Polymer Modification of SARS-CoV-2 Spike Protein Impacts its Ability to Bind Key Receptor

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ABSTRACT. The global spread of SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2) has caused the loss of many human lives and severe economic losses. SARS-CoV-2 mediates its infection in humans via the spike glycoprotein. The receptor binding domain of the SARS-CoV-2 spike protein binds to its cognate receptor, angiotensin converting enzyme-2 (ACE2) to initiate viral entry. In this study, we examine how polymer modification of the spike protein receptor binding domain impacts binding to ACE2. The horseradish peroxidase conjugated receptor binding domain was modified with a range of polymers including hydrophilic *N,N*-dimethylacrylamide, hydrophobic *N*-isopropylacrylamide, cationic 3-(*N,N*-dimethylamino)propylacrylamide, and anionic 2-acrylamido-2-methylpropane sulfonic acid polymers. The effect of polymer chain length was observed using *N,N*-dimethylacrylamide polymers with degrees of polymerization of 5, 10 and 25. Polymer conjugation of the receptor binding domain significantly reduced the interaction with ACE2 protein, as determined by an enzyme-linked immunosorbent assay. Stability analysis showed that these conjugates remained highly stable even after seven days incubation at physiological temperature. Hence, this study

provides a detailed view of the effect specific type of modification using a library of polymers with different functionalities in interrupting RBD-ACE2 interaction.

KEYWORDS. Spike protein, RAFT, Grafting to, Protein-polymer conjugation, protein-protein interactions

Novel coronavirus disease 2019 (COVID-19) has become a global pandemic with over 535 million cases and more than 6.3 million deaths as of June 19 , 2022. [1]Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of COVID-19.[2, 3] SARS-CoV-2 is an enveloped positive-sense, single-stranded RNA virus and belongs to the β-coronavirus genus in the coronavirus family.[4] It shares high genetic sequence identity with SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV).[4] The virus is transmitted person to person[5] and includes a diverse clinical manifestation ranging from mild cases to severe cases, with a mortality rate between 9.3% to 19.7% in 2020 of hospitalized patients.[3, 6, 7]

SARS-CoV-2 consists of four structural proteins, the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The S, E, and M proteins are primarily involved in virus assembly and host cell entry while the N protein is needed for RNA synthesis.[8-10] The S protein on the surface of SARS-COV-2 protein consists of two functional units which includes a receptor binding domain (RBD) containing S1 unit and the membrane fusion domain containing S2 unit.[11, 12] The infection of coronavirus into host cells is initiated by binding of the S1 RBD to the cellular surface receptor angiotensin-converting enzyme 2 (ACE2).[13] The interaction between the RBD of SARS-CoV-2 and ACE2 has been reported to be between 5 and 20 times stronger than for SARS-CoV due to significant increases in salt-bridges and hydrogen bond interactions between these proteins. [14, 15] Subsequently, the S2 unit fuses the host cell and viral membranes to enable transfer of the viral genome into the host cells. Hence, the interaction between spike RBD protein and ACE2 receptor

plays a significant role for viral entry into host cells.[16-18] Therefore, inhibiting the interaction between RBD and ACE2 protein could be used to prevent SARS-CoV-2 infection.[19, 20]

Control over polymer structure through reversible deactivation radical polymerization (RDRP)[21] methods such as atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer polymerization (RAFT) has enabled important biochemical applications and biomaterials.[22-26] The ability to control primary polymer structure across a range of biologically compatible functional groups is a significant strength of RAFT and ATRP. These polymers have been conjugated to various proteins for both biocatalytic and pharmaceutical applications.[27-31] Polymers can be attached to proteins using several strategies including grafting-to, where preformed polymers are attached to proteins, and grafting-from, where polymers are grown off the protein surface from an initiating or transfer agent.[32] Due to the changes in overall biohybrid structure upon bioconjugation, polymer bioconjugation is an effective way of modulating protein performance.

Several molecules have been targeted to the S protein *in vitro*. The protease inhibitor camostat mesylate and cathepsin L inhibitor E-64d have been shown to block SARS-CoV and SARS-CoV-2 cellular entry.[13] SARS-CoV-2 entry into 293/hACE2 cells are also found to be reduced by a potent inhibitor of phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), Apilimod.[13, 33] Moreover, SARS-CoV-2 S protein mediated cell-cell fusion has been prevented by fusion inhibitors EK1C4,[34] IPB02[35] and nelfinavir mesylate.[36] The SARS-CoV-2 S protein has also shown to be inhibited by several SARS-CoV specific neutralizing antibodies including S309, m396 and CR3022.[4, 37] However, these antiviral agents are associated with disadvantages such as toxicity, short half-life, or acute side effects. These limitations may interrupt their further application in clinical settings, creating an urge to find new and effective therapeutics to treat COVID-19. Recently,

bioconjugation has been used for diagnostic, therapeutic as well as vaccine development. In diagnostic applications, one research group showed that SARS-CoV-2-based peptide conjugation with polyacrylamide polymers could be used to detect antibodies.[38] Antibodies specific for SARS-CoV-2 has also been conjugated with gold nanoparticles for SARS-CoV-2 spike protein detection.[39] Bioconjugation approaches for SARS-CoV-2 also have potential therapeutic application. One study has showed that the lipid conjugation of a peptide derived from the C-terminal heptad repeat of SARS-CoV-2 as a promising candidate for SARS-CoV-2 infection.[40] Conjugation of neutralizing antibodies with nanoparticles has also shown potential in inactivating SARS-CoV-2.[41] Additionally, bioconjugation methods can facilitate SARS-CoV-2 vaccine development.[42-45]

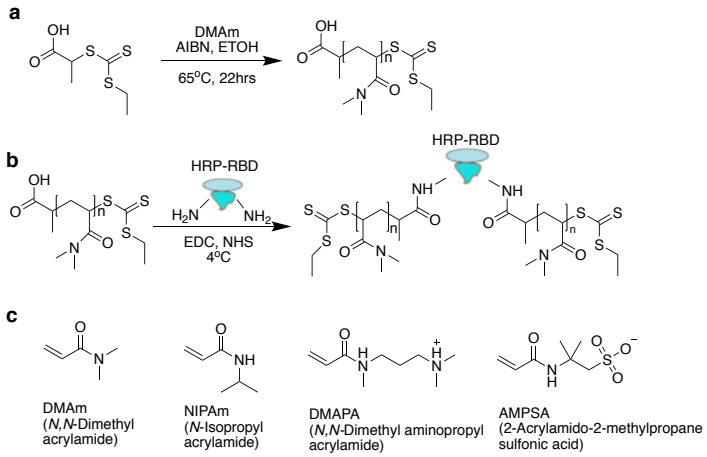
However, understanding how synthetic modification of the RBD impacts its ability to interact with the ACE2 protein is important for downstream inhibition of SARS-CoV-2 infection. If small or minor modifications causes significant reductions in RBD binding to ACE2, then developing future inhibitors that target the RBD-ACE2 interaction *in-vivo* could be a fruitful pathway to new therapeutics against COVID-19 infections.[46] This is because if the binding of RBD to ACE2 is sensitive to changes in the RBD structure or surface features, future therapeutics can target the RBD either through covalent or non-covalent approaches, potentially leading to inhibition of the infection cycle. Polymer modification is an excellent model system to study the impact of modification size and structure, due to the ability to fine tune polymer functionality and molecular weight, through techniques such as RAFT.

In this work, the RBD of spike protein was conjugated with synthetic polymers of different hydrophobicity, charge and chain length using a 'grafting-to' approach. Conjugation with these polymers significantly reduced the interaction between spike RBD protein and ACE2 protein,

suggesting that targeting the interactions between the spike protein and ACE2 or even covalent modification of the S protein could be a fruitful approach. Ongoing efforts to prevent coronavirus impact primarily focused on developing vaccines and therapeutics. However, developing effective therapeutics against SARS-CoV-2 requires a detailed understanding of how modification of SARS-COV-2 protein impacts its interactions with ACE2. Polymers offer an appealing platform from which functionality and size of the attached groups can be systematically varied in a facile manner. From this perspective, our study can provide valuable insight into the development of future inhibitors using downstream modification, by highlighting the sensitivity of the RBD-ACE2 binding to modification and perturbations in the RBD.

Initially, a series of hydrophilic and hydrophobic polymers were synthesized to conjugate with spike protein RBD. RAFT was used with the chain transfer agent 2-(((ethylthio)-carbonothioyl)thio propionic acid (PAETC) (Scheme 1a & 1c).[47, 48] Initially, polymers with a targeted degree of polymerization of 25 units were prepared with differing hydrophilicity *i.e.*, hydrophilic *N,N*- dimethyl acrylamide (DMAm) and hydrophobic *N*-isopropyl acrylamide (NIPAm). Generally, hydrophilic polymers tend to form noncovalent hydrogen bond interactions with amino acid residues at the protein surface while hydrophobic polymers tend to reduce the solubility of protein.[47, 48] Additionally, to investigate the impact of charge properties of polymers on bioconjugate performance, cationic *N,N*-dimethyl aminopropyl acrylamide (DMAPA) and anionic 2-acrylamido-2-methylpropane sulfonic acid (AMPSA) monomers were also incorporated into the DMAm polymers in a 1:1 ratio with a targeted degree of polymerization of 25. The ionizable group containing polymers are predicted to strongly interact with the protein through electrostatics, with a smaller tendency to form hydrogen bonds with the protein.[48] Moreover, to explore the effect of chain length of polymers on the interaction between RBD and ACE2 protein,

DMAm polymers were also synthesized in two additional lengths, DP5 and DP10. The synthesized polymers were characterized using ^{1}H NMR and IR spectroscopy (Figure S1 and S2) as well as size exclusion chromatography to observe monomer conversion and molecular weight distribution respectively (Figure S3 and Table S1).



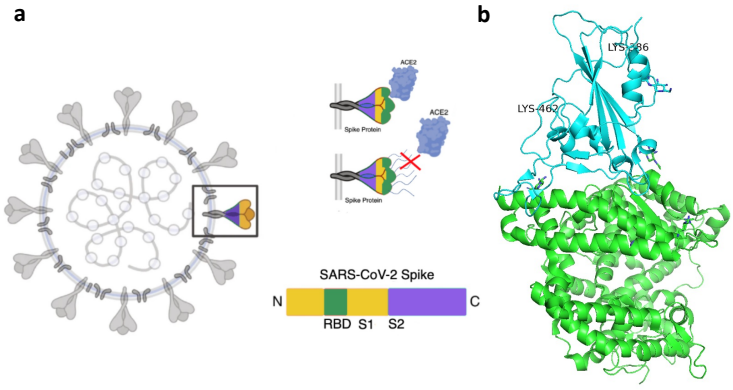
Scheme 1. (a) RAFT polymerization of DMAm. (b) Conjugation of pDMAm to the HRP linked spike RBD protein via a "Grafting to" approach. (c) Respective hydrophilic DMAm, hydrophobic NIPAm, cationic DMAPA and anionic AMPSA monomers used in this study.

This study used a SARS-CoV-2 Spike RBD-ACE2 blocking antibody detection (enzyme-linked immunosorbent assay (ELISA) kit. The kit provides a solution phase sample of the RBD of spike protein linked with horseradish peroxidase enzyme (RBD-HRP) and a 96-well plate coated with ACE2 protein. Each polymer was conjugated to the HRP linked RBD protein using an in situ 1-ethyl-3-dimethylamino)propyl carbodiimide, hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) coupling reaction.[48, 49] The spike RBD domain comprises 8 lysine residues with an additional N-terminal amine group.[50] Horseradish peroxidase enzyme comprises 6 lysine residues.[51] The ratio of protein to polymer for conjugation was maintained at 1:20. The conjugation reaction is illustrated in scheme 1b. Accordingly, conjugation was confirmed using sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE), which showed a complete conjugation of polymer to protein (Figure S4). We also explored the conjugation efficiency to HRP alone using the similar conjugation conditions. There was negligible conjugation of the polymer to the isolated HRP protein (Figure S5), indicating that all polymers will be attached to the RBD section of RBD-HRP.

To characterize the conjugates, a centrifugal ultrafiltration protocol was used to determine the conjugation efficiency by taking UV-vis spectra of conjugates before and after filtration. This technique was able to remove excess polymer from the conjugate solution, as seen by the purification of a polymer only solution at the same polymer concentration used in conjugation (Figure S6). The UV-vis spectra of all conjugates before purification were dominated by polymer end group (~306 nm) indicating a substantial amount of free polymer present in the sample. In contrast, the UV-vis spectra of conjugates after purification showed substantial reduction of absorbance at 306 nm indicating removal of free polymers from the sample. Hence, the absorbance ratio, 280:306 nm was calculated to quantify the grafting density of polymers on protein surface. The ratio indicated covalent binding of ~2 polymers on protein surface (Table S3 & Figure S7). The lysine reactivity of spike RBD protein was also determined using a workflow described in Carmali et *al.* (2017).[52] Following this workflow, the structure of novel coronavirus spike receptor-binding domain in complex with its receptor ACE2 (Protein Data Bank Identification: 6LZG)[50] was used to calculate the solvent accessible surface area using GETAREA. Additionally, PROPKA[53, 54] was used to estimate pKa values, and Adaptive Poisson-Boltzmann Solver[55] to generate electrostatic map. From this analysis, it was predicted that 2 of the 8 lysine residues, Lys462 and Lys386, present in the RBD are fast reacting. Of these, Lys462 is located about 25 Å from the ACE2 binding site and Lys386 is located about 37 Å from the ACE2 binding site.

Therefore, it might be plausible that modification at Lys462 could lead to inhibition of interaction of the spike-RBD with ACE2 (Scheme 2b).



Scheme 2. (a)Spike RBD-ACE2 interaction inhibition via conjugation with polymers and (b)Spike receptor-binding domain complexed with its receptor ACE2 (PDB ID:6LZG).

This work used ELISA to evaluate the interaction of the polymer modified RBD with ACE2. For the assay, native SARS-CoV-2 S1 RBD linked to HRP (RBD-HRP) and its conjugates with different polymers i.e., $pDMAm_{25}$, $pDMAm_{10}$, $pDMAm_{5}$, $pDMAm_{12.5}$-$DMAPA_{12.5}$, $pDMAm_{12.5}$-$AMPSA_{12.5}$, and $pNIPAm_{25}$ were incubated with sample diluent for 1 hour at 37 ℃. The incubated samples were then added to microwell plates coated with ACE2 protein. The polymer modified RBD-HRP will bind with the ACE2 protein to varying degrees depending on the polymer modification of the RBD. Followed by incubation for 1 hour at 37 ℃ and removal of unbound RBD-HRP in and its conjugates after washing, the colorimetric HRP substrate TMB (3, 3', 5, 5'-tetramethylbenzidine) was added. The addition of TMB, followed by stop solution forms a yellow color and the measured absorbance at 450 nm is proportional to the ability of the native or modified RBD to interact with the ACE2 protein. For example, if polymer conjugation does not block the ACE2 binding surface of the RBD, it will bind with the ACE2 protein coated in the well and yield

a high signal. Conversely, if polymer conjugation occludes the ACE2 binding surface, RBD will not be able to bind with ACE2 protein, hence will yield low signal (Scheme S1).

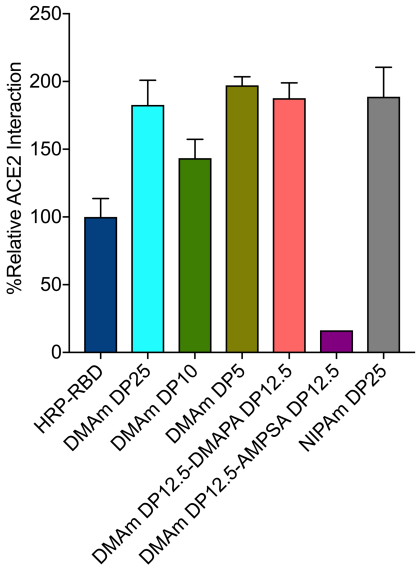


Figure 1. Percent relative ACE2 interaction of HRP linked spike RBD protein in presence of DMAm DP25, DMAm DP10, DMAm DP5, DMAm DP12.5-DMAPA DP12.5, DMAm DP12.5-AMPSA DP12.5 and NIPAm DP25 polymers. The percent relative ACE2 interaction is determined as $\%\, relative\ ACE2\ interaction = \left(\frac{\frac{ACE2\ interaction\ signal\ of\ sample}{HRP\ activity\ signal\ of\ sample}}{\frac{ACE2\ interaction\ signal\ of\ wild\ type}{HRP\ activity\ signal\ of\ wild\ type}}\right) \times 100\%$. Each column represents the average of triplicates$\pm$SD.

Initially, the potential for free polymers in solution to inhibit the interaction of HRP linked RBD protein with ACE2 protein was evaluated. RBD-HRP protein equilibrated with the concentration of polymers that was used in their conjugation was incubated in the wells containing ACE2. The data in Figure 1 indicate that free polymers appear to enhance the interaction of RBD-HRP with ACE2, possibly because of molecular crowding effect.[56, 57] The high concentration of the polymer in solution may occupy a larger proportion of volume in solution, hence reduces the available

volume of solvent for RBD-HRP protein. This may increase the effective concentration of RBD-HRP protein, favoring the association of RBD-HRP protein with ACE2 protein. An exception was observed for DMAm DP12.5-AMPSA DP12.5 polymer which may have a denaturing effect on the protein. Previous studies indicate that anionic polymers tend to unfold protein structure, which agrees with this observation.[47, 52]

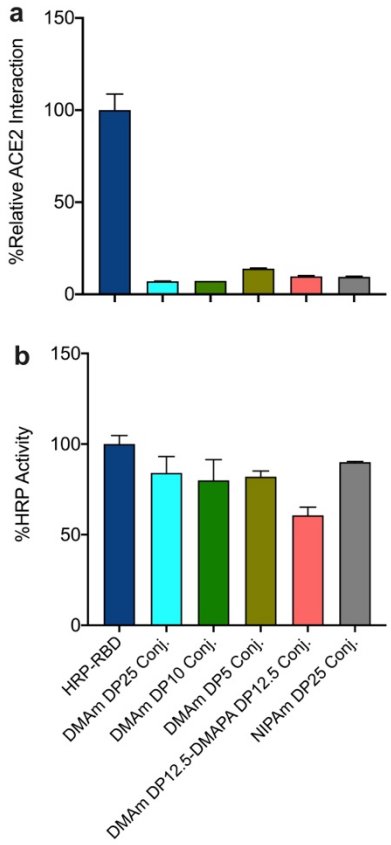


Figure 2. (a)Percent relative ACE2 interaction of HRP linked spike RBD protein and its conjugates with DMAm DP25, DMAm DP10, DMAm DP5, DMAm DP12.5-DMAPA DP12.5 and NIPAm DP25 polymers. (b) Percent HRP activity of HRP linked spike RBD protein and its conjugates with DMAm DP25, DMAm DP10, DMAm DP5, DMAm DP12.5-DMAPA DP12.5 and NIPAm DP25 polymers. In (a) and (b), the percent relative ACE2 interaction is determined as

$\%\ relative\ ACE2\ interaction = \left(\frac{\frac{ACE2\ interaction\ signal\ of\ sample}{HRP\ activity\ signal\ of\ sample}}{\frac{ACE2\ interaction\ signal\ of\ wild\ type}{HRP\ activity\ signal\ of\ wild\ type}}\right) x\ 100\%$ and the percent HRP activity was determined as *%HRP activity*= $\frac{HRP\ activity\ of\ sample}{HRP\ activity\ of\ wild\ type}\ x\ 100\%$. Each column in (a) and (b) represents the average of triplicates±SD.

The ACE2 interaction assay for the conjugates showed considerable inhibition of the binding between RBD-HRP and ACE2, thereby indicating probable occlusion of the ACE2 binding site within the RBD-HRP protein due to polymer conjugation (Figure 2a). All systems showed at least an 80% reduction in binding efficiency between HRP linked RBD protein and ACE2, with up to 95% reduction in RBD affinity to ACE2. As a control study, an HRP activity assay was used to evaluate conjugate's HRP catalytic performance regardless of ACE2 interaction.

The HRP based control study showed that even though the binding between RBD-HRP and ACE2 was significantly altered, the HRP activity was essentially unaffected in all cases (Figure 2b). This suggests that polymer conjugation did not denature the HRP as the enzyme was still active and able to generate a robust colorimetric response. However, the conjugation with charged polymer, including cationic polymer, DMAm DP12.5-DMAPA DP12.5, and anionic polymer, DMAm DP12.5-AMPSA DP12.5 resulted in almost 40% and 65% loss of HRP activity, respectively (Figure S10).

This was consistent with this polymer control assay (Figure 1) where the anionic polymer may induce unfolding of protein structure, reducing activity, and binding profiles. Additionally, chain length effects on HRP activity and RBD-HRP binding to ACE2 were probed using three different lengths $pDMAm_5$, $pDMAm_{10}$ and $pDMAm_{25}$. Interestingly, the inhibitory effect on HRP linked RBD protein and ACE2 interaction becomes more prominent with longer chain lengths. However,

it is important to note that a significant reduction of RBD-HRP binding to ACE2 occurs even with the smallest length of the polymer, i.e., $pDMAm_5$, which showed greater than 85% reduction in binding of RBD-HRP with ACE 2. Since these conjugations were performed targeting the lysine residues of RBD protein, and the lysine residues predicted to be fast reacting are in close proximity with the ACE2 binding site (Lys462 and Lys386 are 25 Å and 37 Å away from the ACE2 binding site respectively), these conjugations are likely to have a prominent effect on inhibiting ACE2 interaction. To further investigate chain length effects, we wanted to conjugate the chain transfer agent, 2-(((ethylthio)-carbonothioyl)thio) propionic acid (PAETC). Unfortunately, significant protein loss was observed from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis and %HRP activity assay (Figure S11a and S11b). This is most likely due to the PAETC conjugation increasing the hydrophobicity of conjugates and caused protein precipitation (Figure S11a & S11b).

The likely mechanism by which the conjugates, with the exception of DMAm DP12.5-AMPSA DP12.5, act is through sterics. As identified by distance estimates, Lys462 and Lys386 are close to the ACE2 binding site of RBD. Attaching synthetic polymers at these Lys462 or Lys386 close to the ACE2 binding site, regardless of functionality, could lead to steric occlusion and reduced binding between RBD and ACE2. It is notable that the results are consistent regardless of whether charged, uncharged hydrophilic or hydrophobic functionality is present in the polymer. The inability for a wide range of free polymers at high concentration to inhibit the RBD/ACE2 interaction further suggests that specific polymer-RBD interactions were not present. Together these data support the steric occlusion mechanism.

This also suggests that for downstream applications and inhibitors of RBD, a viable pathway is to attach either relatively small molecules (molecular weight ~500) through to large antibodies near the binding site of RBD and provide steric occlusions that limit RBD and ACE2 interactions.

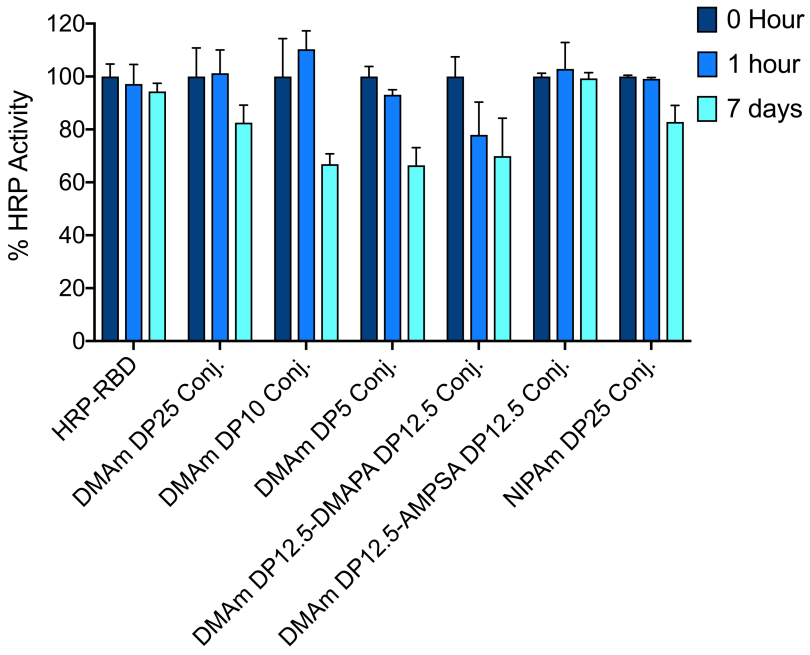


Figure 3. Percent HRP activity of spike RBD-HRP linked protein and its conjugates with DMAm DP25, DMAm DP10, DMAm DP5, DMAm DP12.5-DMAPA DP12.5, DMAm DP12.5-AMPSA DP12.5 and NIPAm DP25 polymers followed by incubation at 0 hour, 1 hour and 7 days. The percent relative ACE2 interaction is determined as $\% \, HRP \; Activity = \left(\frac{HRP \; activity \; signal \; of \; sample \; at \; 0 \; hour}{HRP \; activity \; signal \; of \; sample \; at \; 0 \; hr \; or \; 1 \; hr \; or \; 7 \; days}\right)$ x 100%. Each column represents the average of triplicates±SD.

To determine the stability of RBD-HRP protein and its conjugates with different polymers, a thermal stability approach was performed. The RBD-HRP protein and its conjugates with DMAm DP25, DMAm DP10, DMAm DP5, DMAm DP12.5-DMAPA DP12.5, DMAm DP12.5-AMPSA DP12.5 and NIPAm DP25 polymers were incubated at 37 ℃ for 1 hour or 7 days. Subsequently,

the HRP activity assay as described earlier was performed (Figure 3). The RBD-HRP protein retained 97% activity after 1 hour and 94% activity after 7 days incubation. On other hand, HRP activity assay for all conjugates showed at least 93% residual activity after 1 hour incubation except DMAm DP12.5-DMAPA DP12.5 conj. (78% residual activity). Although conjugation with some functional polymers showed increased %HRP activity after 1 hour incubation compared to 0 hour incubation, this is within typical experimental variability. The residual activity of all conjugates however was at least 70% after 7 days incubation. Hence, these data indicate that the conjugates remained stable even after 7 days incubation at a physiological relevant temperature, with no systematic drift in the relative activity of the conjugates during incubation. This is consistent with earlier work, showing that polymer deconjugation is unlikely over the timescale of 7 days, when EDC based coupling is used.[58] Circular dichroism experiments were performed to monitor the secondary helical content of the conjugates (Figure S12). The secondary helical content of the conjugates is essentially the same for the native and for the respective conjugates.

In summary, a series of bioconjugates were synthesized by attaching a range of well-defined functional polymers i.e., hydrophilic *N,N*-dimethyl acrylamide, hydrophobic *N*-isopropyl acrylamide, cationic *N,N*-dimethyl aminopropyl acrylamide, and anionic 2-acrylamido-2-methylpropane sulfonic acid polymers to the RBD-HRP fusion protein. Free polymers, with exception of anionic 2-acrylamido-2-methylpropane sulfonic acid polymers, increase the binding of RBD to ACE-2 provides a higher stringency environment that may be useful for the search for small molecule compounds that disrupt the RBD-ACE2 interaction. In contrast to their free polymer analogues, bioconjugates of RBD with the water-soluble polymers displayed up to 95% reduction of RBD-HRP interactions with the ACE2 protein. with the greater inhibition observed with longer chain polymers. Additionally, these bioconjugates demonstrated significant thermal

stability even after exposure to physiological conditions for extended periods. These results provide promising routes for modification of the RBD, such as those within an inactivated SARS-CoV-2 virion used for vaccines, to prevent interaction between the RBD and ACE2. Hence, our results suggest that simple covalent modification by protein-polymer conjugation can disrupt interactions that are crucial for viral life cycle, thereby showing the potentiality of bioconjugation for altering vital physiologically relevant interaction. This observation may could guide future development of inhibitors targeting RBD-ACE-2 interaction.

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M.S.R., N.D.A.W., R.C.P., and D.K. designed the research. M.S.R., B. M. C. and N.D.A.W. performed the research. M.S.R., N.D.A.W., R.C.P., and D.K. wrote the manuscript. All authors were involved in editing the manuscript.

Supporting Information.

Materials and experimental methods, Supplemental polymer functionality and molecular weight data, bioconjugate conjugation and structural data, and statistical analysis of bioconjugate performance.

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Data Availability Statement

Raw data for this manuscript will be made available on the Miami University Scholarly Commons.

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