Tissue Regeneration, Drug Delivery, Nanotechnology

3D Bioprinting Spatiotemporally Defined Patterns of Growth Factors to Tightly Control Tissue Regeneration

Growth factors used for tissue regeneration applications often do not perform well in later stages of clinical trials, betraying their early promise. One example is the large initial release of bone morphogenic protein (BMP)-2 from collagen powder/sponges over 21 days that is nonphysiological. A slower- and sustained-release profile may reduce the incidence of adverse effects such as heterotopic ossification, and more physiological recovery is highly desirable. Fracture healing involves different stages, involvement of BMP and vascular endothelial growth factors (VEGFs), and interaction between blood vessels and bone cells. VEGF expression peaks around day 5/10 before decreasing, whereas BMP-2 increases constantly until day 21.

Novel engineering methods can be used to control the release of such growth factors and osteoinductive hydroxyapatite (HA) and laponite nanoparticles into regenerative implants for tight temporal control over growth factor release. In this study, VEGF and BMP-2 factors were tuned by printing functionalized alginate inks with distinct spatiotemporal release profiles to enhance the regeneration of critically sized bone defects. To produce printable biomaterial inks, various weights of methylcellulose were added to RGD alginites. The temporal release profile of growth factors (e.g., VEGF) were tuned by adding clay and hydroxyapatite nanoparticles (nHAs). While methylcellulose increased VEGF release, laponite (clay nanoparticles) slowed the release of VEGF. The alginate, methylcellulose, and nHA blend was then referred to as the vascular bioink, releasing VEGF for more than 10 days. The authors then demonstrated that spatial gradients of VEGF could be maintained for more than 14 days by printing VEGF at different spatial presentations.

When gradient VEGF implants were compared with homogeneous and no VEGF conditions, vessels were observed following 2 weeks of subcutaneous implantation in mice. In the no-VEGF group, the blood vessels were absent. Unlike the homogeneous VEGF group, the gradient group showed blood vessels in the implant center. To modulate the slower release of BMP-2, laponite was added to alginate–methylcellulose bioinks. To assess the effect of slow-release BMP-2 bioinks, fast-release (alginate–methylcellulose) bioinks were bioprinted with bone marrow-derived mesenchymal stem cells (BMSCs) and compared with the slower-release candidates. Eight weeks after implantation in a segmental defect, greater amounts of mineralized tissue were observed in the slow-release compared with the fast-release group using micro-computed tomography (micro-CT) and histological analysis. Thereafter, VEGF and BMP-2 were co-released from printed constructs to heal a critically sized bone defect. Using micro-CT angiography, combined VEGF and BMP-2 (composite) groups led to a significant increase in vessel volume compared with the VEGF gradient group. Comparatively, there was also greater vessel connectivity and greater maturity (verified by α-SMA and vWF expression).

In terms of cartilage and new bone deposition, the composite group also performed better (Safranin O staining, micro-CT imaging) with dense, cortical-like bone present, comparable to native bone. A greater quantity of bone marrow was similarly observed in the composite group. Thus, the authors’ approach to identifying suitable spatiotemporal presentation of growth factors to regenerate large bone defects is described herein. They demonstrated improved angiogenesis and neo-bone formation while reducing off-target effects and envision this approach in regenerating other tissue types. (Freeman, F.; et al. Sci. Adv. 2020, 6, eabb5093.)

3D Printing of Personalized Thick and Perfusable Cardiac Patches and Hearts

Cardiac tissue engineering integrates cardiac cells with biomaterials and is a promising approach for regenerating infarcted hearts. An obstruction to this approach is the selection of biomaterials with requisite properties, with
biocompatible degradation products and minimal immune response. The Dvir group has demonstrated a personalizable approach for engineering autologous cardiac patches. A biopsy of fatty tissue is obtained from patients before the cells are reprogrammed into pluripotent stem cells, whereas the extracellular matrix (ECM) is processed into a hydrogel. One drawback about this approach is the lack of blood vessel networks that match patient vasculature. Other approaches in recent times were unable to print full, thick vascularized cardiac patches.

In this study, advanced 3D printing methods were used to generate thick, vascularized, perfusable cardiac patches to match the patient’s immunological, biochemical, and anatomical properties. They further extend this to printing whole hearts with major blood vessels. From omental fatty tissue, patient cells were reprogrammed into iPSCs (OCT4+) and then differentiated into cardiomyocytes (CMs) and endothelial cells (ECs). Computerized tomography (CT) was then used to visualize the 3D structure and the orientation of major blood vessels. Computer-aided design (CAD) was then used to model the smaller vessels using mathematical equations to simulate oxygen transport and consumption. The personalized hydrogels were then mixed with the iPSC-derived endothelial, cardiac, and fibroblast cells, together with gelatin as a sacrificial ink. A 3D printer was able to create 2 mm thick vascularized patches with high cell viability. Incubation at 37 °C led to gelatin liquefying to leave 300 µm lumens within the cardiac patches. These patches were physically robust, holding their shape, while liquid could be infused into the open lumens. These patches were positive for actinin (CM cells) and CD31 (EC cells), with the ECs and fibroblasts assembling into their native configuration. The printed vascularized cardiac patch was also contractile, exhibiting calcium transients to demonstrate its functionality. These were transplanted in rat omentum displaying elongated and aligned cells with massive striations, indicating their contractility and functionality. For organs or tissues with larger dimensions, an alternative strategy was used since these gels have unsuitable mechanical properties. To preserve the integrity of the delicate structures and sensitive cells, a gel support comprising alginate microparticles and xanthan gum is utilized for free-form printing and safe enzymatic or chemical degradation. The authors managed to generate thick, vascularized, perfusable tissue. Triaxial lumen structures allowed the perfusion of dye, indicating the potential for blood transfer. The authors then printed large (20 mm height, 14 mm diameter) volumetric tissue with anatomical architectures. These structures had similar mechanical properties to decellularized rat hearts. Sarcomeric actinin structures were also observed in the heart tissue internal compartment.

In summary, the authors demonstrate a fully personalized method to print cardiac tissue and organs obtained from patient source tissue. These may be further tested in long-term in vitro and in vivo animal studies to evaluate the true therapeutic value of the printed cardiac tissue. (Noor, N.; et al. Adv. Sci. 2019, 6, 1900344.)

**Cryo-Shocked Cancer Cells for Targeted Drug Delivery and Vaccination**

Acute myeloid leukemia (AML) has a dismal prognosis and its standard of care, chemotherapy, is only partially successful with frequent disease relapse. Stem cell transplantation is another approach but suffers from a lack of donors and the risk of mortality. Since AML originates in the bone marrow, targeted drug delivery could improve efficacy and reduce collateral toxicity to nonhematopoietic tissue. AML cells exhibit marrow homing abilities; hence, the Gu lab repurposed them for drug delivery while neutralizing their disease-causing properties. They utilized cryo-shocking to render the AML cells (liquid nitrogen-treated [LNT] cells) nonviable before loading them with drugs (doxorubicin [DOX]). Following 12 h in liquid nitrogen, LNT cells were thawed at 37 °C. These shrank in size but maintained their nucleus and cytoskeleton, with a rougher cell surface. Calcein-AM/EthD-1 showed the lack of viability, while cell counting kit-8 (CCK8) and annexin V–propidium iodide (PI) showed that the cells were nonproliferative. In mouse models, the AML cells did not form a tumor and exhibited no circulating cancer cells.

In LNT cells, most of the proteins expressed by live AML cells were present. Particularly, CXCR4 and CD44 were detected in both cells through confocal imaging and flow cytometry. LNT and viable AML cells exhibited similar accumulation efficiency in bone marrow following intravenous infusion. The intact nuclear and cytoplasmic structures allowed DOX intercalation up to 65 µg per 10^7 LNT cells. DOX was released in a sustained manner with 81% released over 10 h. While a higher amount of DOX from LNT was required for AML cytotoxicity compared with free DOX, LNT delivery led to longer detection of DOX in blood and bone marrow. DOX-loaded LNTs were able to reduce AML tumor growth.

LNTs were then investigated for enhancing antigen uptake and mature antigen-presenting cells (APCs). LNTs upregulated CD40, CD80, CD86, and major histocompatibility complex II (MHC-II). CD4+ T cells and CD8+ T cells were also increased in peripheral blood. DC maturation and T-cell-activated cytokines—interferon-γ (IFN-γ), tumor necrosis factor–α (TNF-α), and interleukin-6 (IL-6)—were similarly detected following LNT and adjuvant treatment (monophosphoryl lipid A [MPLA]). The combination of LNT/DOX and adjuvant nearly eliminated AML tumors.
after 21 days. Increased inflammatory cytokines IFN-γ and TNF-α suggested boosted immunity from the combined therapeutic.

The authors then evaluated LNT cells as a prophylactic cancer vaccine by immunization and adjuvant 1–3 weeks prior to inoculation of AML cells. The tumor bioluminescence intensity was significantly smaller compared with the control group. Whereas all control mice (without preimmunization) perished, 71% of mice were tumor-free 90 days following the challenge. Furthermore, blood serum levels of IFN-γ, TNF-α, IL-12, and IL-6, as well as CD3+ and CD8+ T cells, were significantly higher in LNT/adjuvant-treated mice. In summary, the authors used a simple liquid nitrogen treatment procedure to negate the tumorigenicity yet preserve cellular integrity. This renders the AML non-pathogenic and allows it to be exploited for drug delivery and antitumor immune responses. While a highly interesting approach, this will warrant further efforts in regard to manufacturing, quality control, and clinical application.

(Ci, T.; et al. J. Sci. Adv. 2020, 6, eabc3013.)

Decoy Nanoparticles Protect against COVID-19 by Concurrently Adsorbing Viruses and Inflammatory Cytokines

The COVID-19 pandemic has shown the importance of rapidly developing therapeutic strategies for ongoing or preparing for future disease outbreaks. The spike (S) protein (containing S1 and S2 subunits) has a vital role in viral infection. The S1 subunit engages with human angiotensin-converting enzyme II (ACE2). The infected host organism then mounts a sufficient immune response, with significantly increased inflammatory cytokines to eliminate pathogens and promote tissue repair. This in turn could spur the inflammatory state, leading to immune dysfunction—cytokine release syndrome (“cytokine storm”). While most patients display mild symptoms, ~20% progress to severe symptoms, including pneumonia, septic shock, and even multiple organ failure. Therefore, treatments that block SARS-CoV-2 progress through ACE2 could be highly promising.

While antivirals such as remdesivir are showing encouraging signs for early intervention, few target late-stage infection-associated cytokine release syndrome (CRS). Monoclonal antibodies for IL (interleukin)-6 and GM-CSF (granulocyte–macrophage colony-stimulating factor) have been suggested as therapeutic candidates, but it is challenging to suppress CRS due to the complexity of cytokine targets and their interactions. From previous nanotechnology experience, the authors have shown that protein synthesis and display enabled the generation of nanovesicles expressing native proteins on their surface. They hypothesize that engineering and displaying ACE2 on nanovesicles can compete with and bind SARS-CoV-2 to create nanodecoys. Briefly, the engineered nanodecoys are created by transfecting ACE2 on 293T cells before fusing them with THP1 cells. The final product contains ACE2, IL-6R, and GM-CSF moieties, facilitating the neutralization of viruses and secreted inflammatory cytokines.

Immunostaining and flow cytometry demonstrated positive ACE2 transfection in 293T cells. To extract cell membrane material, the intracellular material was removed by hypotonic lysis, mechanical disruption before gradient centrifugation. Both ACE2 and THP vesicles were then mixed, sonicated, and repeatedly extruded through nanopores to form nanodecoys. Confocal microscopy demonstrated successful fusion since ACE2 and THP1 were prelabeled with different fluorescence dyes. Western blots showed the successful combination and preservation of the different biological components (ACE2, CD130, CD116). Each 1 μg of nanodecoys was found to contain 140 pg of ACE2.

In an in vitro infection model (huh-7 cells), pseudotyped SARS-CoV2 (PsV) was incubated with either nanodecoys or their component vesicles (293T-, THP1-, ACE2-Ves). Only ACE2-Ves and nanodecoys inhibited PsV infection, while 293T- and THP1-Ves were unable to do so. In addition to SARS-CoV-2, pseudotyped SARS-CoV and SARSr-CoV were also inhibited by the nanodecoys. This demonstrates the promising broad-spectrum antiviral properties of nanodecoys and their potential against emerging coronavirus outbreaks. The nanodecoys’ antiviral properties were further repeated on Vero-E6 monkey kidney cells and human Caco-2 cells using authentic SARS-CoV-2 with reduced levels of N protein and a reduction of viral copy number (using quantitative real-time reverse transcription PCR [qRT-PCR]). The nanodecoys further removed inflammatory cytokines—20 μg removed 160 pg of IL-6 and 25 pg of GM-CSF—suggesting their potential for suppressing CRS.

In lipopolysaccharide (LPS)-stimulated macrophages, IL-6 and GM-CSF became highly upregulated, before nanodecoys significantly decreased cytokine levels. The nanodecoys were then tested on mouse subjects and introduced by inhalation while being retained in the lungs after 72 h. The mice were then subject to acute lung inflammation (ALI) by intratracheal LPS inhalation. Following the ALI challenge, mice were treated with nanodecoys, before lung bronchoalveolar lavage fluid (BALF) was obtained to assess cytokine levels. Promisingly, the nanodecoys effectively decreased IL-6, GM-CSF, and total protein levels in BALF. Histological examination showed a decreased extent of lung injury, thereby protecting the mice to a certain degree. Importantly, the nanodecoys did not give rise to any short- and long-term systemic toxicity, supporting their biocompatibility. Thus, the authors demonstrate a versatile nanodecoy as a potential COVID-19 therapeutic. This technology can be further customized in order to cater for high-affinity ACE2 variants for the S protein, and may be amenable to gene editing, vesicle purification, and large-scale production of nanodecoys.
Environmental Exposure Enhances the Internalization of Microplastic Particles into Cells

Microplastics have emerged as a significant ecological issue, highly present in ecosystems. Biomolecules and microorganisms often attach to microplastic surfaces, increasing their likelihood of organism ingestion in zooplankton, bivalves, and vertebrates. These particles enter the gastrointestinal tract, spreading through the circulatory system, tissues sparking inflammatory responses and necrosis. Microplastics are anticipated to translocate through cells, including immune cells such as macrophages. The rate of microplastic internalization is influenced by particle size (nanomicron size) as well as other factors, such as the biomolecules attached to the microplastic surface. Within seconds of exposure, these biomolecules coat the surfaces and evolve to contain varying compositions of high- or low-affinity binding. These coatings, also known as coronas, comprise carbohydrates, lipids, and proteins, which suggests that their composition may influence internalization behavior.

The authors test the effect of freshwater and saltwater environments on model spherical microplastic polystyrene particles (3 µm) on murine macrophage cell lines (J774A). The polystyrene microplastic particles were incubated in ultrapure water (negative controls), IgG-opsonized particles (positive controls), and compared with particles incubated in an artificial pond (fresh water) or water from a marine environment (saltwater). Raman analysis showed that pristine particles were smooth, freshwater treatment had heterogeneity, salt water had the appearance of salt crystals, and the IgG particles were homogeneously rough. Raman analysis showed that freshwater treatment gave rise to coronas containing carbohydrates (C–O–C bands), amino acids (C–N–S bands), nucleic acids (PO4 band), lipids (C–H and C–H2 bands), and proteins (C–H and C=O bands). Pristine and saltwater treated particles did not give rise to the above biomolecule signatures. The strong salt signatures may mask the presence of biomolecules. Further analysis by x-ray photoelectron spectroscopy showed that biomolecules were also present on the saltwater-incubated particles.

In summary, the authors demonstrate how the environment can influence microplastics, giving rise to the formation of “eco-coronas” by biomolecules adsorbed on their surface. This occurred following incubation in both freshwater and saltwater systems. This suggests that eco-coronas have a significant influence on macrophage internalization and need to be taken into account during toxicity evaluation of environmental microplastics. (Ramsperger, A.; et al. Sci. Adv. 2020, 6, eabd1211.)

COVID-19

Mosaic Nanoparticles Elicit Cross-Reactive Immune Responses to Zoonotic Coronaviruses in Mice

Given that bat-origin coronaviruses have caused three severe disease outbreaks to date, developing suitable vaccine candidates is of great interest. Vaccine candidates for SARS-CoV-2 include the spike trimer (S) after one or more of its receptor-binding domains (RBDs) adopt an “up” position for binding with a host receptor. Through understanding the mechanics of viral entry via S binding with angiotensin-converting enzyme 2 (ACE2), many vaccine candidates are designed to target the RBD. Targeting multiple antigens (multivalent) can enhance immune cell responses and provide longer-lasting immunity compared with “monovalent” antigen vaccines. One approach is to use nanoparticles to couple engineered proteins (SpyCatcher) to prepare multimerized SARS-CoV-2 that generates high neutralizing antibody titers. While a promising approach against SARS-CoV-2, the authors propose that this SpyCatcher system can generate mosaic nanoparticles to simultaneously display human and animal RBDs.

They identified 29 diverse RBDs from a wide range of geographies (China, Bulgaria, Kenya, etc.) of diverse species origin (bat, pangolin, human, etc.); these had 68%–95% of sequence identity. From these, eight RBDs were used to create three mosaic nanoparticles. Mice were immunized using soluble SARS-CoV-2 spike trimer (SARS-2S), nanoparticles (NPs) displaying SARS-2 RBD, three mosaic NPs (mosaic-4a, mosaic-4b, mosaic-8), or unconjugated NPs. Sera from mice immunized with unconjugated NPs, anti-SARS-2S trimer, and anti-SARS-2 RBD serum responses were similar. In vitro neutralization assays using pseudotyped virus assays on 293T-ACE2 target cells correlated with IgG ELISA (enzyme-linked immunosorbent assay) results. While SARS-2S trimer immunized mice showed no binding or neutralization except for an autologous response to SARS-2, sera from the RBD-NP immunized mice neutralized against four strains, demonstrating increased immunogenicity. The mosaic NPs achieved this without a diminished anti-SARS-2 response from homologous SARS-2 NPs.

The serum responses were then compared, matched (RBDs present on NPs) versus mismatched (RBDs not present on NPs), and they demonstrated that even without
the particular RBD being present, antibody titers (e.g., for SARS-2) did not generate lower titers compared with the homotypic SARS-2 NP immunization. This demonstrates the common evolutionary origin of epitopes between the RBDs. The mosaic NPs also demonstrated a 7- to 44-fold more potent neutralization sera compared with homotypic SARS-2 immunization. Among the candidates, mosaic-8 induced higher antibody titers against mismatched RBDs than homotypic SARS-2 NPs, demonstrating the effectiveness of the co-display approach for generating broad coronavirus immunity from a single prime. From IgG+ B cells, antibody cross-reactivity was observed for RBDs having at least 70% sequence identity overlap. Antibody cross-reactivity was then compared in convalescent plasma from SARS-CoV-2 infections. While most of these bound to SARS-2 RBD, binding to other sarbecovirus RBDs (RaTG13, SHC014, WIV1, Rs4081, and BM-4831) was found to be significantly weaker, with many human plasma IgGs showing no discernible binding. Neutralization with SARS, SHC014, and WIV1 pseudoviruses also demonstrated weak or low neutralization.

Importantly, the mosaic NP candidates described in this study could generate neutralizing Abs against emerging coronaviruses that could spill over to human populations. This makes them a prime vaccine candidate against newly discovered zoonotic coronaviruses. (Cohen, A.; et al. *Science* **2021**, *371*, 735–741.)

### A Smartphone-Read Ultrasensitive and Quantitative Saliva Test for COVID-19

While SARS-CoV-2 has ravaged the world, assays are highly sought after to expand COVID-19 testing capacity. Ideally, these possess the following attributes: (1) easily collected samples, (2) have greater sensitivity than current methods, (3) allow viral quantification to monitor treatment, and (4) can be performed with little training to obtain reliable results. Although nasal and nasopharyngeal swabbing is the most common method of sample collection, saliva samples have good correlation with nasopharyngeal (saliva) levels (3.6- to 124-fold) compared with nasal samples. In 103 paired saliva and nasal samples, saliva samples identified higher numbers of positive cases (44 vs 28) and identified all the individuals detected by RT-qPCR with both saliva and nasal sample positive results. The authors then combined the CRISPR-FDS onto a microchip and developed a smartphone fluorescence microscope to read the assay. The integrated system used 0.5–3 mL of saliva, lysed, heated (>37 °C) before the sample was combined with RPA and the CRISPR reagents. The device exhibited linearity between 1 and 10^7 copies/µL with a load of detection (LOD) of 0.05 copies/µL. It also demonstrated equivalence with RT-qPCR. Saliva and nasal swab samples from 31 individuals demonstrated reasonable correlation too ($r^2 = 0.8029$).

CRISPR-FDS performed in seven rhesus macaque subjects with COVID-19 infection obtained higher oropharyngeal (saliva) levels (3.6- to 124-fold) compared with nasal samples. In 103 paired saliva and nasal samples, saliva samples identified higher numbers of positive cases (44 vs 28) and identified all the individuals detected by RT-qPCR with both saliva and nasal sample positive results. The authors then combined the CRISPR-FDS onto a microchip and developed a smartphone fluorescence microscope to read the assay. The integrated system used 0.5–3 mL of saliva, lysed, heated (>37 °C) before the sample was combined with RPA and the CRISPR reagents. The device exhibited linearity between 1 and 10^7 copies/µL with a load of detection (LOD) of 0.38 copy/µL using the smartphone device, giving a 1.3% false-positive rate with saliva from 103 blinded saliva samples.

Beyond the current iteration of the CRISPR-FDS, an on-chip process can be developed with sample barcoding, and the heating element can be controlled by a smartphone app with secure, wireless data reporting, which can support public telehealth efforts and facilitate the aggregation of data. Such convenient and integrated testing systems may improve disease surveillance and infected individual containment. (Ning, B.; et al. *Sci. Adv.* **2021**, *7*, eabe3703.)

### SARS-CoV-2 D614G Variant Exhibits Efficient Replication Ex Vivo and Transmission In Vivo

Mutant strains of SARS-CoV-2 are associated with increased pathogenicity. The D614G (Asp614→Gly) is associated
with higher viral loads in the upper respiratory tract, although without altered disease severity. In pseudotyped studies, the D614G substitution increased infectivity in cell lines and increased sensitivity to neutralization. Structural studies also suggest improved binding ability to the angiotensin-converting enzyme (ACE) 2 receptor. To further understand the acquisition of the D614G mutation, the authors generated a mutant containing the nanoLuciferase (nLuc) gene. To examine the effect of the mutation on SARS-CoV-2 cellular entry, the virus mutants were introduced to cells in culture media containing neutralizing antibodies (Abs). Acquiring the luciferase signals suggests that D614G led to a 3.7- to 8.2-fold higher expression compared with control cells, suggesting easier entry.

The mutant virus was then challenged in primary human nasal epithelial and large airway epithelial cells and small airway epithelial cells. The D614G mutant showed a higher viral titer compared with wildtype (WT) viruses. Competitive assays on large (proximal) airway epithelial (LAE) cells showed the dominance of D614G mutants after four continuous passages at 72 h intervals. These findings suggest that the D614G mutant enhances viral replication fitness in primary epithelial cells that strongly express the human ACE2 receptor. Electron microscopy further visualized virions on the surface of the primary human airway cells, although the number of spike proteins was not significantly different between the two viral strains and similar spike–nucleocapsid ratios from Western blot analysis. The D614G strain also did not alter neutralization effects from neutralizing monoclonal Abs from convalescent human serum.

The mutant strain was then challenged in hACE2 transgenic mice and Syrian hamsters. Analysis of lung lesions, inflammatory reactions, etc., suggests similar severity of both strains. In vivo, the D614G strain similarly became dominant after 3 days in hamster subjects but was not different in hACE2 mice. To examine mutant transmissibility, eight pairs of hamsters with a naive and infected animal were kept. After the monitoring period of 4 and 6 days, all naive subjects were infected as seen from positive nasal wash samples. However, at day 2, WT-infected hamsters had no infection and viral shedding, while it was detected in the D614G mutant group. This supports the notion that the mutant exhibits significantly faster transmission kinetics compared with WT in hamster subjects. The D614G mutant alters the spike into an “up” conformation to increase binding with ACE2 receptor binding and infectiousness. While increasing efficient entry, the virus titers did not increase in cell lines.

Histopathological samples revealed similar severity between WT and the mutant, but demonstrated greater transmissibility. This could be due to a lower minimum infectious dose between subjects or variations in moisture droplet transfer. Studies demonstrating differences in antibody neutralization between mutant and WT strains may render therapeutics and vaccines ineffective. This unequivocally demonstrates how mutant infectious strains are a major public healthcare risk worth monitoring closely. (Hou, Y.; et al. Science 2020, 370, 1464–1468.)

**Antibody-Like Proteins That Capture and Neutralize SARS-CoV-2**

A category of tools to help against the COVID-19 pandemic (and further pandemics) identify high-affinity antibodies (Abs) or Ab-like proteins (ALPs) against virus proteins and then develop suitable diagnostics and neutralizing proteins against them. Several approaches currently exist, including identifying cross-reactive antibodies, albeit with lower affinity. Isolating monoclonal Abs from infected patients is also another approach; however, significant resource challenges exist in collecting B cells and identifying and producing effective antibodies on a large scale. In vitro selection is advantageous, as it does not involve time-consuming animal immunization, and it is permissive for immunoglobulin proteins (single-chain variable fragments, single-domain Abs) and nonimmunoglobulin proteins (lipocalin, fibronectin domains, Z domains, ankynin repeat motifs, etc.). These increase the likelihood of obtaining high-affinity ALPs. The authors utilize a cell-free mRNA method to generate highly diverse protein libraries on the order of $10^{12}$ to $10^{13}$. These are larger than ALP libraries expressed in *Escherichia coli* with a range of $10^9$ to $10^{11}$.

Their modified mRNA method involves high-speed in vitro selection by adding peptide template DNA and skipping mRNA transcription and synthesizing protein/mRNA/puromycin linker (PµL) complexes. This method completed six rounds of selection in ~14 h and performed selection against vascular endothelial growth factor receptor 2 (VEGFR2), obtaining peptides with angiogenic inhibitory activity. This method, transcription–translation coupled with association of PµL (TRAP) display, identifies novel targets for human EGFR1 (HER1) and HER2 with a nanobody and a monobody as a backbone protein. This method was further applied to identify monobodies against the S1 subunit of the SARS-CoV-2 spike protein. To prevent promoter-independent transcription events, 2’-methoxy (OMe) modifications were used in the TRAP display. The mRNA/PµL complex formation efficiency increased from 11% to 60% (mPµL). Against EGFR1 and HER2, high-affinity binders were obtained from the monobody, whereas midaffinity ones were obtained from the nanobody library.

To construct the library, the authors focused on monobodies. They used the S1 subunit of the SARS-CoV-2 spike protein and the receptor-binding domain (RBD–horseradish peroxidase–biotin) before starting the TRAP display selection. In the first round, an mRNA template was used to maximize monobody library diversity. From the second round, the
TRAP display boosted selection speed, as well as selection against each target. The third round gave rise to binder monobodies against both targets. Three further rounds were performed at 10 times lower target concentration (2 nM) before a total of six rounds were performed. The monobodies were obtained by the fourth day. From the selection procedure, nine clones were obtained for further study, with the ability to bind to the S1 and RBD targets through an ELISA (enzyme-linked immunosorbent assay). Of the candidates, seven clones exhibited subnanomolar to nanomolar affinity against S1.

When tested against the S1 subunit of SARS-CoV, four monobodies did not bind. Another four bound to both targets, while one exhibited low affinity to both. The monobodies were then tested for their ability to interfere with the S1/ACE2 binding to mimic cell–virus interactions. ELISA results showed that monobody clones 4, 6, 9, and 10 inhibited S1 and ACE2 binding at a concentration of 100 nM. Clones 4, 6, and 10 further inhibited the interaction at a 10 nM concentration. Testing combinations of the monobodies in ELISA, the authors found that they recognized three epitopes, with 16 pairs for further ELISA. Among the clone candidates, clone 10 was used as a capture monobody and clone 12 as a detection monobody, giving rise to the highest signal-to-noise ratio using an ELISA format. The monobody candidates were then used for magnetic “pull-down” from cultivated samples, enhancing the limit of detection. At concentrations from 0.1 to 10,000 particles/µL, a linear dynamic range was observed when compared with PCR and the detection limit increased from 1 to 0.1 particles/µL.

Monobody pull-down of SARS-CoV-2 from patient samples was also successfully and significantly enhanced by the pull-down and demonstrated higher sensitivity compared with traditional quantitative real-time reverse transcription PCR (qRT-PCR). The monobody clone 6B was then found to inhibit SARS-CoV-2 infection, causing VeroE6 cells to release smaller amounts of SARS-CoV-2 mRNA found in supernatants using RT-qPCR. In its dimeric form, the neutralizing activity was further enhanced and comparable to high-affinity human neutralizing antibodies. This demonstrates the usefulness of the TRAP display in selecting highly-effective ALPs. Taking 3 days from receipt of the SARS-CoV-2 S1 subunit to identify the monobody sequences, the improved TRAP display is highly effective and speedy in identifying novel therapeutic biomolecules in emerging pandemics. (Kondo, T.; et al. Sci. Adv. 2020, 6, eabd3916.)

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