

Detecting Arginine Post-Translational Modifications in p38MAPK α and Vimentin Proteins Using Quantum-Si's Next-Generation Protein Sequencing Technology

SUMMARY

Proteins undergo a diverse array of post-translational modifications (PTMs) to their amino acid side chains, which strongly affect protein function and mediate intricate cellular events. Measuring the diversity, dynamics, and functional consequences of PTM states of proteins across the proteome is essential to understanding the role of proteins in health and disease. However, the diversity of proteoforms in the human proteome remains largely unmapped, and the discovery and detection of PTMs is highly challenging.¹ New, more sensitive methods for PTM detection will greatly aid biomarker discovery, drug discovery, and the development of precise and personalized approaches to medicine.

Modifications of the arginine side chain are of biomedical interest. Methylation and citrullination of arginine residues in human proteins have been shown to play key roles in disease states such as cardiovascular disease, autoimmune disease, and cancer.²⁻⁶ Here, we demonstrate the application of Platinum[®], Quantum-Si's next-generation protein sequencing technology,⁷ to the detection of arginine methylation and citrullination in p38MAPK α and Vimentin proteins with single-molecule resolution and sensitivity.

Quantum-Si's protein sequencing technology offers a sensitive platform for PTM detection and discovery with the power to address the critical need for accessible methods to study the role of PTMs in human health and disease.

Q-SI TECHNOLOGY

Quantum-Si's benchtop Platinum[®] instrument enables protein sequencing from biological samples in a simple user-friendly workflow. Our technology utilizes dye-tagged N-terminal amino acid recognizers and semiconductor chip technology to detect the binding characteristics and binding order of N-terminal amino acids, resulting in unique kinetic signatures that can be used to differentiate and identify amino acid residues and PTMs. A more detailed overview of the workflow and technology can be found in our [Science Paper](#).

p38MAPK α : p38 mitogen-activated protein kinase α

INTRODUCTION

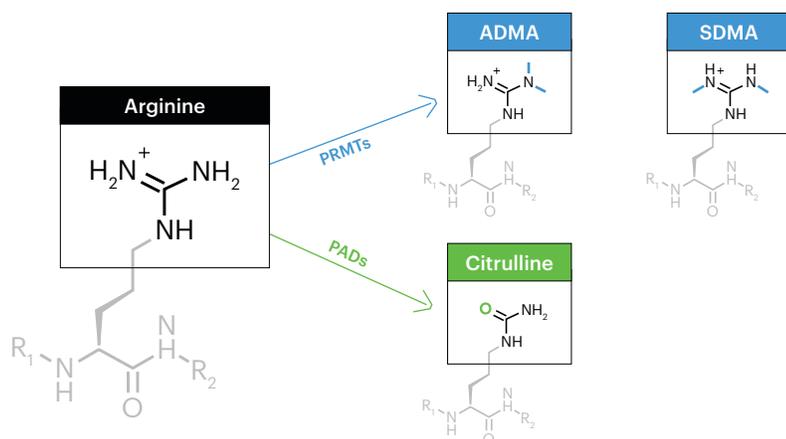
Arginine plays a critical role in protein structure and function due to the unique properties of the guanidinium group that forms the terminus of its side chain. This group is both positively charged and capable of forming extended hydrogen bond networks and cation- π interactions with other amino acids and with nucleic acids. Arginine, therefore, often mediates key interactions between protein binding partners or between proteins and DNA.

The two most common arginine PTMs, dimethylation and citrullination, alter the arginine side chain and change its properties (Figure 1), potentially resulting in important downstream effects on cellular processes. Dimethylation retains arginine's positive charge but increases its size and hydrophobicity and blocks hydrogen bond formation. Citrullination eliminates arginine's positive charge, resulting in a neutral side chain, greatly impacting protein conformation and function. Dimethylation and citrullination of arginine are carried out by enzymes and may be part of the normal regulation of cellular processes or involved in disease states. Arginine dimethylation is catalyzed by protein arginine methyltransferases (PRMTs). PRMTs transfer two methyl groups either asymmetrically onto the same nitrogen atom, resulting in asymmetric dimethyl arginine (ADMA), or symmetrically onto opposite nitrogen atoms, resulting in symmetric dimethyl arginine (SDMA). Arginine citrullination is catalyzed by protein arginine deiminases (PADs). PADs carry out the hydrolysis of arginine's positively-charged guanidinium group, resulting in a neutral ureido group.

Arginine PTMs have emerged as important targets in biomedical research. Methylated arginine residues and their respective PRMTs have been implicated in diseases such as cardiovascular disease and cancers.^{2,3} Research has demonstrated that arginine citrullination is critically involved in immune system function, skin keratinization, myelination, and gene expression regulation.⁴⁻⁶ Notably, the removal of arginine's positive charge in some cases can cause proteins to activate the immune system, contributing to autoimmune diseases.⁵

REFERENCES

1. Aebersold R, Agar JN, Amster IJ, et al. How many human proteoforms are there?. *Nat Chem Biol.* 2018;14(3):206-214. doi:10.1038/nchembio.2576
2. Couto E Silva A, Wu CY, Citadin CT, et al. Protein Arginine Methyltransferases in Cardiovascular and Neuronal Function. *Mol Neurobiol.* 2020;57(3):1716-1732. doi:10.1007/s12035-019-01850-z
3. Chen L, Liu S, Tao Y. Regulating tumor suppressor genes: post-translational modifications. *Signal Transduct Target Ther.* 2020;5(1):90. Published 2020 Jun 10. doi:10.1038/s41392-020-0196-9
4. James EA, Pietropaolo M, Mamura MJ. Immune Recognition of α -Cells: Neoepitopes as Key Players in the Loss of Tolerance. *Diabetes.* 2018;67(6):1035-1042. doi:10.2337/dbi17-0030
5. Nguyen H, James EA. Immune recognition of citrullinated epitopes. *Immunology.* 2016;149(2):131-138. doi:10.1111/imm.12640
6. Wegner N, Lundberg K, Kinloch A, et al. Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. *Immunol Rev.* 2010;233(1):34-54. doi:10.1111/j.0105-2896.2009.00850.x
7. Reed BD, Meyer MJ, Abramzon V, et al. Real-time dynamic single-molecule protein sequencing on an integrated semiconductor device. *Science.* 2022;378(6616):186-192. doi:10.1126/science.abo7651
8. Witze ES, Old WM, Resing KA, Ahn NG. Mapping protein post-translational modifications with mass spectrometry. *Nat Methods.* 2007;4(10):798-806. doi:10.1038/nmeth1100


FIGURE 1

Post-translational modifications of arginine. The arginine side chain consists of a positively-charged guanidinium group at the terminus of a flexible aliphatic chain. In arginine dimethylation, protein arginine methyltransferases (PRMTs) transfer two methyl groups—either asymmetrically to the same nitrogen atom (ADMA) or symmetrically onto opposite nitrogen atoms (SDMA). These modifications increased size and hydrophobicity and block hydrogen bonding. In arginine citrullination, protein arginine deiminases (PADs) carry out the hydrolysis of arginine's positively-charged guanidinium group, resulting in a neutral ureido group (a transformation referred to as deimination). This transformation results in a negligible mass increase of 0.9840 Da, though the loss of positive charge can dramatically alter protein conformation and function.

CHALLENGES FOR THE DETECTION OF ARGININE PTMS

Research into these arginine PTMs has been particularly challenging because they are difficult to detect and differentiate with current proteomic methods.⁸ Mass spectrometry (MS) is the most frequently utilized tool for detecting protein PTMs. However, MS cannot easily distinguish between ADMA and SDMA because they are constitutional isomers with identical masses.⁹ Likewise, deimination of arginine to citrulline results in a negligible mass increase of 0.9840 Da. This mass difference can easily be confused with a ¹³C isotope or misinterpreted as deamidation of nearby asparagine or glutamine residues.¹⁰ In addition, MS techniques for arginine PTM detection, specifically arginine modifications, require highly specialized knowledge and training and advanced analysis methods.

Enzyme-linked immunosorbent assay (ELISA), another common method for PTM detection, uses antibodies specifically generated to detect a modified protein of interest.

REFERENCES

- Afjehi-Sadat L, Garcia BA. Comprehending dynamic protein methylation with mass spectrometry. *Curr Opin Chem Biol.* 2013;17(1):12-19. doi:10.1016/j.cbpa.2012.12.023
- Vitorino R, Guedes S, Vitorino C, Ferreira R, Amado F, Van Eyk JE. Elucidating Citrullination by Mass Spectrometry and Its Role in Disease Pathogenesis. *J Proteome Res.* 2021;20(1):38-48. doi:10.1021/acs.jproteome.0c00474
- Larsen SC, Sylvestersen KB, Mund A, et al. Proteome-wide analysis of arginine monomethylation reveals widespread occurrence in human cells. *Sci Signal.* 2016;9(443):rs9. Published 2016 Aug 30. doi:10.1126/scisignal.aaf7329
- Egelhofer TA, Minoda A, Klugman S, et al. An assessment of histone-modification antibody quality. *Nat Struct Mol Biol.* 2011;18(1):91-93. doi:10.1038/nsmb.1972
- Jeong HJ, Lee SJ, Lee HJ, et al. Prmt7 promotes myoblast differentiation via methylation of p38MAPK on arginine residue 70. *Cell Death Differ.* 2020;27(2):573-586. doi:10.1038/s41418-019-0373-y
- Van Steendam K, Tilleman K, De Ceuleneer M, De Keyser F, Elewaut D, Deforce D. Citrullinated vimentin as an important antigen in immune complexes from synovial fluid of rheumatoid arthritis patients with antibodies against citrullinated proteins. *Arthritis Res Ther.* 2010;12(4):R132. doi:10.1186/ar3070
- Li FJ, Surolia R, Li H, et al. Citrullinated vimentin mediates development and progression of lung fibrosis. *Sci Transl Med.* 2021;13(585):eaba2927. doi:10.1126/scitranslmed.aba2927

Although arginine PTMs are estimated to be widespread in human cells,¹¹ commercially available antibodies against arginine PTMs are limited to specific sites on a few highly studied proteins. The requirement to generate new antibodies, along with complex workflows, high costs, limited antibody reproducibility,¹² and other challenges associated with ELISA assay development, will likely hinder discovery and further study of novel arginine PTM sites.

Continued development toward novel methods is needed to facilitate direct detection of arginine PTMs in proteins. Next-generation protein sequencing on Quantum-Si's Platinum[®] offers a single-molecule sequencing solution to the detection of ADMA, SDMA, and citrulline that is not based on mass-to-charge ratio or antibody specificity, but rather on the kinetic signature of binding between recognizers and N-terminal amino acids (NAAs).

Quantum-Si's Platinum instrument harnesses the power of single-molecule protein sequencing to gain insights into these PTMs with single molecule resolution, overcoming current technological gaps, and providing direct detection of arginine PTMs.

METHODOLOGY & WORKFLOW

PTM detection on Platinum consists of isolating peptides and subjecting them to a single-molecule protein sequencing reaction.⁷ Proteins are first digested into peptide fragments and conjugated C-terminally to macromolecular linkers. The peptide complexes are immobilized on Quantum-Si's semiconductor chip, resulting in single peptide molecules with exposed N-termini ready for sequencing. During the sequencing reaction, the surface-immobilized peptides are exposed to a solution containing dye-labeled NAA recognizers that bind on and off to their cognate NAAs with characteristic kinetic properties. Aminopeptidases in solution sequentially remove individual NAAs to expose subsequent amino acids for recognition. Fluorescence lifetime, intensity, and kinetic data are collected in real time and analyzed to determine amino acid sequence and PTM content.

Sequencing profiles of peptides are visualized as kinetic signature plots—simplified trace-like representations of the time course of complete peptide sequencing containing the median pulse duration (PD) for each recognition segment (RS) and the average

duration of each RS and non-recognition segment (NRS). When recognizers bind to NAAs, they also make important contacts with nearby downstream residues in the sequence, influencing the average PD of binding events between recognizers and target peptides. This kinetic sensitivity to nearby downstream residues provides a wealth of information on peptide sequence composition and is extremely beneficial for mapping traces to their proteins of origin. A summary of the workflow for sequencing of peptides and detection of PTMs is presented in Figure 2.

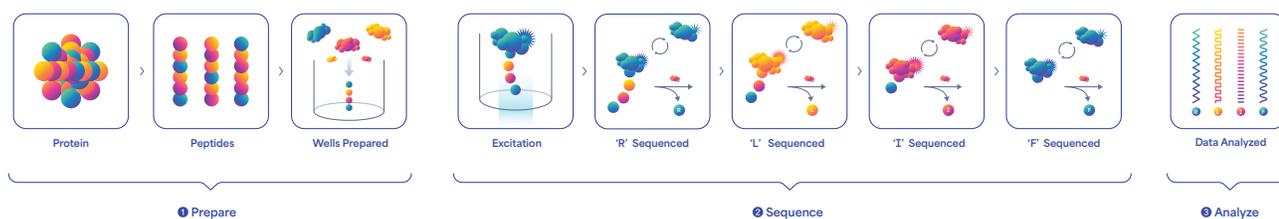


FIGURE 2

The Platinum Protein Sequencing Workflow. Proteins are digested and functionalized into individual peptides that are immobilized on a semiconductor chip with N-terminal amino acid (NAA) recognizers and aminopeptidases. Binding kinetics and order of binding are then used to determine the peptide sequence. Analysis software is used to identify proteins using the sequencing information.

RESULTS & DISCUSSION

DETECTION OF ARGININE DIMETHYLATION

We seek to demonstrate the detection and differentiation of arginine, ADMA, and SDMA using next-generation protein sequencing with single-molecule resolution. We focused on a key segment of the signaling protein p38MAPK α . Dimethylation of arginine residue 70 of p38MAPK α in myoblast cells by PRMT7 is a critical regulatory step in the activation of myoblast differentiation in humans.¹³

We generated synthetic peptides corresponding to residues 69 to 76 of p38MAPK α in three versions containing either arginine, ADMA, or SDMA at position 2: YRELRLLLK, YR_{ADMA} ELRLLK, and YR_{SDMA} ELRLLK. We sequenced each peptide on the Quantum-Si Platinum[®] instrument using three recognizers with specificity for 7 NAAs (F, Y, W, L, I, V, and R) and analyzed data to identify RSs, determine the mean PD of each RS, and characterize the kinetic signature of each peptide. Each peptide displayed a distinguishable pattern due to the distinct kinetic influences of arginine,

ADMA, and SDMA on recognizer binding (see example traces in Figure 3).

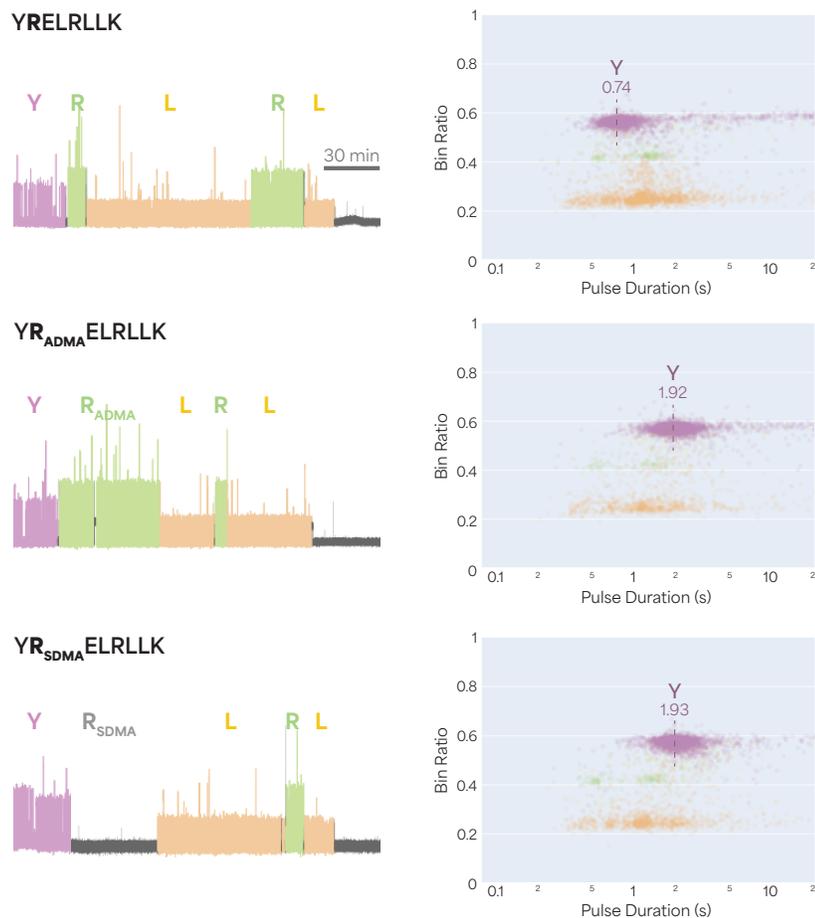


FIGURE 3

Kinetic signatures distinguish three synthetic p38MAPK α -derived peptides containing arginine (top), ADMA (middle), and SDMA (bottom) at position 2. Full length peptide sequences are indicated. Left: Example protein sequencing traces. Arginine and ADMA residues exhibited binding with the R recognizer, whereas SDMA exhibited no binding. Right: Kinetic profile from sequencing experiments of the synthetic peptides. The mean PD of tyrosine recognition increased for ADMA and SDMA.

Arginine and ADMA residues exhibited binding with the R recognizer, whereas SDMA exhibited no binding (Figure 3). This result indicates that symmetric dimethylation of arginine—in contrast to asymmetric dimethylation—reduces the affinity of the R recognizer for N-terminal arginine, providing a clear kinetic difference between these isomeric arginine PTMs. Quantum-Si's NAA recognizers contact residues at position 2 and 3 from the N-terminus when they bind to their target NAAs; therefore, modification of

these downstream residues can influence recognizer binding affinity.⁷ We observed a strong influence of arginine dimethylation on recognition of the upstream tyrosine residue in these peptides by the FYW recognizer. The median PD of tyrosine recognition increased from 0.74 s for YRE to 1.92 s and 1.93 s for YR_{ADMA}E and YR_{SDMA}E, respectively (Figure 3). The influence that these dimethylated arginine residues have on the recognition of preceding NAAs serves as a powerful feature of protein sequencing with single-molecule sensitivity and precision. These results demonstrate the capacity for unprecedented sensitivity in detection of arginine dimethylation using Platinum's next-generation protein sequencing technology.

DETECTION OF ARGININE CITRULLINATION

We next sought to demonstrate that differential binding kinetics can be used to rapidly differentiate citrullinated arginine residues from native arginine residues. We generated synthetic peptides corresponding to residues 47 to 62 of MLRV in two versions containing either arginine or citrulline at position 2: NDLRDTFAALGRVNVK and NDLCitDTFAALGRVNVK. We sequenced each peptide on the Quantum-Si Platinum instrument using five recognizers with specificity for 11 NAAs (F, Y, W, L, I, V, R, A, S, N, and Q). Each peptide displayed a highly distinguishable kinetic signature due to the influence of the different arginine and citrulline side chains on recognition (Figure 4). Citrullination eliminated N-terminal arginine recognition by the R recognizer, as demonstrated by the example traces (Figure 4) and the level of coverage (Figure 4, green box). Citrullination at position 4 also resulted in a large increase in the median PD of recognition of the N-terminal leucine located at the preceding position by the LIV recognizer. Median PD increased from 0.44 s for LRF to 0.72 s for LCitF (Figure 4, orange box). These results demonstrate the capability of Quantum-Si next-generation protein sequencing technology in detection and digital quantification of arginine citrullination with the capacity to overcome the challenges facing conventional methods.

DETECTION OF ARGININE CITRULLINATION IN VIMENTIN

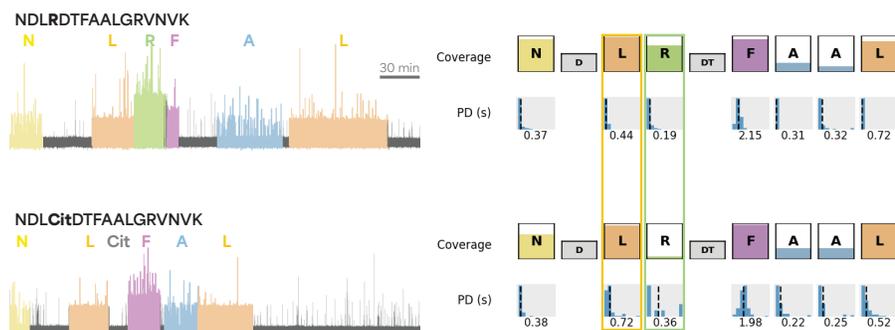


FIGURE 4

Kinetic signatures distinguish two synthetic MLRV-derived peptides containing arginine (top) and citrulline (bottom) at position 4. Full length peptide sequences are indicated. Left: Example protein sequencing traces. Arginine exhibited binding with the R recognizer, whereas Cit exhibited no binding. Right: Coverage and mean PD of the recognized residues. The mean PD of leucine recognition increased for Cit (orange box), while the coverage of the arginine was eliminated (green box).

We next sought to demonstrate the detection of arginine citrullination in vimentin, a type III intermediate filament protein that provides structural support to cells and is involved in maintaining cell shape and integrity. Citrullinated vimentin has been implicated in various autoimmune diseases, such as rheumatoid arthritis, as it can trigger an immune response leading to inflammation and tissue damage.^{15,16} This protein when digested with LysC produces the peptide VRFLEQQNK in which citrullination happens at position 2.

We generated two synthetic peptide sequences containing either arginine or citrulline at this position—VRFLEQQNK and VCitFLEQQNK—and sequenced each peptide on the Quantum-Si Platinum[®] instrument using five recognizers with specificity for 11 NAAs (F, Y, W, L, I, V, R, A, S, N, and Q). Each peptide displayed a highly distinguishable kinetic signature as a result of the different binding kinetics of arginine and citrulline side chains (Figure 5). For the VRFLEQQNK peptide, the R residue was detected, whereas the preceding V residue was not detected due to the V residue's mean PD being too short for recognition. For the VCitFLEQQNK peptide, citrullination eliminated the recognition of the R residue and resulted in the recognition of the preceding V residue due to an increase in the mean PD.

Next, we digested and sequenced the full vimentin protein with the synthetic peptide VCitFLEQQNK spiked in at equal peptide concentration. The spike-in citrullinated peptide can be clearly distinguished from the non-citrullinated peptide of the vimentin protein (VRFLEQQNK), as each peptide displayed a distinct kinetic signature (Figure 5). Example sequencing traces demonstrated that for peptide VRFLEQQNK, the first V residue was not detected, while the second R residue was clearly detected. In contrast, for the spike-in peptide VCitFLEQQNK, citrullination led to the recognition of the first V residue, while the recognition of the second R residue was eliminated. These results demonstrate that Quantum-Si Platinum[®] sequencing instrument can detect arginine citrullination in vimentin, an important protein in autoimmune diseases.

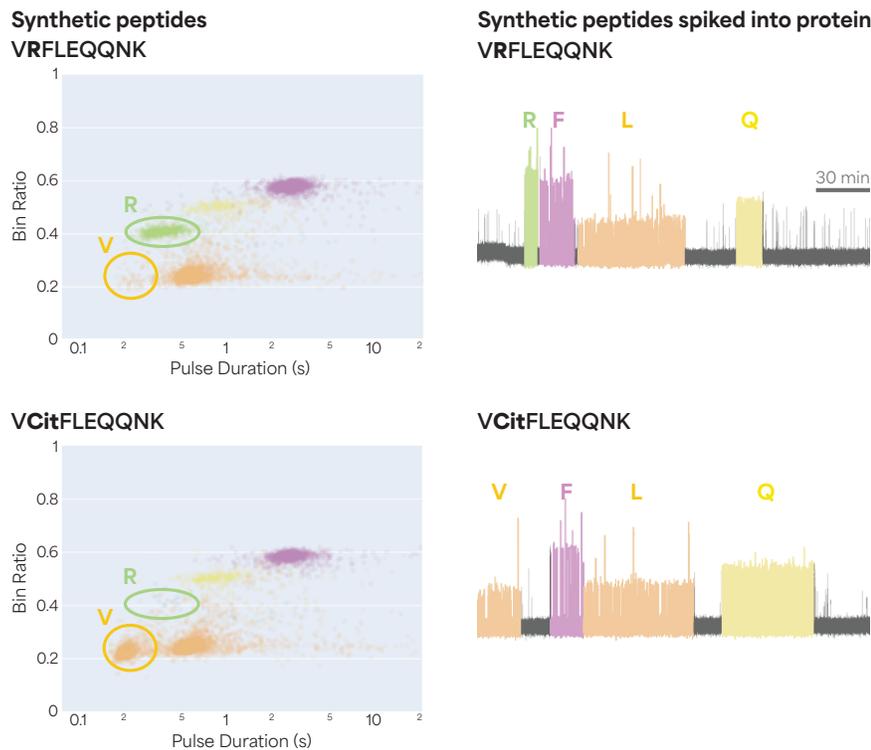


FIGURE 5

Kinetic signatures distinguish peptides of vimentin protein containing arginine and citrulline at position 2. Full length peptide sequences are indicated. Left: Kinetic profile from sequencing experiments for two synthetic peptides of vimentin. Right: Example protein sequencing traces of the non-citrullinated peptide of vimentin (top) and the spike-in synthetic citrullinated peptide (bottom).

CONCLUSION

In this application note, we directly detect arginine PTMs using Quantum-Si's next-generation protein sequencing platform. Arginine PTMs play important roles in human health and disease but have been challenging to study. Current proteomic methods such as mass spectrometry and ELISA have been capable of indirect identification of these arginine PTMs using highly specialized techniques or limited to a small set of specific proteins on the basis of antibody availability and other challenges. The ability to directly detect PTMs via next-generation protein sequencing demonstrated here offers great potential for accelerated biomedical research and for a wide range of commercial applications in drug discovery and biomarker development.