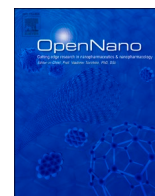




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Research Article

Passivating the Omicron SARS-CoV-2 variant with self-assembled nano peptides: Specificity, stability, and no cytotoxicity

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ABSTRACT

The SARS-CoV-2 Omicron variant is called a “variant of concern” (VOC) which has spread all over the world at a faster rate than even the first SARS-CoV-2 outbreak despite travel restrictions. In order to combat the health consequences from a SARS-CoV-2 Omicron variant infection, the objective of the present *in vitro* study was to develop self-assembled nano peptides to attach to the virus and inhibit its attachment and entry into mammalian cells for replication. For this purpose, two amphipathic peptides containing hydrophobic and hydrophilic peptides and an unnatural amino acid (such as 2-aminoisobutyric acid (U)) were designed to attach to the less mutated virus envelope rather than more frequently mutated S-protein region: NapFFTLUFLTUTEK KKK and NapFFMLUFLMUMEK KKK. These peptides were synthesized using the solid phase peptide synthesis method and were characterized for mammalian cell infection using well-established pseudo virus assays. *In vitro* results showed that the two self-assembled nano peptides significantly inhibited the ability of the SARS-CoV-2 Omicron variant virus to infect mammalian cells and replicate with IC₅₀ values of 0.5 and 360 mg/ml for NapFFTLUFLTUTEK KKK and NapFFMLUFLMUMEK KKK, respectively. Most impressively, 1 mg/ml of NapFFTLUFLTUTEK KKK resulted in a 2 log reduction in pseudovirus replication after just 15 min at a viral load of 10⁶ copies/ml. Results further confirmed that the peptides continued to passivate the SARS-CoV-2 Omicron variant for up to one week and were stable in cell culture media before being added to the virus. Mechanistically, *in vitro* results showed selective binding of the peptides to the SARS-CoV-2 Omicron variant envelop protein over the more frequently mutated spike protein up to one week demonstrating the stability of the peptides. Cytotoxicity studies with fibroblasts also showed no toxicity when exposed to the peptides for 72 h. In summary, the present results strongly suggest that the two peptides developed in this study should be further researched for a wide range of anti-SARS-CoV-2 virus applications, including the present Omicron and future mutations.

1. Introduction

According to the US Centers for Disease Control (CDC) website [1], numerous SARS-CoV-2 variants appeared during the winters of 2020–2022. Currently, the world is battling the most contagious of them all, the Omicron SARS-CoV-2 variant. Specifically, B.1.1.7 (20I/S01Y.V1) appeared in September 2020, B.1.351 (20H/S01Y.V2) in October 2020, P.1 (20J/S01Y.V3) in January 2021, as well as

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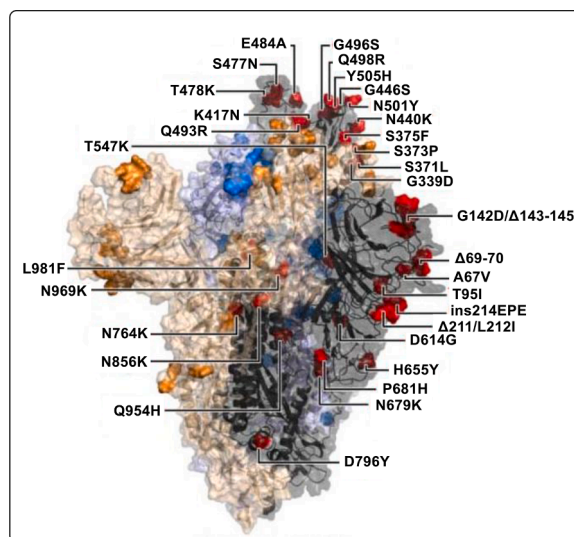


Fig. 1. Structure of the most mutated region (S protein) of the Omicron SARS-CoV-2 variant (adapted from modified from COVID-19 Genomics UK Consortium).

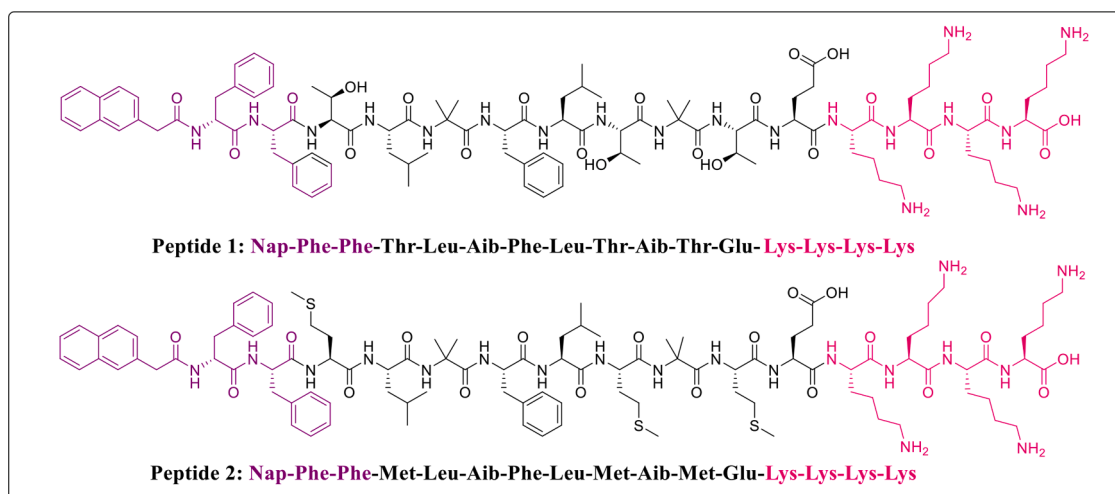


Fig. 2. The synthetic structure of two amphipathic peptides designed here that target the SARS-CoV-2 envelop protein, especially the SARS-CoV-2 Omicron variant.

B.1.617.2 (Delta Plus) and Omicron (B.1.1.529) in late 2021. It is believed that Omicron variant spread from Gauteng to the world faster than any prior SARS-CoV-2 variant [1]. According to the University of Hong Kong, and despite this faster rate of spreading even in the presence of travel restrictions, the Omicron variant replication rate in the lung is 10 times lower than the Delta variant. It was also determined that the Omicron variant spreads in countries with relatively high vaccination rates ranging from 69 and 77%, like Denmark and the UK [2,3]. Collectively, from the above statistics, it is clear that there is an urgent need to find a new therapeutic solution for SARS-CoV-2 and all of its variants rather than the traditional approach of developing new boosters and asking the public to take new boosters for every new variant. This is especially true since the CDC showed that less people have taken a booster shot than the original vaccine. Specifically, more than around 211 million people are fully vaccinated in the US, however, only around 86 million have received the primary vaccine with a booster [4].

In order to develop a new strategy for passivating SARS-CoV-2 and its variants, we have developed peptides that specifically target the envelop region of SARS-CoV-2 which has not mutated as frequently as the spike protein (Fig. 1). Since the Omicron variant's most mutated region is on the spike S-protein (such as at A67V, del69-70, T95I, G142D, del143-145, del211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, and L981F as shown in Fig. 1a), we designed two peptides that target the envelope protein, which is the least mutated region among all SARS-CoV-2 variants.

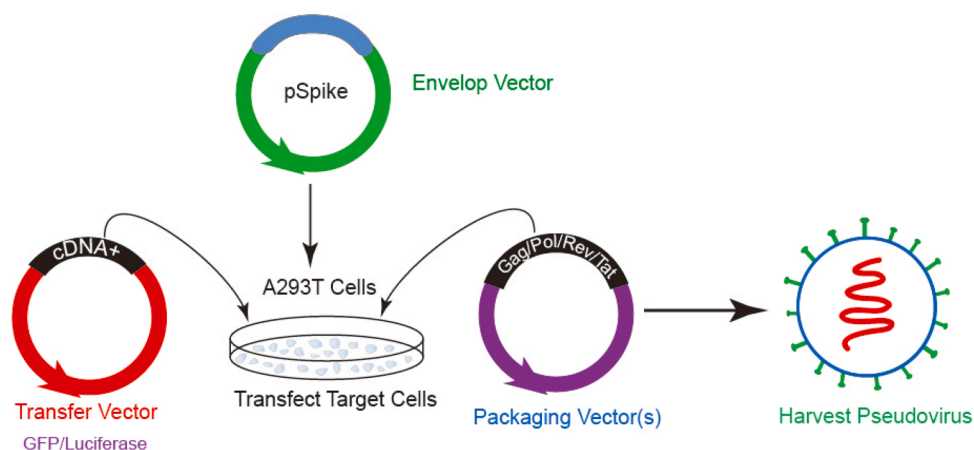


Fig. 3. Creative Diagnostics (NY, USA) Creation of the SARS-CoV-2 GFP Omicron Pseudo Virus Used in this Study. (Adapted from <https://www.creative-diagnostics.com/news-human-ace2-stable-cell-line-hek293t-85.htm>).

As shown in our previous work, NapFFTLUFLTUTEKKKK and NapFFMLUFLMUMEKKKK are two peptides we designed without the hydrophilic region 4 lysine residues that inhibited the Delta SARS-CoV-2 variant computationally [5]. These peptides were designed complementary to a specific sequence on the viral envelope and which we incorporated into the most popular self-assembled motif Nap with two phenylalanine residues (NapFF) [6]. To increase the degree of hydrophobicity since this region is very hydrophobic, we further incorporated α -aminoisobutyric acid (Aib, U) [7,8]. The incorporation of Aib (U) into the peptides has shown many antiviral and antibacterial properties including enhancing biomolecule delivery systems, such as the delivery of RNA into cells [9]. Aib is the only unnatural amino acid incorporated onto both peptides that contain an α,α -dimethyl group that cannot be encoded. Aib has many advantages when incorporated into peptides, such as enhancing the interaction between the peptide and cell membrane, [10,11] increasing the permeability into cells in general, [12] and resistance to protease digestion [13].

These two peptides are very hydrophobic in nature and to increase their polarity for use in a biological milieu, we added four lysine residues (Lys)₄ to create NapFFTLUFLTUTEKKKK and NapFFMLUFLMUMEKKKK as shown in Fig. 2. As this study demonstrates for the first time, these two amphipathic peptides exhibited excellent inhibition of the Omicron SARS-CoV-2 variant using well-established *in vitro* pseudo variant experiments.

2. Materials and methods

2.1. Peptide synthesis and characterization

The peptides were synthesized manually under a nitrogen bubbler gas using Fmoc solid peptide synthesis chemistry [14]. The peptides were purchased from GL Biochem (Shanghai, China). Their structures were confirmed using proton nuclear magnetic resonance (¹H-NMR) and liquid chromatography–mass spectrometry (LC-MS) to confirm their molecular mass. We observed fractions of NapFFTLUFLTUTEKKKK as [M + 2H]²⁺ at m/z of 985.3, [M + 3H]³⁺ at m/z of 657.4, and [M + 4H]⁴⁺ at m/z of 493.3. For NapFFMLUFLMUMEKKKK, we observed fractions as [M + 2H]²⁺ at m/z of 1030.4, [M + 3H]³⁺ at m/z of 687.4, and [M + 4H]⁴⁺ at m/z of 515.9 as shown in Fig. 4.

2.2. *In vitro* pseudovirus experiments

The SARS-CoV-2 Omicron variant pseudo virus and mammalian cells were purchased from Creative Diagnostics (NY, USA) for this assay. The protocol used for this experiment can be found at: <https://www.creative-diagnostics.com/news-human-ace2-stable-cell-line-hek293t-85.htm>.

For the pseudovirus, we used the Lentiviral SARS-CoV-2 Omicron variant pseudovirus. To understand the mechanism of SARS-CoV-2 cell entry, it is essential to study how Spike proteins interact with the Angiotensin-Converting Enzyme 2 (ACE2) receptor. However, such studies are hampered by the danger of producing and manipulating live coronavirus. Live SARS-CoV-2 has to be handled under biosafety level 3 conditions, which has hindered the development of vaccines and therapeutics. Pseudo-viruses are useful virological tools because of their safety and versatility, as the pseudovirus is restricted to a single round of replication and can be handled using biosafety level 2 (BSL-2) containment practices.

The pseudotyped Luciferase/GFP rSARS-CoV-2 Spike displays an antigenically correct spike protein pseudotyped upon replication but also contains incompetent virus particles that contains a heterologous lentiviral (HIV) core capable of a single round of infection carrying a genome that expresses either a GFP or luciferase optical reporter gene upon infection. Pseudotyped Luciferase/GFP rSARS-CoV-2 Spikes are produced in HEK-293T cells using three separate plasmids, encoding the spike protein, a lentiviral gag polyprotein, and a reporter gene that can be used to test the ability of serum, antibodies, and drugs to neutralize the infectivity of the SARS-CoV-2

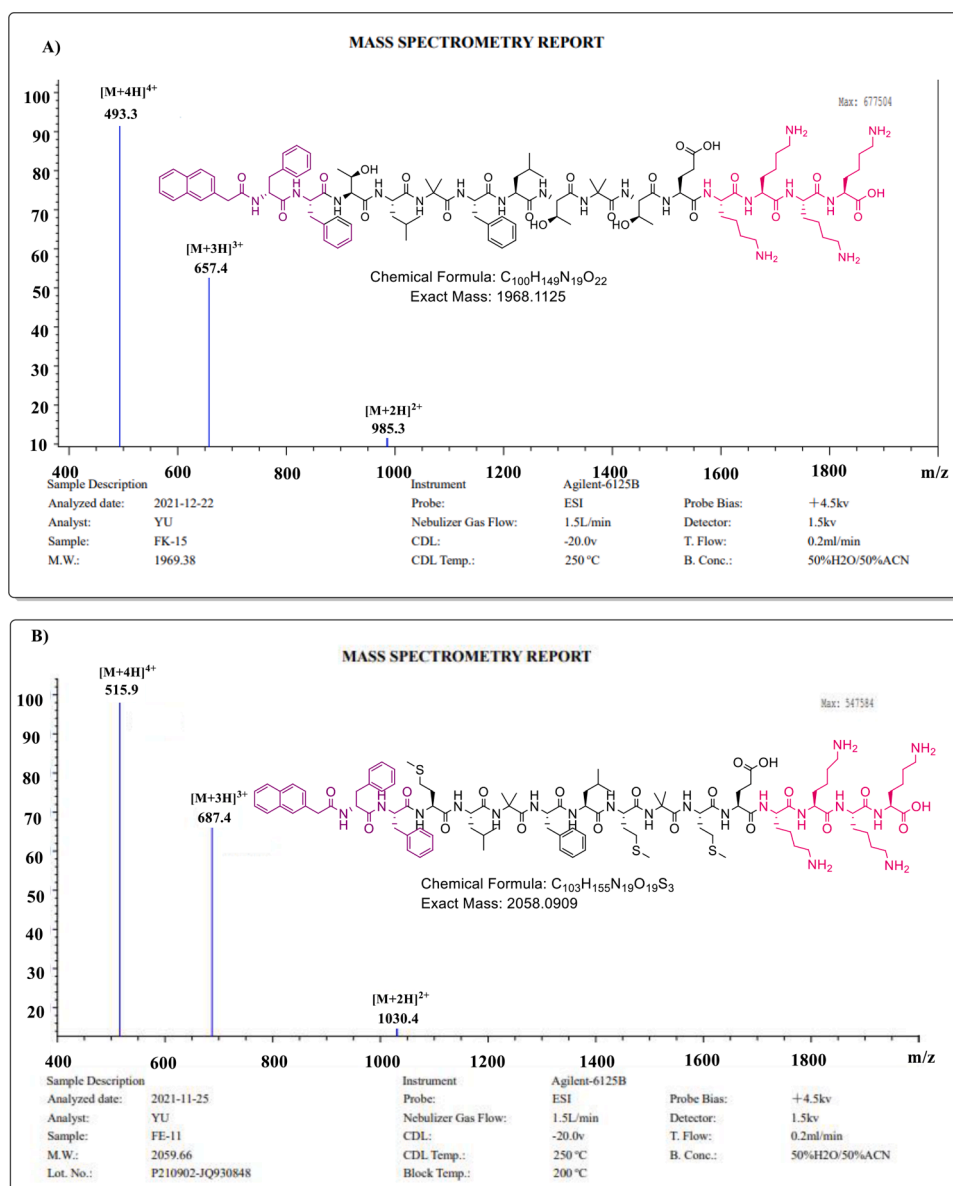


Fig. 4. LC-MS spectrum of the peptides NapFFTLUFLTUTEK and NapFFMLUFLMUMEEK.

spike protein (Fig. 3).

HEK293T cells were used in this project (Creative Diagnostics). This cell line was constructed by the transduction of the human angiotensin I converting enzyme 2 (ACE2) into HEK293T cells, followed by stable cell selection. HEK293T is derived from HEK293 and is commonly used in scientific research. The HEK293T-human ACE2 cell line can be used for *in vitro* screening and characterization of drug candidates against SARS-CoV.

The peptides of interest to this study were added at various concentrations (from 0 to 0.001 mg/ml) to various concentrations of a SARS-CoV-2 Omicron pseudo virus (10 to 10^6 copies/ml) added to a model mammalian cell line (seeded at 10^4 cells) (Creative Diagnostics). Standard cell culture medium (DMEM+10% FBS) was also added to the wells. The peptides were then allowed to interact with the pseudovirus and cells for 15 min to 1 week under standard incubator conditions. After the prescribed time period, the samples were analyzed using a fluorimeter. All experiments were conducted in triplicate and repeated at three different time periods with appropriate controls, including no peptides, no cells, and no pseudovirus. Differences between fluorescence intensity was assessed using ANOVA and student t-tests with $p < 0.01$ considered statistically significant. IC50 values (i.e., the concentration of the peptides needed to inhibit 50% replication compared to controls) were also determined following the same methods described above simply using different peptide concentrations.

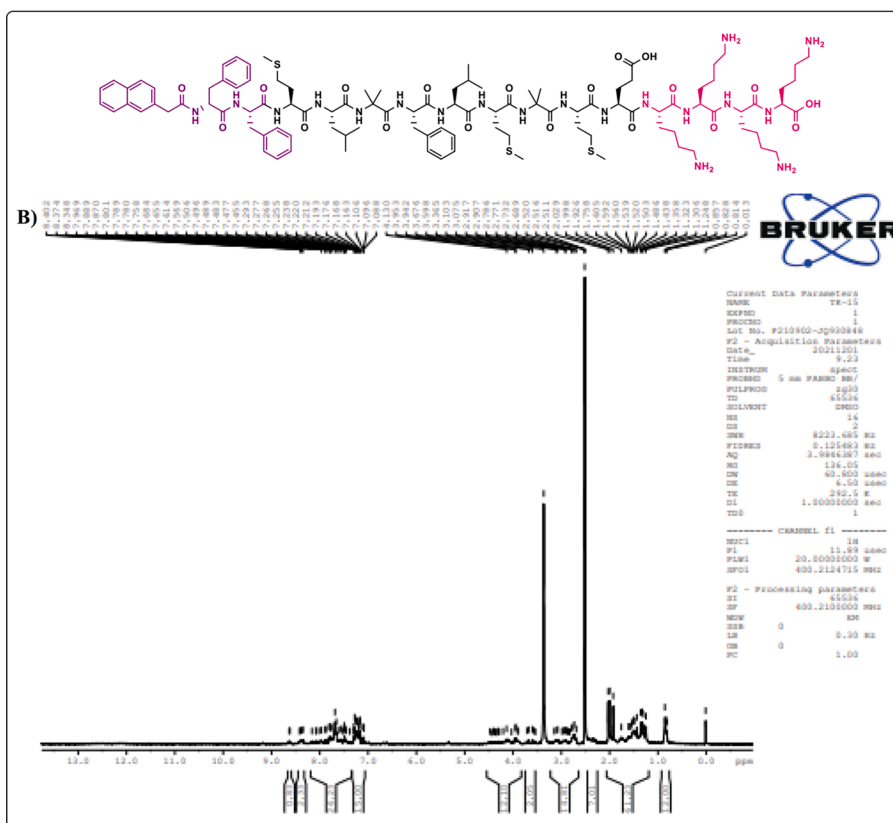
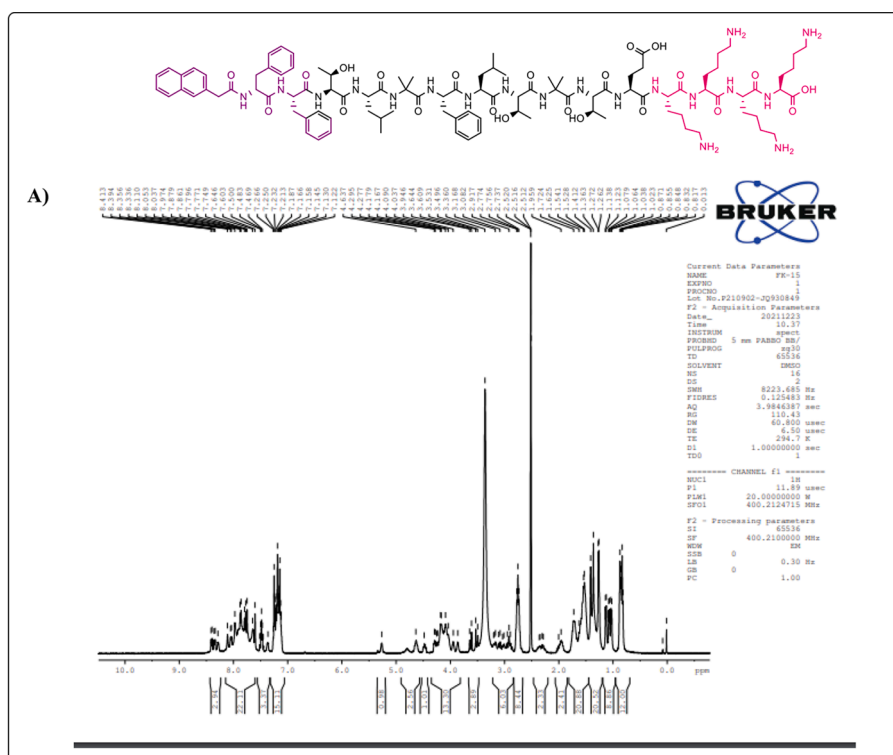


Fig. 5. ¹H-NMR structure of the peptides NapFFTLUFLUTEKKKK and NapFFMLUFLUMEKKKK.

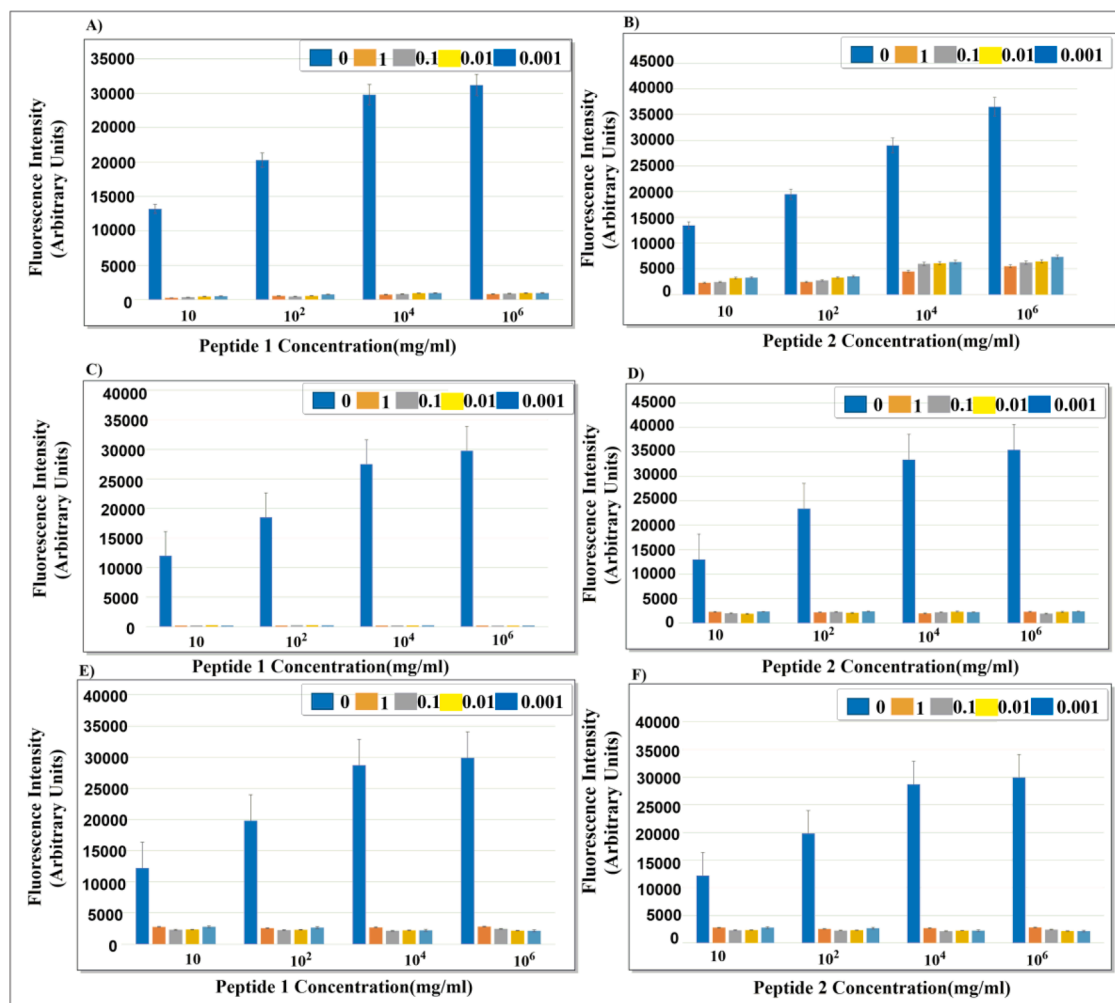


Fig. 6. The peptides were tested on the human ACE2 stable cell line-HEK93. (A) Peptide 1 incubated with the pseudo virus for 15 min, (B) Peptide 2 incubated with the pseudo virus for 15 min, (C) Peptide 1 incubated with the pseudo virus for a day, (D) Peptide 2 incubated with the pseudo virus for a day, (E) Peptide 1 incubated with the pseudo virus for a week, and (F) Peptide 2 incubated with the pseudo virus for a week. All peptide groups were statistically ($p < 0.01$) less than the no peptide (control). Time = 15 min, one day, and one week. Data = mean \pm SEM; $N = 3$. All controls, no cells and no pseudo virus showed no to minimal fluorescence within experimental error. Cell density was constant and pseudo virus concentration corresponds to copies/ml (x axis). Concentration in the caption is mg/ml.

2.3. Mechanism of peptide SARS-CoV-2 attachment

Further, to determine the mechanism by which the peptides attach to the Omicron SARS-CoV-2 variant, the peptides were added at various concentrations for up to one week to just the Omicron SARS-CoV-2 spike, and envelope proteins and attachment measured using fluorescence intensity (Creative Diagnostics).

2.4. Cytotoxicity assays

Lastly, the cytotoxicity of the peptides was determined by adding the peptides at various concentrations to a fibroblast cell line (ATCC 1213) cultured to confluence in well plates for 72 h in standard cell culture media (DMEM + 1% FBS) under standard incubator conditions. At the conclusion of the time period, cell viability was determined using a Live/Dead assay kit (Molecular Probes).

2.5. Statistical analysis

All experiments were conducted in triplicate and repeated at least three different times. Statistical differences between means were determined using student t-tests.

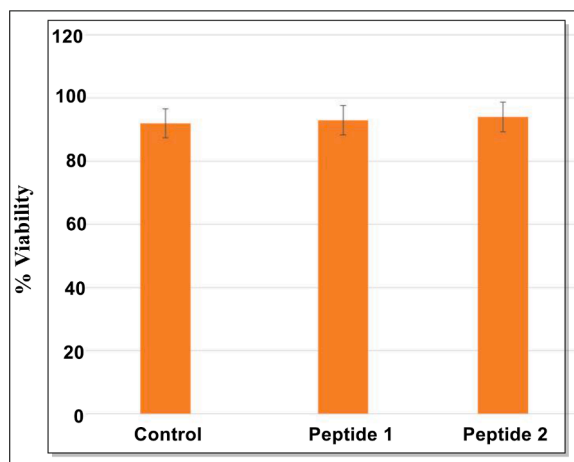


Fig. 7. Viability assays of peptides 1 and 2 on a fibroblast cell line for 72 h. Data = mean \pm SEM; $N = 3$; peptide concentration was 1 mg/ml. All values are statistically the same. Control = no peptide.

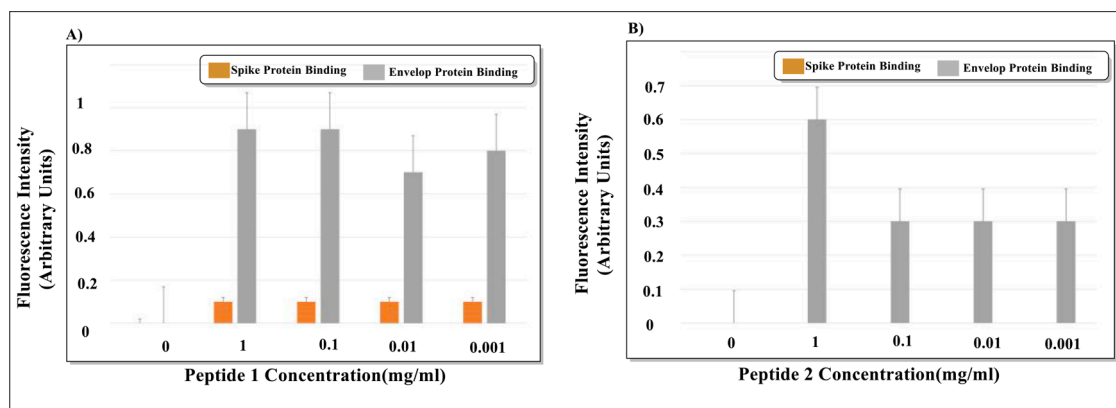


Fig. 8. Specificity assays of peptide A) 1 and B) 2 towards the SARS-CoV-2 Omicron variant spike or envelope protein at concentrations ranging from 0 to 0.001 mg/ml. Data = mean \pm SEM; $N = 3$; Time: 15 min; peptide concentration was 1 mg/ml. Peptides binding to the envelop protein were statistically ($p < 0.01$) greater than the spike protein and control (no peptides).

3. Results and discussion

The synthetic two amphipathic peptides NapFFTLUFLTUTEK⁴ and NapFFMLUFLMUMEK⁴ were successfully synthesized and purified using preparative liquid chromatography. The structure of these two peptides were confirmed using LC-MS and H-NMR spectra as shown in Figs. 4 and 5.

There are peptides developed by others that target the S-protein of SARS-CoV-2 such as EK1, EKL1C, and EK1C4 [15]. However, we designed two peptides that target the viral envelope protein. As described, the peptides were tested on an infected human ACE2 stable cell line-HEK93 and results showed that they effectively inhibited Omicron pseudovirus (PsV) mammalian cell infection as shown Fig. 6. Specifically, 1 mg/ml of NapFFTLUFLTUTEK⁴ resulted in a 2 log reduction in pseudo virus replication after just 15 min at a viral load of 10^6 copies/ml (Fig. 6); its IC_{50} value after 15 min at a viral load of 10^6 copies/ml was 0.5 mg/ml. Similarly, 1 mg/ml of NapFFMLUFLMUMEK⁴ resulted in a 1 log reduction in pseudo virus replication after just 15 min at a viral load of 10^6 copies/ml (Fig. 6); its IC_{50} value after 15 min at a viral load of 10^6 copies/ml was 360 mg/ml. As a demonstration of the stability of these peptides in cell culture media, results further showed that peptides 1 and 2 passivated Omicron for up to one week in culture. This addresses the high stability of the peptides to protein adsorption, enzymes, and other factors in the cell culture media after binding to the virus.

Further, the peptides did not exhibit any cytotoxicity to fibroblasts for up to 72 h in culture as shown in Fig. 7.

Lastly, we used specificity assays to test whether the peptides bind to the SARS CoV-2 Omicron spike or envelope proteins to prove our proposal as shown in Fig. 8. Results showed that both peptides bind specifically to the SARS-CoV-2 envelope protein. These are the first two peptides to our knowledge used to target the SARS-CoV-2 Omicron envelope protein showing much progress in passivating a region of the virus which is not mutating, and, thus, should be further studied for a wide range of anti-SARS-CoV-2 Omicron viral applications. Many other peptides target the SARS-CoV-2 Omicron S-protein such as EK1, EK1C4, and EKL1C to inhibit virus

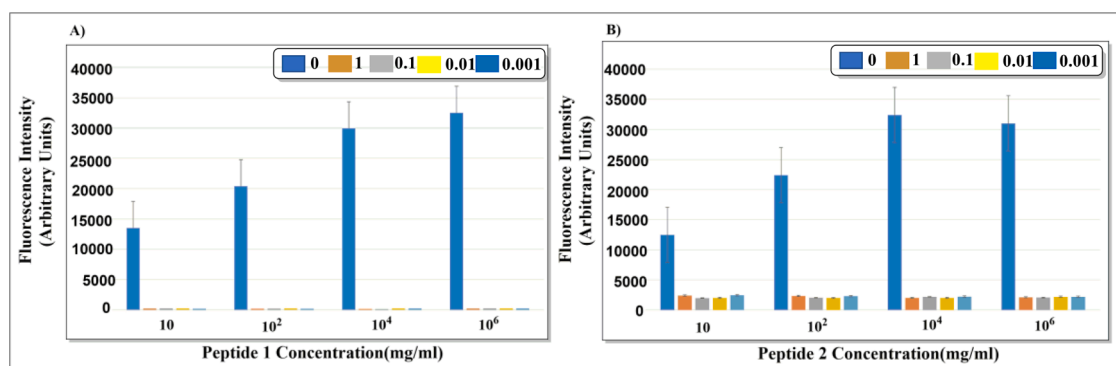


Fig. 9. The stability assay of the peptide (A) 1 and (B) 2 toward the SARS-CoV-2 Omicron variant at concentrations ranging from 0 to 0.001 mg/ml. Data = mean \pm SEM; $N = 3$; Time: a week. Peptides binding to the envelope protein were statistically ($p < 0.01$) greater than the control (no peptides).

replication with IC50 values of 309.4, 8.63, and 26.14 nM, respectively, but the spike protein has been known to mutate, thus, putting into question the role that such peptides can play for future SARS-CoV-2 mutations [15].

The stability of the peptides in the body before they reach the virus was assessed by culturing the peptides in cell culture media for up to one week and then completing the above-mentioned SARS-CoV-2 Omicron variant envelope binding assays, as shown in Fig. 9. From this result, the peptides were found to be stable for up to a week in the cell culture media and still bound to the virus meaning that peptides were not deactivated by proteins, enzymes, or other biomolecules in the cell culture media.

4. Conclusion

In this study, we designed two amphipathic peptides NapFFTLUFLTUTEK and NapFFMLUFLMUMEK that contained the unnatural amino acid Aib to find a new nanomaterial to passivate the highly spreadable SARS-CoV-2 Omicron variant. The Aib unnatural amino acid differs from the natural Alanine amino acid in its extra methyl group at the α position of the C atom. We have previously shown that Aib increases the hydrophobicity of a compound and exhibits antibacterial and anticancer activity. These two peptides were synthesized using a well-known solid phase peptide synthesis strategy. *In vitro* SARS-CoV-2 Omicron pseudo virus studies showed that these two peptides effectively inhibited viral replication and were stable for up to one week in culture. Further, results demonstrated the specific binding of the peptides to the less mutated envelope protein (rather than the more frequently mutated S-protein region) and were not cytotoxic to fibroblasts for up to 72 h in culture. Thus, this study demonstrates that these two peptides should be further studied as a prophylactic or therapeutic for COVID-19, including the present and future mutations of SARS-CoV-2.

CRedit authorship contribution statement

Alaa F. Nahhas: Conceptualization, Writing – original draft, Visualization, Data curation. **Thomas J. Webster:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

A patent related to this paper has been filed with the U.S. Patent Office. A. N. and T. W. are listed as inventors on the patent application. The other authors declare no competing interests.

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