solution to particles for final particle concentration of 2.5 mg/mL particles.

2.2. Pipette the particle suspension in 10 mL aliquots into 75 x 50 mm rectangular petri dishes for one-dimensional stretching or in 15 mL aliquots into 100 x 100 mm square petri dishes for two-dimensional stretching. Remove bubbles by either pipette or pushing bubbles to the side and let films dry overnight in a chemical hood.

2.3. Once films have dried, remove films from plastic dishes with tweezers and cut off edges of films with scissors. Save edges in a 50 mL conical tube to be used as spherical particles.

2.3.1. Load a film onto the automated thin film stretching device by mounting a film onto aluminum blocks. For 1D stretching, mount film onto aluminum blocks of one axis of the stretcher (**Figure 2**) by placing two short edges of film in between two pieces of neoprene rubber. Using an Allen wrench, screw the metal grips on top of rubber to hold the film in place. For 2D stretching, mount all four edges onto four aluminum blocks to stretch on both axes (**Figure 2**).

2.3.2. Measure and record the length of film in between aluminum blocks on one axis for 1D stretching or both axes for 2D stretching. Calculate the distance required to stretch the film in one or two directions based on desired fold-stretch.

2.3.3. Place the film-loaded stretching device in an oven at 90 °C and bring the film to temperature over 10 minutes. Place a large beaker in the oven with a small amount of water.

2.3.4. Stretch film.

2.3.5. When stretching is completed, remove the stretching device from oven and let the film cool to room temperature for 20 minutes.

2.3.6. For 1D stretching, cut the film out of the stretching device at the edges. For 2D stretching, cut out and save the center square of film that is uniformly stretched on both axes. Place films in conical tubes with 2 films per tube and discard the rest of the film.

2.3.7. Add approximately 25 mL of DI water to each conical tube and vortex until the films are dissolved.

2.3.8. Once the films are dissolved, wash particles 3 times with DI water. For microparticles, fill up conical tubes to 50 mL and centrifuge at 3000 x g for 5 minutes, pour out supernatant, add approximately 20 mL of DI water, and vortex to resuspend particles. For nanoparticles, transfer particles to high speed centrifuge tubes and centrifuge at 40,000 x g for 15 minutes, pour out supernatant, add approximately 20 mL of DI water, and vortex to resuspend particles.

2.3.9. After the third wash, resuspend particles in approximately 1 mL of DI water. Record the weight of the microcentrifuge tube and add particles to the tube. Freeze particles in a -80 °C freezer for 1 hour or flash freeze in liquid nitrogen. Once frozen, lyophilize particles overnight.

2.3.10. Once lyophilized, weigh particles in the microcentrifuge tube and subtract recorded weight of the empty tube to determine the weight of lyophilized particles.

3. Surface protein conjugation to create Artificial Antigen Presenting Cells

3.1. Prepare 2-(N-Morpholino)ethanesulfonic acid (MES) buffer. Make a 0.1 M solution of MES in water and adjust the pH to 6.0 by titration with 1M sodium hydroxide (NaOH).

3.2. Resuspend lyophilized PLGA/PBAE micro/nanoparticles at 20 μg/mL in MES buffer by vortexing. Fill a polypropylene microcentrifuge tube with 900 μL of MES buffer and add 100 μL of the particle solution to the tube.

3.3. Prepare EDC/NHS solution. Dissolve 40 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 48 mg N-hydroxysulfoxuccinimide (NHS) in 1 mL MES buffer.

3.4. Add 100 μL EDC/NHS solution to the particles and vortex to mix.

3.5. Incubate the tube on an inverter at room temperature for 30 minutes.

3.6. Spin down microparticles at 5,000 x g for 5 minutes, or nanoparticles at 17,000 x g for 5 minutes. Discard the supernatant and resuspend in 1 mL PBS by vortexing (microparticles) or sonicating at 2-3 W for 5 seconds (nanoparticles).

3.7. For microparticles, add 8 μg of the desired signal 1 protein, and 10 μg anti-mouse CD28 (clone 37.51) to the particles. For nanoparticles, add 16 μg signal 1 and 20 μg anti-mouse CD28. Add PBS to bring the volume in the tube to 1.1 mL.

3.8. Incubate the tube on an inverter at 4 °C overnight.

3.9. The next day, wash the particles. Spin down microparticles at 5,000 x g for 5 minutes, or nanoparticles at 17,000 x g for 5 minutes. Discard the supernatant, and resuspend particles in 1 mL of sterile PBS by vortexing (microparticles) or sonication at 2-3 W for 5 seconds (nanoparticles). Repeat twice.

3.10. Resuspend the particles at the desired concentration in culture medium for immediate use. For long-term storage, resuspend particles at 10 mg/mL in a 100 mM sucrose solution. Freeze, lyophilize, and store the particles at -80 °C.

4. Characterization and evaluation of aAPC

4.1. Characterization of aAPC size and shape (**Figure 3**)

4.1.1. Characterize microparticle size and shape by imaging particles using scanning electron microscopy (SEM). For SEM imaging, spread lyophilized particles onto carbon tape adhered to an aluminum tack and sputter coat with gold-palladium.

4.1.2. Analyze size and aspect ratio of particles using image analysis software. To determine particle size, set scale using scale bar and measure particle diameter. Repeat for approximately 100 particles to determine the average particle diameter and generate a histogram of particle sizes. To determine aspect ratio, measure the distance across the long axis and the short axis of

particles and divide long axis by short axis. Repeat for approximately 50 particles of each shape to determine the average particle aspect ratio for each shape and generate histograms.

4.1.3. Characterize nanoparticle size and shape by imaging particles using transmission electron microscopy (TEM). Analyze size and aspect ratio of nanoparticles using image analysis software as described in 5.1.2. Alternatively, measure spherical nanoparticle size using dynamic light scattering (DLS) or nanoparticle tracking analysis (NTA).

4.2. Protein Conjugation Efficiency

4.2.1. Prepare aAPC according to Methods 1-3, but in Step 3.7 use fluorescently labeled signal 1 protein and anti-mouse CD28. After conjugation and washing, resuspend the aAPC in 1 mL PBS for a final concentration of 2 mg/mL aAPC.

4.2.2. Prepare protein standards in a black polystyrene 96-well microplate. Add 5 μg of fluorescently labeled signal 1 protein to PBS in the first well of the plate and make 10 1:2 dilutions in PBS across the row of the plate. Leave the last well blank. Repeat this step to generate another set of standards with fluorescently labeled anti-CD28.

4.2.3. Pipette 100 μL of the aAPC solution into replicate wells of the black polystyrene microplate in triplicate.

4.2.4. Read the fluorescence on a fluorescence plate reader at the appropriate wavelengths. Use the fluorescence readings from the protein standards to generate standard curves for each fluorescent antibody. Using the standard curve, calculate the concentrations of signal 1 and anti-CD28 in each sample well, and then calculate the amount of surface protein and conjugation efficiency (**Figure 4A, 5A**).

4.3. **Evaluation of aAPC for *in vitro* stimulation of CD8+ T cells**

4.3.1. Prepare B' media (RPMI medium supplemented with L glutamine, 10% FBS, 1% Non-essential amino acid solution, 1 % Sodium pyruvate, 1 % MEM Vitamin solution, 92 μM β-mercaptoethanol, 10 ng/mL ciprofloxacin, and 30 U/mL IL-2.

4.3.2. Sacrifice a Black 6 mouse via carbon dioxide exposure.

~~4.3.2.~~4.3.3. ~~.~~Harvest the spleen from the mouse according to a previously established protocol.[16] Collect the spleen~~and collect it~~ in a 50 mL conical tube with 10- 15 mL PBS. Using a pestle, mash the spleen through a 70 μm cell strainer into a 50 mL conical tube. During mashing, wash the strainer with 40 mL PBS.

4.3.3.4.3.4. Spin the splenocytes at 300 x g for 5 minutes. Pour off the supernatant and resuspend the splenocytes in 4 mL of Ack Lysing Buffer to lyse the red blood cells. Allow the tube to sit undisturbed for 1 minute, then add PBS to bring the volume in the tube to 20 mL.

4.3.4.4.3.5. Spin the cells at 300 x g for 5 minutes. Pour off the supernatant and resuspend the cells in 1 mL of PBS. Count the cells using a hemocytometer.

4.3.5.4.3.6. Spin the cells at 300 x g for 5 minutes and resuspend in the desired volume of cell separation buffer. Isolate CD8+ T cells from the single cell suspension using a CD8+ negative selection T cell isolation kit, following the manufacturer's protocol.

4.3.6.4.3.7. Following magnetic separation, spin CD8+ T cells at 300 x g for five minutes and remove the cell separation buffer. Resuspend cells in 1 mL of PBS and count the cells. Label cells with carboxyfluorescein succinyl ester (CFSE) according to the manufacturer's protocol.

4.3.7.4.3.8. Incubate 8,333 CD8+ T cells and 0.0833 mg (or desired dose) of aAPC in 150 µL B' media in each well of a 96 well U-bottom tissue culture-treated plate.

4.3.8.4.3.9. Incubate at 37 °C for 7 days. After 3-4 days, refresh the culture medium by adding 75 µL fresh medium to each well.

4.3.9.4.3.10. After 3 days of incubation, analyze CFSE labelled cells on a flow cytometer to assess proliferation. Each peak on the flow cytometry CFSE histogram represents a generation of cells due to the CFSE dilution with each successive cell division.

4.3.10.4.3.11. After 7 days, use a hemocytometer to count the number of cells in each well. Prior to counting, stain dead cells with a Trypan Blue solution. Exclude dead cells from final cell counts. Normalize the final cell concentration to the initial concentration to calculate the fold-expansion (**Figures 4 and 5**).

REPRESENTATIVE RESULTS:

A schematic for the automated 2D thin film stretching device is given in **Figure 1**. A schematic and description for a 1D thin film stretching device is given in Ho et. al. [17] The stretcher is constructed from aluminum parts using standard milling and machining techniques.[16] The stretcher is constructed from aluminum parts using standard milling and machining techniques. Similar to the 1D stretcher, the 2D stretcher consists of metallic grips and guide rails. Bidirectional lead screws are used to translate linear to rotational motion. The lead screws are the attached via mechanical taps to identical stepper motors with sufficient torque. The 8 stepper motor control wires can be soldered onto 8 pin heat resistant amphenol connectors for easy attachment to the control console in an oven for thin film stretching. Polytetrafluoroethylene (PTFE) coated wire of sufficient length must be used to connect the stepper motors to the drivers in the control console. The recommended computer control

scheme is given in **Figure 1A**. The two motors must be connected through heat resistant wiring to 2 independent drivers. The two drivers must then be connected to a microcontroller to interface with a computer. The drivers should be connected to the X-Axis and Y-Axis outputs on the microcontroller. The drivers and microcontroller both require an external power supply. Prior to connecting the power supply to these three components it is recommended that a 4 A fuse be inserted in between each of the powered connections to protect the components from current overload. Finally, the microcontroller can be linked via a Parallel Port Input to a computer using a DB25 Male to Male cable. The electronics used to control the stepper motors are heat sensitive and therefore must be placed outside of any heat source (such as an oven) used during operation to heat the thin films to sufficient temperature to enable stretching. Although the recommended motors are heat resistant up to the temperatures specified in this protocol for stretching particles, the motors and drivers will build up additional heat while they are attached to the main power supply. Therefore, it is recommended that the device only be turned on during the period of actual film stretching to minimize potential heat build-up.

PLGA nano- and microparticles were synthesized using the single emulsion techniques described in this protocol and imaged using TEM (**Figure 3A**) and SEM (**Figure 3B**), respectively. Spherical nanoparticles had a diameter of 237.3 ± 4.0 nm, as measured by DLS and 224 nm as measured by NTA (**Figure 3C)**. Microparticles were synthesized by homogenization at 5000 rpm to generate spherical particles with an average diameter of 3 ± 1 μm (**Figure 3D**). The particles were stretched using the automated film stretching device at 90 °C in one dimension to generate prolate ellipsoidal nano- and microparticles and stretched at 70 °C in two dimensions to generate oblate ellipsoidal particles. The aspect ratios of the microparticles of all three shapes were analyzed by measuring the long axis and short axis distance of particles and dividing the two. Spherical microparticles had an aspect ratio of 1.05 ± 0.04, while 1D stretched prolate ellipsoidal particles had a larger aspect ratio of 3.6 ± 0.8 (**Figure 3E**). 2D stretched oblate ellipsoidal particles had an aspect ratio of 1.2 ± 0.2, roughly maintaining an aspect ratio of one.

EDC/NHS reaction chemistry was used to conjugate a fluorescently labelled peptide-loaded MHC IgG dimer and anti-CD28 antibody to the surface of stretched and spherical PLGA particles. Conjugation efficiency results demonstrate similar amounts of protein on the surface of spherical and ellipsoidal micro-aAPC (**Figure 4A**) and nano-aAPC (**Figure 5A**), and demonstrate that protein coupling during aAPC synthesis occurs in a concentration-dependent manner. To evaluate the effect of shape on aAPC functionality, spherical and prolate ellipsoidal aAPC conjugated with gp100-loaded MHC IgG dimer and anti-CD28 were used to stimulate PMEL transgenic CD8+ T cells. T cells were labelled with CFSE and evaluated by flow cytometry after 3 days to assess proliferation (**Figure 4B, 5B**). Prolate ellipsoidal aAPC were found to induce higher levels of T cell proliferation at sub-saturating doses than spherical aAPC, with the best separation achieved at a 0.01 mg dose. After 7 days, the T cells were manually counted. Prolate ellipsoidal aAPC more effectively stimulated T cells compared to their spherical counterparts at the microscale (**Figure 4C**) and nanoscale (**Figure 5C**), and dose-dependent T cell expansion was observed.

Figure Legends:

Figure 1: Schematic representation of automated thin film stretcher. (A) Schematic of control console for thin film stretcher. (B) Schematic of mechanical hardware for thin film stretcher. (Left) Overhead view of mechanical hardware. (Right) Cross-section of gripping mechanism for thin films.

Figure 2: Photographs of assembled automated thin film stretcher to stretch polymeric particles into anisotropic shapes. The 2D thin film stretching device is composed of two axes with aluminum mounts that grip the film. The two axes contain lead screws in opposing directions so that they move apart from each other. To automate the stretching procedure, a USB linked microcontroller is connected to two stepper motor drivers that relay signals to unipolar stepper motors through a thermal cable.

Figure 3: Size and aspect ratio analysis of spherical and ellipsoidal PLGA particles. (A) TEM and (B) SEM images of spherical, 1D stretched prolate ellipsoidal, and 2D stretched oblate ellipsoidal PLGA (A) nanoparticles and (B) microparticles. (C) Spherical nanoparticles were sized by NTA and determined to be 224 nm in diameter. SEM images of PLGA microparticles were analyzed for (D) size distribution of spherical particles and (E) aspect ratios of all particle shapes. (C) Reproduced and adapted with permission from *Small* [13] Copyright Wiley-VCH 2015.

Figure 4: Characterization and functional assessment of spherical and prolate ellipsoidal micro-aAPC. (A) Conjugation efficiency of fluorescently-labelled peptide-loaded MHC IgG dimer and anti-CD28 antibody to the surface of spherical and prolate ellipsoidal microparticles. (B) CD8+ T cells were labelled with CFSE and incubated with spherical and 1D-stretched micro-aAPC at 0.01, 0.1, and 1 mg doses, or non-cognate controls. After 3 days, cells were evaluated by flow cytometry to assess proliferation. (C) T cells were also evaluated after 7 days by manual counting. Cell counts were normalized to the initial count to calculate fold-expansion. For comparison between prolate ellipsoidal and spherical fold expansion, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. Error bars represent standard error of the mean (SEM) for 3 replicates. Reproduced and adapted with permission from *Biomaterials*[12] Copyright Elsevier 2014.

Figure 5: Characterization and functional assessment of spherical and prolate ellipsoidal nano-aAPC. (A) Conjugation efficiency of fluorescently-labelled peptide-loaded MHC IgG dimer and anti-CD28 antibody to the surface of spherical and prolate ellipsoidal nanoparticles. (B) CD8+ T cells were labelled with CFSE and incubated with spherical and prolate ellipsoidal nano-aAPC of varying fold-stretch at 0.01, 0.1, and 1 mg doses. After 3 days, cells were evaluated by flow cytometry to assess proliferation. (C) T cells incubated with prolate ellipsoidal particles of varying fold stretch (ranging from 1.5 to 3.5)~~(C) T cells~~ were also evaluated after 7 days by manual counting. Cell counts were normalized to an untreated condition to calculate fold-expansion. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ compared to spherical. Error bars represent standard error of the mean (SEM) for 3 replicates. Reproduced and adapted with permission from *Small* [13] Copyright Wiley-VCH 2015.

DISCUSSION:

This protocol details a versatile method for the precise generation of anisotropic polymeric particles. The thin film stretching technique described here is scalable, highly reproducible and inexpensive. Alternative techniques for generating anisotropic particles suffer from many limitations, including high cost, low throughput, and limited particle size. The thin film stretching approach is also advantageous because the particles are modified to be anisotropic after synthesis, and, as a result, is compatible with a wide range of particle sizes and synthesis techniques. **Figure 1** details the setup of the automated two-dimensional stretching device. This device can also be used without the electronic components by manually turning the screws until the film has reached the desired degree of stretching. However, we have found that the automated process is much more consistent and rapid than manual operation. [15] Various techniques have been developed to synthesize anisotropic particles, such as microfluidic approaches [17-19], layer-by-layer coating [21~~20~~], and other bottom-up synthesis approaches. [21,22] However, these approaches do not enable strong control over particle geometry and are not as versatile in terms of shapes that can be generated and particle materials that can be used. A popular top-down method for fabricating nonspherical particles is Particle Replication in Non-Wetting Templates (PRINT). [24~~23~~] Although PRINT enables precise control over particle shape, it requires expensive machinery and is not as accessible and simple to implement as the thin film stretching method.

The single emulsion technique can be used to fabricate PLGA particles of various sizes, ranging from the nano to microscale. [12,13] By varying homogenization speed or sonication amplitude, microparticle and nanoparticle size, respectively, can be modulated. Once spherical particles have been generated, the thin film stretching method described here can be used to deform the particles into various shapes. [15] In this protocol, we describe the generation of prolate and oblate ellipsoidal particles by stretching in one or two dimensions, respectively. Spherical particles are cast into a thin plastic film, which is heated above the glass transition temperature of PLGA and stretched in one or two dimensions to deform the particles. The aspect ratio of the particles is highly controllable. By tuning the degree of film stretch, the aspect ratio of the particles can be modulated, and we have found that measured particle aspect ratio is highly correlated with the predicted value. [12,13] Various other shapes can be generated by modifying the temperature during stretching or the degree of stretching. For example, biconcave discoidal particles resembling the shape of red blood cells can be generated by stretching microparticles 1.5-fold in two dimensions at 90 °C. [15] This film stretching technique has also been used to transform spherical polystyrene particles into many anisotropic shapes including worms, barrels, and rectangular discs. [21~~20~~] The film stretching device can be used by manually controlling the screws or the device can be automated as shown in **Figure 1** to make the process more efficient and consistent. [15] This simple technique reliably produces anisotropic particles that retain their shape under physiologic conditions. [24] Furthermore, this method has been applied to other polymeric materials, in addition to PLGA, such as polycaprolactone (PCL) and hybrid particles made of PLGA and poly(beta-amino ester) (PBAE).

This protocol also describes how PLGA particles of varied size and shape can be conjugated with the surface proteins required for CD8+ T cell activation to act as aAPC. Proteins can be covalently conjugated to anisotropic and spherical PLGA micro- and nanoparticles by EDC/NHS mediated coupling of primary amines on proteins to carboxyl groups on the particle surface. The efficiency of protein conjugation can be measured by coupling fluorescently labeled protein to the surface of particles as described in this protocol, and we have found that this technique couples protein to particles at 15-20% efficiency. [12,13] Prolate ellipsoidal micro- and nanoparticle aAPC are more effective than their spherical counterparts at activating CD8+ T cell proliferation and expansion *in vitro.* [12,13] Ellipsoidal aAPC have enhanced binding to and interaction with T cells due to their larger surface area for contact. [12] Anisotropic particles also have superior properties over spherical particles *in vivo* due to their enhanced biodistribution and resistance to phagocytosis. [13] This platform is highly modular and has the potential to be adapted to many other drug delivery applications. Using this procedure, polymeric particles of tunable shape and size can be generated and the particle surface can be conjugated with any protein of interest.

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