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LDRD Project Number: 223169
LDRD PROJECT TITLE: A targeted opsonization platform for programming innate immunity against rapidly evolving novel viruses
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ABSTRACT

Recent work has shown that artificial opsonins stimulate the targeted destruction of bacteria by phagocyte immune cells. Artificial opsonization has the potential to direct the innate immune system to target novel antigens, potentially even viral pathogens. Furthermore, the engagement of innate immunity presents a potential solution for the spread of pandemics in a scenario when a vaccine is unavailable or ineffective. Funded by the LDRD late start bioscience pandemic response program, we tested whether artificial opsonins can be developed to target viral pathogens using phage MS2 and a SARS-CoV-2 surrogate. To direct opsonization against these viruses we purified antibody derived viral targeting motifs and attempted the same chemical conjugation strategies that produced bacterial targeting artificial opsonins. However, the viral targeting motifs proved challenging to conjugate using these methods, frequently resulting in precipitation and loss of product. Future studies may be successful with this approach if a smaller and more soluble viral-targeting peptide could be used.

INTRODUCTION AND EXECUTIVE SUMMARY OF RESULTS:

Context of the ongoing pandemic:
The SARS-CoV-2 pandemic has disrupted global economies and healthcare systems for more than a year with currently no clear end in sight. Operation Warp Speed engaged private-public synergy and resulted in the fastest vaccine production and distribution to date. Currently, millions of Americans are fully vaccinated or have immunity granted by natural infection. Despite this, the high rate of mutation for SARS-CoV-2 has resulted in the evolution of variants that evade the immune system and enable it to persist in the population. There is clearly a need for novel therapeutics that combat viruses in new ways, especially those with the capability for facile adaptability so that medical scientists and physicians are better equipped to keep pace with the staggeringly rapid generation of immune-escaping viral progeny inherent in such viruses as SARS-CoV-2.
Immune response background, call for therapeutics that engage innate immunity :

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LABORATORY DIRECTED RESEARCH & DEVELOPMENT
WHERE INNOVATION BEGINS

When a body is exposed to a novel virus, the adaptive immune response requires weeks to generate antibodies that recognize and counter the pathogen [1]. This period of susceptibility is the primary reason why novel viruses can become pandemics. Currently our best countermeasure against novel viruses is to have prior exposure to the pathogen, either administration of vaccines or natural prior to exposure of the pathogen.

Both vaccination and prior natural exposure result in the buildup of pathogen-specific antibodies. However, when vaccines are unavailable or ineffective, there are typically no reliable therapies available to reduce the increasing numbers of virus. In this case, our first line of defense is the innate immune system [2]. A key component of innate immunity is opsonization, which is the targeting of immune cells to engulf and destroy invaders (phagocytosis). However, the inherent small size of viruses along with the extreme variability of viral surface features evades the innate immune response. Therefore, therapeutics that could direct the innate immune response against novel viruses would be valuable in contexts where there is no prior/adaptive immunity.

Artificial Opsonization, a new way to engage the innate immune response:

Recent work has shown that "artificial opsonins" stimulate the targeted destruction of bacteria by phagocyte immune cells [3, 4]. In this study, opsonins are produced by attaching many copies of

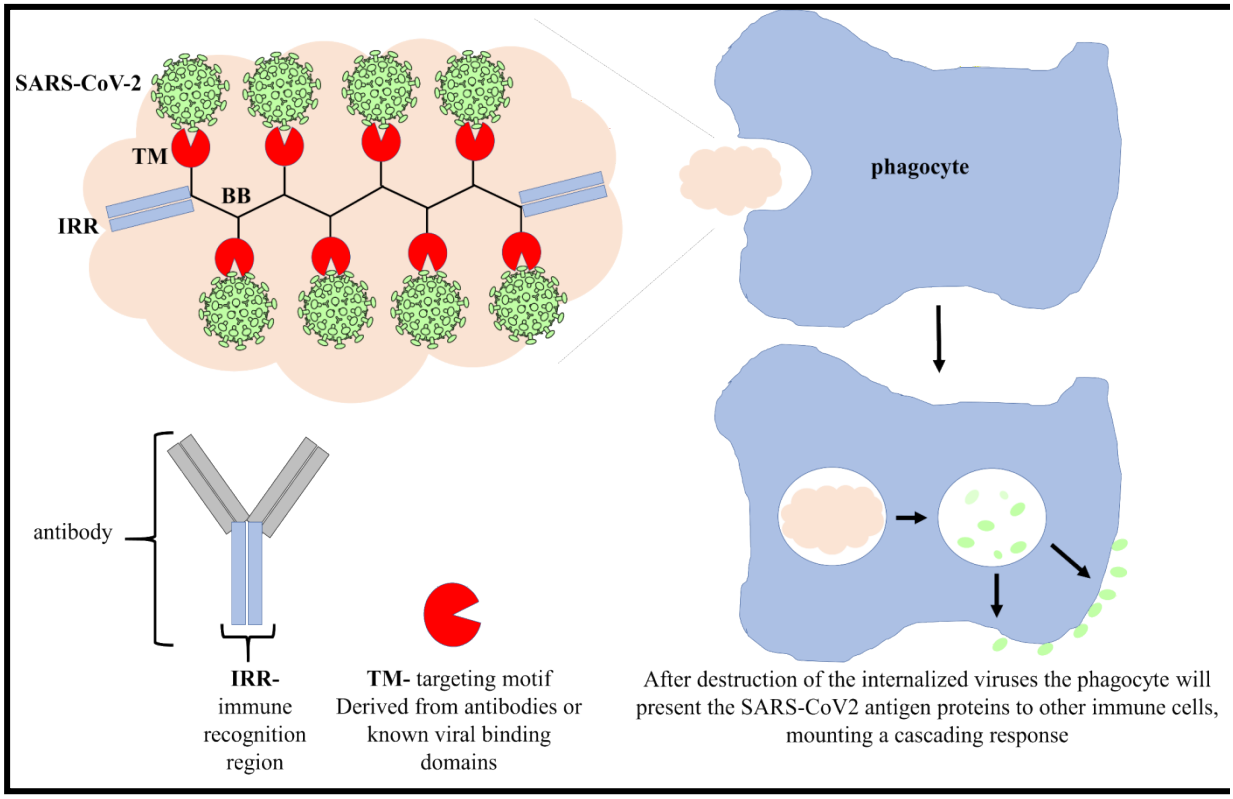


Figure 1. Artificial opsonin components and expected outcomes. Top left: the artificial opsonin bound to SARS-CoV-2. TM- targeting motif derived from antibody scFvs or nanobodies. BB- a PEG-polylysine graft designed to be a scaffold that presents the TM at high valency. IRR- the immune recognition region derived from human IgG-Fc. Right side of figure: phagocyte recruited to internalize opsonin. After destruction of the

a targeting motif (TM), which binds the pathogen, to an immune-inert chemical backbone (BB). This construct is covalently linked to an immune recognition region (IRR), which is an antibody fragment that directs phagocytes to engulf and destroy the target (Fig. 1). In the Katzenmeyer et al. study, multiple copies of vancomycin were used as the targeting motif. Vancomycin binds the cell wall of Gram-positive bacteria, specifically interacting with Ala-D-Ala linkages in the peptidoglycan. Nearly 100% of a clinically relevant *S. epidermidis* cells were opsonized in the 2011 paper and the 2017 study showed a biofilm forming strain of *S. epidermidis* lead to oxidative burst and chemokine production. Opsonins that were unbound to bacteria did not recruit neutrophil response. Taken together, this indicates that artificial opsonization may be a novel therapeutic strategy, and with simple changes could further be adapted to target viruses, if

viral binding protein (i.e., TM) is used instead of the vancomycin. Additionally expect that the IRR, like the TM, could be exchanged with other recognition regions that allow us to direct specific recruitment of different types of immune cells. Therefore, the "plug and play" modularity of the artificial opsonin technology could provide a platform for directing specific innate immune responses to counter novel pathogens.

Adapting artificial opsonization to target viruses:

Here, as a proof of concept, we sought to test whether this platform could be developed to target viral pathogens using phage MS2 and SARS-CoV-2. To direct opsonization to SARS-CoV-2, we obtained a purified a cameloid nanobody TM(s) that binds the surface-exposed spike protein on the novel CoV. For MS2 opsonization we obtained purified an anti-MS2 scFv.

DETAILED DESCRIPTION OF RESEARCH AND DEVELOPMENT AND METHODOLOGY:

Production of targeting motifs:

Two versions of the anti-MS2 scFv was produced by Genscript. In brief, *E.coli* BL21 Star™ (DE3) competent cells were transformed with the recombinant plasmid. A single colony was inoculated into LB medium containing kanamycin; cultures were incubated in 37 °C at 200 rpm. Once cell density reached to OD=0.6-0.8 at 600 nm, 0.5 mM IPTG was introduced for induction. SDS-PAGE and Western blot were used to monitor the expression.

Version 1:

Full length protein:

> U312PGD090-1_anti ms2 PelB_ss_scFv_corrected seq vector: pET30a

NdeI--ATG--**anti ms2 PelB_ss_scFv_corrected seq**--TEV--His tag--Stop codon--HindIII

Protein Length=290

MKYLLPTAAAGLLLLAAQPAMAAEVKLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQTPEKRLEWVATISTG
GGYTYFPDSVKGRFTISRDNAKNALYLQMKSLRSEDTAMYYCARQGDFGDWYFDVWGAGTTVTVSSGGGGSGGGGSG
GGGSTDVLMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVSNRFSGVPDRFSGSGS
GTDFTLKISRVEAEDLGVYFCSQSTHVPWTFGGGTKLEIKRGAAAENLYFQGGHHHHHH..

Version 2:

> U312PGD090-4_anti MS2 scFv vector: pET28a-MBP

His tag--MBP tag-- HindIII--Linker--TEV protease site--**anti MS2 scFv**--Stop codon--XhoI

Protein Length=655

MGSSHHHHHHGTKTEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHD
RFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAK
GKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNK
GETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKD
KPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTGTDYDIP
TTKLGSGSSGSGENLYFQG**SAEVKLVESGGGLVKPGGSLKLSCAASGFTFSSYAMSWVRQTPEKRLEWVATISTGGG
YTYFPDSVKGRFTISRDNAKNALYLQMKSLRSEDTAMYYCARQGDFGDWYFDVWGAGTTVTVSSGGGGSGGGGSGGG
GSTDVLMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGT
DFTLKISRVEAEDLGVYFCSQSTHVPWTFGGGTKLEIKR**. .

The SARS-CoV-2 targeting nanobody was produced and purified by ProMab and the sequence could be provided upon request and required NDA/IP approval.

Material acquisition:

Poly-L-lysine hydrobromide (MW 30,000-70-000), acid-dPEG$_5$-NHS ester, *N*-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), 10x phosphate buffered saline (PBS), protein standard mix 15-600 kDa, bovine serum albumin (BSA), and Amicon 10K MWCO centrifugal concentrators were all purchased from Millipore-Sigma. Econo-Pac 10DG desalting columns were purchased from Biorad, anti MS-2 scFV targeting motif from Genscript, nanobody from Promab, purified human IgG-Fc fragment from Bethyl Labs.

SEC Chromatogram:

Size exclusion chromatography was performed on an ÄKTA Start protein purification system using a HiLoad 16/600 Superdex 200 pg column. All purifications were performed in 1x PBS buffer with an injection volume of 1 mL and a flow rate of 1 mL/min.

UV- spectroscopy:

UV spectra were taken on a NanoDrop One spectrophotometer in Protein A280 mode with a sample volume of 2 uL.

Bioconjugation procedure:

For Bioconjugation reaction, the targeted protein (BSA or ScFv or Nanobody) was reacted with the NHS-terminated PEG moiety where amide bond will be formed with the reaction of available lysine groups. The free COOH end of the PEG moiety is further functionalized with NHS and EDC chemistry where because of the kinetics of the reaction the free end of PEG carboxylic acid can be easily functionalized. Excess NHS and EDC are removed via desalting column. This conjugated unit is further reacted with Polylysine (PLL) Unit to form the multivalent conjugate. The ratio of Polylysine to the conjugated unit is crucial to maintain the multivalency. Unreacted proteins or salts are removed by SEC chromatogram. This indiscriminate conjugate strategy ensures low valency of the multivalent conjugate, a crucial requirement to keep the multivalent species in solution.

Protocol for HL-60 positive control assay:

The following protocol was used to grow HL-60 neutrophils

Pilot growth assay.
Three days growth and count at day 3 and day 4 (Cells per mL) and fold change in cells/mL.

Preparation for experiment.

Day 1

Set up 3 flasks, seeded at 3 different HL-60 concentrations (2-fold difference, 5E5, 1E6, 2E6 cells per mL).
Grow in IMDM media supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.
Prepare to passage by dilution in fresh medium every 2–3 days.

Day 3 (PMA addition)

For experiments, plate 2E5 cells/well at the specified concentration in 24-well tissue culture polystyrene well-plates in medium supplemented with 100 nM phorbol-12-myristate-13-acetate (PMA;EMD Chemicals) to induce differentiation of precursor cells into neutrophil-like cells.
Incubate for 24 hours at 37°C with 5% CO_2 prior to performing an experiment.

Day 4. Media exchange
HL-60 neutrophil cells should be differentiated and adherent (check with phase contrast) at 2×10^5 cells/well in 24-well tissue culture plates. Remove PMA media and replace with fresh media.

Day 5. Experiment.

Opsonization of positive control

1. 1-10 µL of FITC-IgG beads (Cayman Chemical #500290) is added to neutrophils at 1:500 dilution In 50 µL tissue culture plates containing 0.5 mL of neutrophil suspension (2E5 cells/well)

a. Add Cytochalasin D to negative control wells at a final concentration of 10 μM to inhibit actin polymerization and prevent phagocytosis.

Measurement protocol 2 (trypan blue/flow) (well 3)

1. Rinse with cold HBSS to arrest phagocytosis (Centrifuge at 200xg 3 min), resuspending in 500 μL.
2. Add 0.4% Trypan blue (Jenn: 25uL of 0.4% to 96 wells ~0.1% final) to distinguish intracellular opsonins from exterior by quenching the extracellular fluorescence
3. Centrifuge at 500xg (5 min), image

RESULTS AND DISCUSSION:

Production of viral targeting motifs:

To facilitate MS2 targeting by the artificial opsonin we tried two versions to identify the best pilot expression strategies. Version one secretes the scFv to the periplasm using the PelB signal sequence. Version two uses maltose binding protein (MBP) as a solubility carrier to facilitate cytoplasmic expression. The solubility carrier is used to prevent formation of inclusion bodies since normally the oxidative environment of the periplasm is required for proper folding of scFvs. Version 2 resulted in greater amounts of soluble product and was selected for large culture volume over expression and purification on a Ni column. 3.8 mg was produced at a concentration of 0.38 mg/mL and a purity of >80%. 10 mL (22 mg total) of the SARS-CoV-2 nanobody was produced at 90% purity (not shown).

-1: anti ms2 PelB_ss_scFv_corrected seq (~30.864 kDa)_BL21 Star™ (DE3)

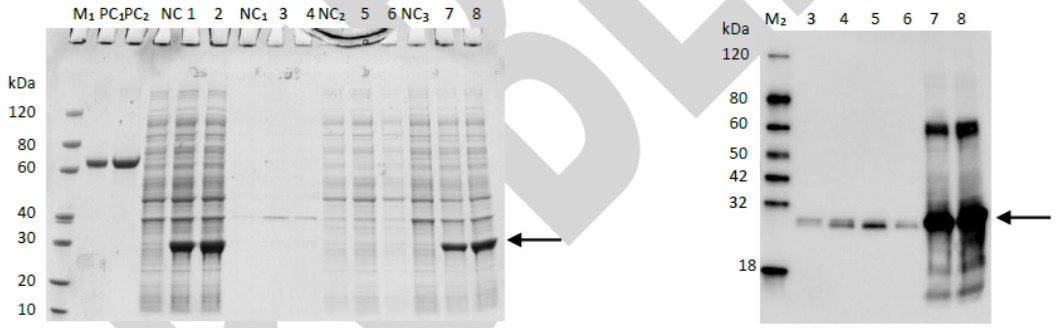


Fig.1 SDS-PAGE (left) and Western blot (right, using anti-His antibody (GenScript, Cat.No.A00186)) analysis of anti ms2 PelB_ss_scFv_corrected seq in *E.coli* expression in construct pET-30a(+).

Lane M_1: Protein marker
Lane M_2: Western blot marker
Lane PC_1: BSA (1 µg)
Lane PC_2: BSA (2 µg)
Lane NC: Cell lysate without induction
Lane 1: Cell lysate with induction for 16 h at 15 °C
Lane 2: Cell lysate with induction for 4 h at 37 °C
Lane NC_1: Periplasmic space of cell without induction
Lane 3: Periplasmic space of cell with induction for 16 h at 15 °C
Lane 4: Periplasmic space of cell with induction for 4 h at 37 °C
Lane NC_2: Supernatant of cell lysate without induction
Lane 5: Supernatant of cell lysate with induction for 16 h at 15 °C
Lane 6: Supernatant of cell lysate with induction for 4 h at 37 °C
Lane NC_3: Pellet of cell lysate without induction
Lane 7: Pellet of cell lysate with induction for 16 h at 15 °C
Lane 8: Pellet of cell lysate with induction for 4 h at 37 °C

Figure 1. Expression of anti-ms2 scFv, version 1

-4: anti MS2 scFv (~70.894 kDa)_BL21 Star™ (DE3)

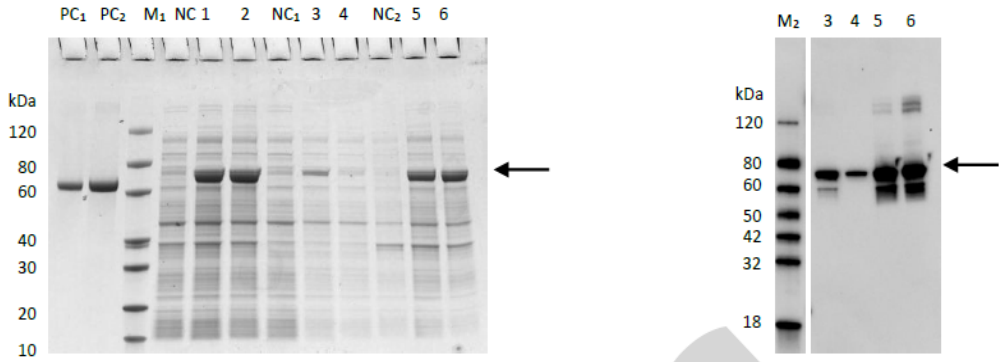


Fig.2 SDS-PAGE (left) and Western blot (right, using anti-His antibody (GenScript, Cat.No.A00186) or using Mouse-anti-MBP mAb (GenScript, Cat.No.A00190)) analysis of anti MS2 scFv in *E.coli* expression in construct pET28a-MBP.

Lane M_1: Protein marker
Lane M_2: Western blot marker
Lane PC_1: BSA (1 μg)
Lane PC_2: BSA (2 μg)
Lane NC: Cell lysate without induction
Lane 1: Cell lysate with induction for 16 h at 15 °C
Lane 2: Cell lysate with induction for 4 h at 37 °C
Lane NC_1: Supernatant of cell lysate without induction
Lane 3: Supernatant of cell lysate with induction for 16 h at 15 °C
Lane 4: Supernatant of cell lysate with induction for 4 h at 37 °C
Lane NC_2: Pellet of cell lysate without induction
Lane 5: Pellet of cell lysate with induction for 16 h at 15 °C
Lane 6: Pellet of cell lysate with induction for 4 h at 37 °C

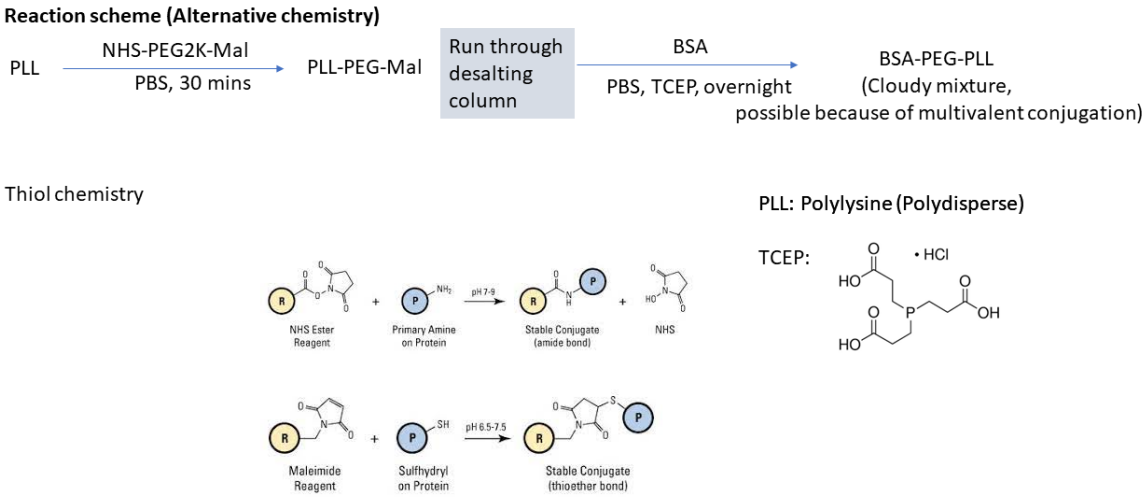
Figure 2. Expression of anti-ms2 scFv, version 2

Bioconjugation of targeting motifs (TMs) and IgGs to produce artificial opsonins:
Protein functions can be integrated and enhanced by conjugating a number of protein molecules into one, due to synergistic and multivalent effects. With a precise control of ligand number, density, and three-dimensional arrangement, the multivalent display provide a great opportunity to study the binding affinity of multiple ligands on one molecular entity to receptors.[5] Multivalent representation of proteins, however, is not trivial due to the choice of proper scaffold and solubility issues. Due to the time-constraint and supply-chain disruption we adopted an

indiscriminate conjugation strategy in contrast to the site-specific[6] conjugation strategy. The other underlying rationale is to establish the proof-of-concept work to lay the groundwork for more time consuming efforts around more sophisticated conjugation chemistries.

We have attempted an artificial opsonin constructed from multivalent conjugates of poly(L-lysine)-graft-poly(ethylene glycol) with ScFv (PLL-PEG-ScFv) and human IgG-Fc (PLL-ScFv-IgG). We also attempted a multivalent construct out of nanobody (NB-PEG-PLL) and with IgG (PLL-NB-IgG). As a negative control, we synthesized the bovine serum albumin (BSA) analog BSA-PEG-PLL and BSA-PLL-IgG.

As a conjugation strategy, we have attempted two different procedures. The first procedure based on thiol chemistry as described [3, 4] by Katzenmeyer and Bryers was ineffective in our case as the multivalent conjugates formed was not soluble in the reaction medium and cannot be solubilized with DI water. Scheme 1 outlines the approach.

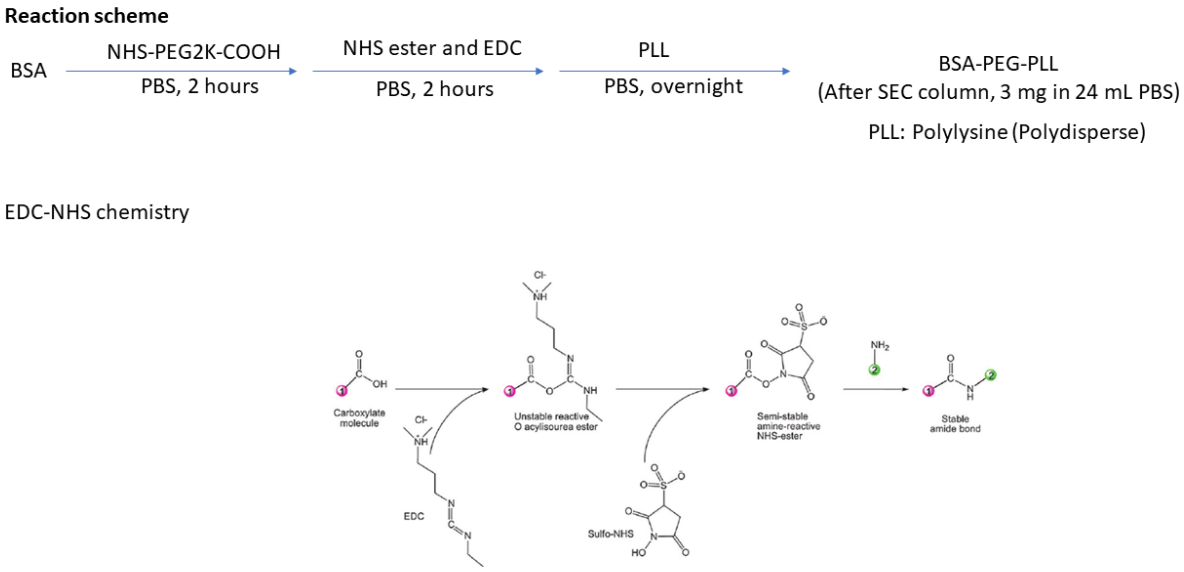


Scheme 1: The thiol chemistry adopted for multivalent constructs

After multiple rounds of experimentation, we settled with EDC-NHS chemistry[7, 8] as described in the Scheme 2. We are able to show successful BSA-PEG-PLL and BSA-PLL-IgG construct as purified by SEC chromatogram (Figure 3).
The conjugation attempt to construct the anti-MS2 scFv was not successful as we lost the protein to the Amicon filter during the buffer exchange. ScFv is prepared in Tris buffer, which is not an ideal medium for the bioconjugation chemistry we adopted in PBS solution. To change the buffer from Tris-HCl to the PBS buffer, we used the Amicon filter for diafiltration. The losses are

possibly due to the hydrophobicity of scFv or affinity for the filter. Multiple attempts to recover the protein was unsuccessful.

The other round of conjugation was attempted with SARS-CoV-2 Nanobody. The same bioconjugation protocol as described in the Scheme 2 was adopted. Due to the lower extinction coefficient of the nanobody construct, we relied on the PLL-NB-IgG extinction coefficient as our guiding principle to collect PLL-NB-IgG.x



Scheme 2. EDC-NHS chemistry adopted for multivalent constructs

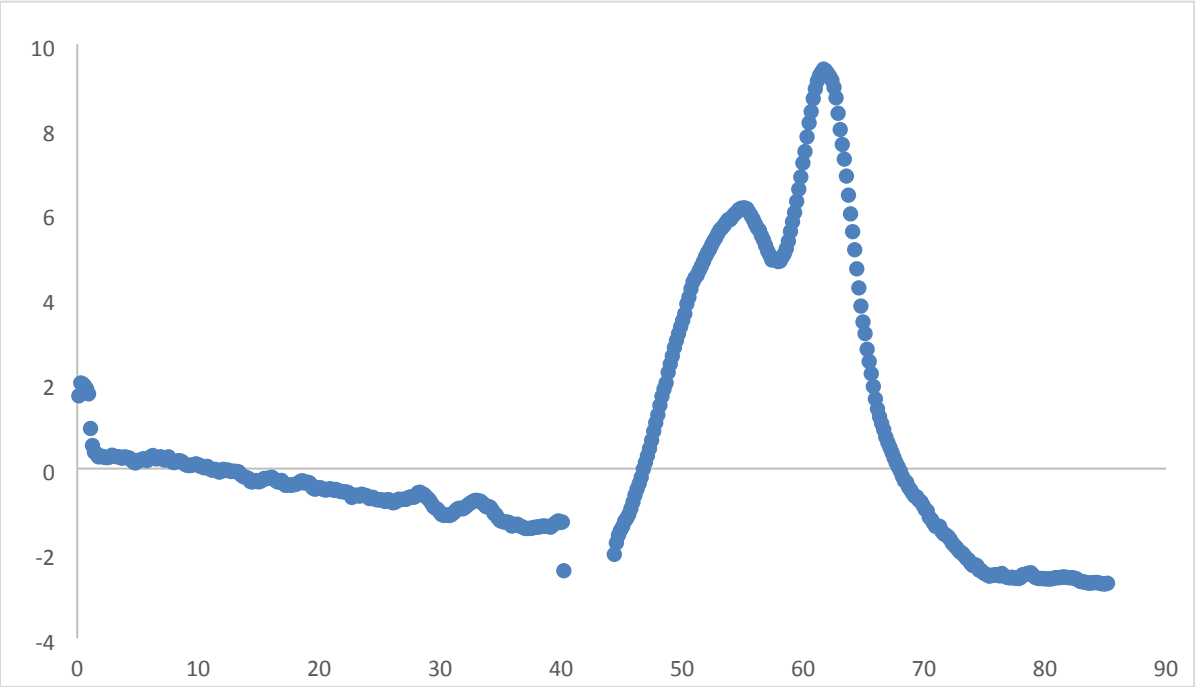


Figure 3. SEC Chromatogram showing the multivalent BSA conjugates

Positive control phagocytosis assay: growing neutrophils and opsonizing IgG-FITC beads

To prepare for testing whether the artificial opsonins stimulate neutrophil phagocytosis we cultured and differentiated HL-60 cells and tested the efficiency of opsonization of IgG-FITC-labeled beads. The first test was done using a co-culture of VEROS cells (used to help CX7 imager obtain focal plane). HL-60s were PMA treated to induce differentiation into neutrophil-like cells. Test groups included PMA treated, un-treated, and cells treated with phagocytosis inhibitor cytochalasin-D. Surprisingly all test groups showed evidence of phagocytosis (data not shown) for unknown reasons. A subsequent test showed that PMA treatment resulted in damaged cells. It was not clear why the same test on different days yielded different results.

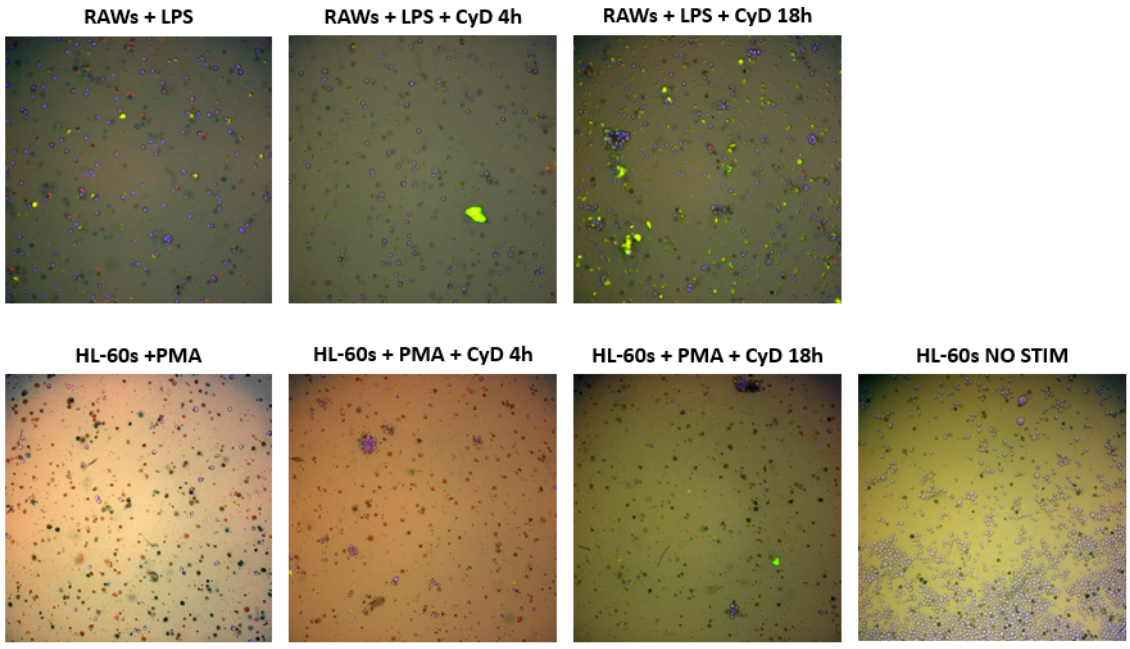


Figure 4. Comparison of HL-60 cells with/without PMA treatment to RAWs cells

Taken together, this suggests that the HL-60 neutrophils may be sensitive to small changes in handling and/or the methods used to prepare these cells are not-yet optimized.
One last test, including the SARS-CoV-2 targeting opsonins is planned for the last funding day of this project.

ANTICIPATED OUTCOMES AND IMPACTS: This should be > 700 words (no upper limit) without any addendum materials. If the impact is well articulated in the Addendum section, then the word count can be reduced to >300 words. The impact should include a description of next step(s), summary of publications and anticipated publications, conference

presentations, potential new R&D deriving from what was learned, IP development, potential impact and path forward for NNSA and DOE, etc.

The challenges we faced with producing and conjugating scFvs and nanobodies essentially halted progress on this project. As such we have no IP development, derivative R&D, presentations or expected publications at this time. We feel that we formed a strong team that would be well positioned to tackle projects converging on conjugation, immunology, and virology. We suggest that the next steps for this project should be exploring the identification and isolation of new small soluble peptides that bind the surfaces of viruses. Potentially, other paths forward include troubleshooting strategies that could make conjugation (site specific conjugation strategies) or protein concentration more facile.

CONCLUSION:

We faced unexpected challenges on this project that included the difficulty in producing high quantities of viral-targeting motifs and difficulties conjugating sufficient quantities of these targeting motifs to the PEG-polylysine backbones. These steps are essential for producing artificial opsonins and testing whether this technology could be deployed against viruses. Another unexpected challenge was the difficulty in handling HL-60 neutrophils. Despite our team's expertise in immunology and cell culturing, these proved much more difficult to work with compared to VERO or RAWS cells. Our team feels that given more time we would be able to troubleshoot and solve both the above challenges. However, these setbacks delayed us to the end of our funding period and did not facilitate further investigation. We suggest that the major limiting step for this technology is the lack of small soluble viral targeting peptides. It would be especially advantageous if such peptides can be expressed using bacterial cytoplasmic expression strategies, unlike scFvs and nanobodies which require disulfide chemistry (periplasmic expression) for proper folding. If more facile methods for targeting viruses are identified, it would support further investigation of whether artificial opsonization can be used against viruses.

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