Article

Boronic Acid-Linked Cell-Penetrating Peptide for Protein Delivery

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ABSTRACT: Studying functional protein delivery into live cells is important, ranging from fundamental research to therapeutics. Cell-penetrating peptides (CPPs) are known to deliver proteins with applauded efficacy and have gained importance for applications in protein therapeutics and exploration of versatile cellular mechanisms. The primary aim of the work is to design a CPP as a tool and delivery vehicle for macromolecules, including proteins. In this work, boronic acid-linked cyclic deca arginine (cR10) is reported as an efficient CPP that exhibited 3-fold higher delivery of chemically synthesized ubiquitin (Ub) than pristine cR10-linked Ub, examined with live U2OS cells. As a futuristic plan, an artificial intelligence machine learning-based rationale has been designed and proposed.
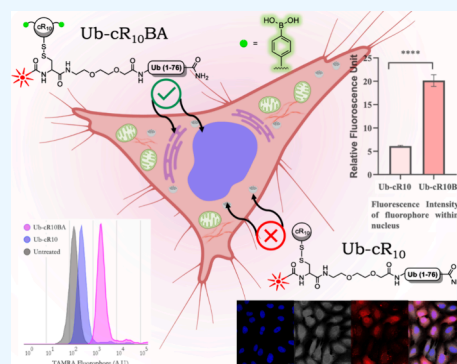
■ INTRODUCTION

Intracellular delivery of proteins related to different critical cell functions has a substantial role in deepening our understanding of the fundamentals of chemical biology.[1] Live cell delivery of proteins is challenging due to their inherent physical properties. The delivery has two primary challenges: (i) protein must be transported into the cytosol through the cell membrane, which is less accessible for pristine protein due to shape, size, and other intrinsic controlling factors. Usually, small molecules can cross the membrane comparatively in a more accessible way; however, large molecules like proteins cannot cross due to various complex issues. (ii) By some means, although proteins are internalized, the major problem lies in entrapment by endosomes, and endosomal uptake suggests "nondelivery" of the proteins.[2] Incidentally, the late endosomes, i.e., lysosomal entrapment, would end up with protein degradation. Thus, protein delivery within the cytosol, i.e., for bioavailability, needs assistance, and it is the prime target of this work.

Physical methods are reported to assist protein delivery, such as sonoporation, microinjection, electroporation, bead loading, etc.[3] These methods can bypass endocytosis, possibly via membrane manipulation, and eventually could damage the cell. Therefore, it is less applicable to living organisms. Other methods, such as zinc finger or DNA binding proteins, supercharged proteins, or nanoparticle/liposome-based delivery, are also known.[4−7] Among different protocols, cell-penetrating peptides (CPPs) have gained popularity due to efficient delivery, ease of operation, etc. The first set of CPPs derived from natural proteins is a transactivator of transcription (TAT) and penetratin. Later, several synthetic CPPs with modularity in structural aspects have been reported to surpass the applicability of conventional CPPs.[8] With better cell permeability, CPPs can translocate successfully the CPP-cargo

complex to the cytosol.[9−17] There has been a lot of effort invested in exploring the mechanism of CPP-cargo translocation within the cell; however, the exact pathway remains hypothetical/elusive.[18] Cellular delivery of CPP-cargo, which follows endocytosis, generally exhibits endosomal escape, although the escape mechanism is still unclear and case-dependent. CPPs are known with cationic functional groups (i.e., arginine,[9,10] guanidinium proline,[13] etc.); however, hydrophobic, amphipathic, or anionic CPPs are also known.[19,20] One possibility of CPP-cargo delivery is electrostatic interactions between cationic CPPs and anionic cell surface proteoglycans.[21]

While one of the possibilities leads to the CPPs' interactions with glycans, Raines et al. reported an exciting finding where formations of boronate esters with the glycocalyx enhance cellular uptake.[22] The hypothesis of CPP's interactions with glycans and Raines et al.'s reports generates curiosity in attempting to use a scaffold/additive on CPPs, which has known affinity toward carbohydrates and is hitherto unexplored. To fill the knowledge gap by combining the effect of boronic acid and CPP, cR10 (cyclic deca arginine) modified with boronic acid was designed and synthesized toward its utility in delivering a model protein. At the same time, only cR10 (without any modification) was used as a control to elucidate the efficacy of the designed CPP.

The electrostatic attraction between the positive charges of CPPs and the negative charges of the cell surface would help in

Scheme 1. (a) Synthetic Scheme of cR10BA; (b) Synthetic Scheme of cR10
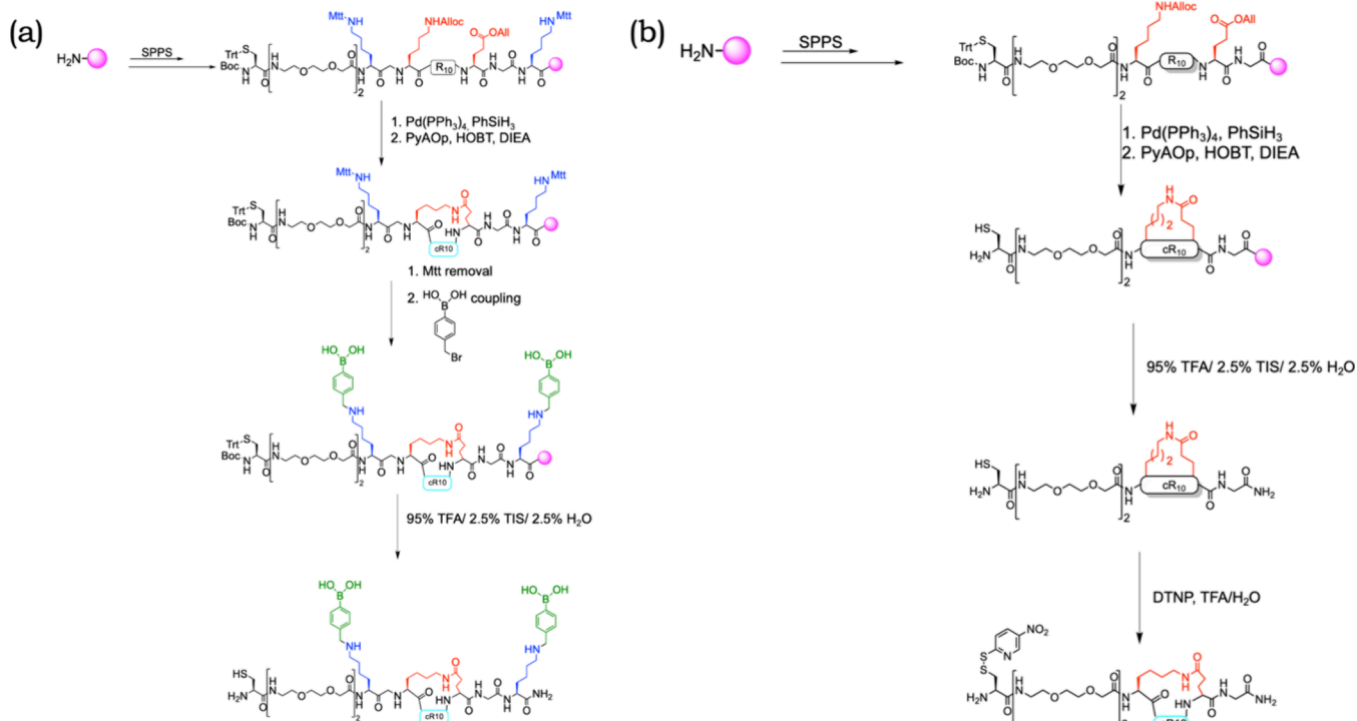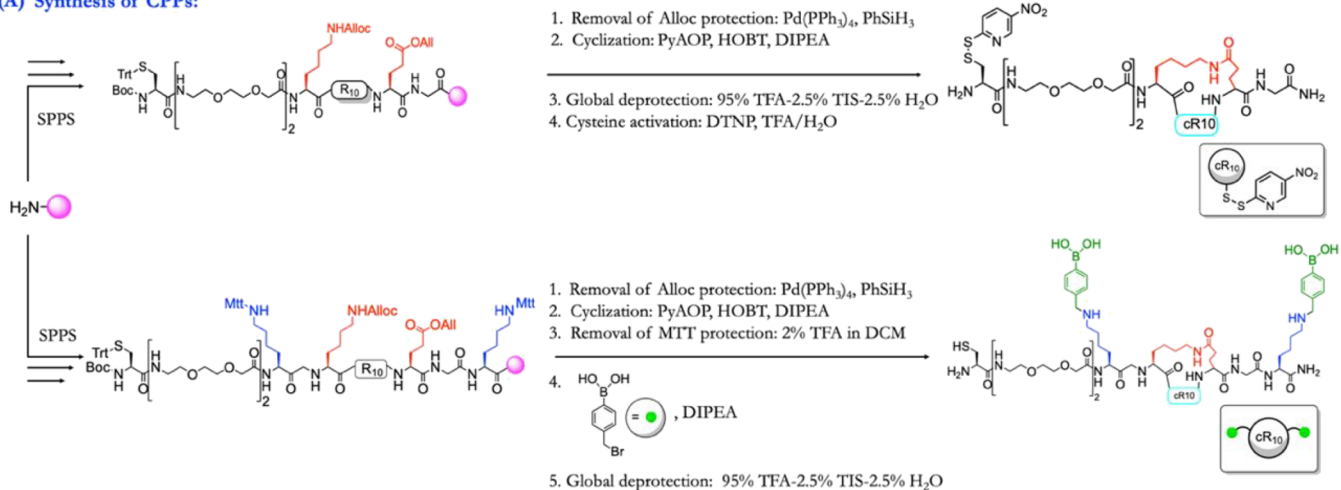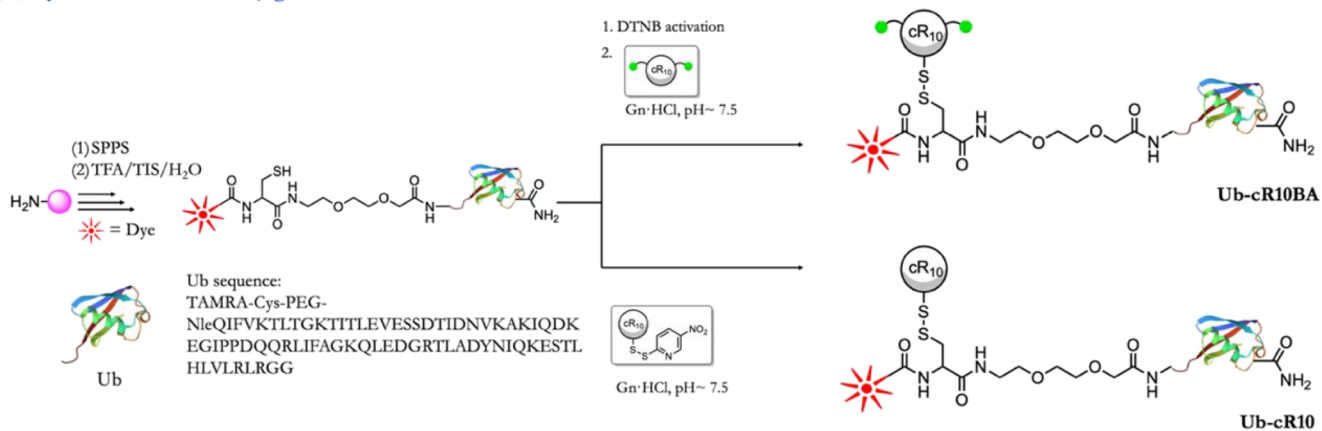
Figure 1. (A, B) General synthesis schemes of the probes.

the cellular delivery of the CPP-cargo. Indeed, it could be principally envisioned that in the case of BA containing CPPs, the CPP would face more attraction with the cell surface glycans, leading to an accelerated mobility of CPPs toward the cell than mere CPPs.

The state-of-the-art showed that as a synthetic CPP, cR10 was pioneered by Cardoso et al. as a promising choice of CPP to deliver large proteins such as GFP, mCherry, etc.[9,10] Later, the Brik group explored cR10 modified with DABCYL for synthetic mono-Ub delivery[15] followed by synthetic di-Ub delivery.[16] To understand the role of DABCYL in CPPs, Brik et al. carried out several modifications to DABCYL.[17] Myo-inositol hexaki-sphosphate delivery using guanidinium proline containing CPPs was reported by the Wennemers group, which made a significant addition to this field.[14] On the other hand, distinct contributions to this field were made by Raines and co-workers using cR10 linked with cytochrome C and GFP for cell delivery.[23] Ovaa et al. reported a Ub-TAT conjugate with enhanced delivery.[24] Zhuang and co-workers have also reported the delivery of Ub via cR10, although the Ub was predominantly stuck in endosomes and lysosomes.[25] Overall, it would be tremendously interesting to explore cR10 modifications to tune the delivery of the target protein.

As a protein of interest, i.e., cargo, ubiquitin (Ub) was chosen, a 76 amino acid long protein.[24] Ubiquitination, i.e., covalent attachment of ubiquitin to proteins, is the second significant post-translational modification for proteins after phosphoryla-tion.[26] Uni- to polyubiquitination of proteins controls several functionalities of a large family of proteins. Indeed, the ubiquitination/deubiquitination process is dysregulated in several diseases, including cancer, and thus acts as a modulator of cancer progression. The ubiquitination/deubiquitination and its connection with cancer are complex and require more studies. Primarily, to understand the cellular functions of Ub, its cytosolic delivery is necessary to investigate its function or underlying mechanisms that are still unclear. As Ub cannot be delivered to the cell on its own,[17] it needs assistance, and the CPP can be a good contender for it. Considering the importance of Ub delivery to the cell, it has been targeted as cargo to test the hypothesis of improved cytosolic delivery via boronic acid-based CPPs. Artificial intelligence and machine learning (AI-ML)-based methods can be used to predict the future CPP for applications with improved efficiency to understand protein delivery and for futuristic applications.

■ RESULTS AND DISCUSSION

Synthesis. Two CPPs and Ub were synthesized using Fmoc-SPPS manual synthesis, cR10, and cR10BA, coupled to prepare Ub-cR10 and Ub-cR10BA via a cleavable disulfide linkage (Scheme 1). In the current study, the protein of interest (Ub) with an N-terminally attached fluorophore (TAMRA, see the Supporting Information for details) was designed to monitor its cellular localization. During synthesis, an extra Cys residue was introduced after the sequence to facilitate disulfide-linked CPP modification on Ub (as the native Ub sequence does not contain any Cys residues). A PEG linker ([2-[2-(Fmoc-amino)ethoxy]-ethoxy]acetic acid) was incorporated between the Ub sequence and the N-terminal Cys residue as a spacer between the natural and additional residues (i.e., TAMRA and cysteine) with the aim of better solubility and to maintaining the protein's original folding (Figure 1).[10]

Accordingly, the synthesis was carried out manually on Rink Amide resin via Fmoc-SPPS and purified by HPLC to prepare

TAMRA-Cys-PEG-Ub (1−76 AA) (yield ~23%), verified by mass analysis (see the Supporting Information). For the attachment of the Cys-cR10 CPP to the final Ub construct, the activated CPP was dissolved in guanidinium phosphate buffer (pH ~ 7.5) and mixed with TAMRA-Cys-PEG-Ub (Figure 1). Keeping the reaction mixture at 37 °C for 5−7 min, the hetero disulfide-linked fluorescently labeled Ub-cR10 was formed. However, a similar synthetic route for generating Ub-cR10BA did not work out. To synthesize the said scaffold, the Cys residue at the final Ub construct was activated using 5,5′-dithiol-bis-[2-nitrobenzoic acid], DTNB, and activated Ub was added to Cys-cR10BA dissolved in guanidinium phosphate buffer (pH ~7.5) for 2 h to prepare the desired analogue Ub-cR10BA. Thus, disulfide-linked Ub-cR10/cR10BA was further used for the live-cell delivery in U2OS cells, an osteosarcoma cell line monitored via confocal laser scanning microscopy (CLSM) with 40× resolution.

Biological Study. Cellular uptake analysis of the probes (Ub-cR10 and Ub-cR10BA) was performed at 2 μM concentrations in a serum-free medium with 1 h of incubation time at 37 °C. Initially, U2OS cells were treated with the probes for 1 h followed by washing with only PBS and heparin sulfate solution in PBS (see details in the Supporting Information, Section S4 Cell Delivery Experiments).[10,27] Afterward, the cells were washed for another two rounds with PBS followed by Hoechst as a live-cell nuclear staining. Confocal laser scanning microscopy images of Ub-cR10BA exhibited homogeneous distribution throughout the cytosol and outreach to the nucleus. The control probe, Ub-cR10, showed almost no distribution within the cytosol (Figure 2). For small proteins <60 kDa, once
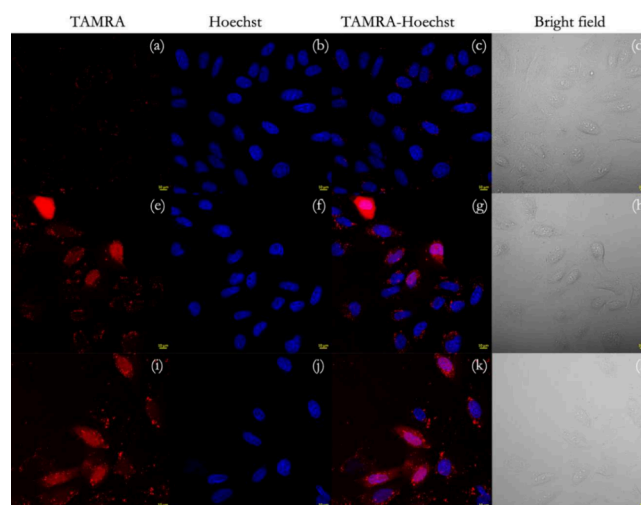
Figure 2. Intracellular delivery of (a−d) Ub-cR10 and (e−l) Ub-cR10BA at 37 °C, concentration: 2 μM, scale bars: 10 μm. Two sets of image are shown for Ub-cR10BA (set-1, e−h; set-2, i−l).

they penetrate the cell membrane, the passive diffusion through the nuclear pore will allow it to reach the nucleus, which reflects its cytosolic delivery.[28−30] As the Ub conjugates have a small molecular weight, they can freely diffuse into the nucleus, reflecting live-cell delivery.

Therefore, to compare the delivery efficiency between Ub-cR10 and Ub-cR10BA, the fluorescence intensity of the fluorophore attached to Ub (TAMRA in the present case) was measured in the nucleus (Figure 3a,b). For each probe, more than 150 cells from three different sets of experiments were
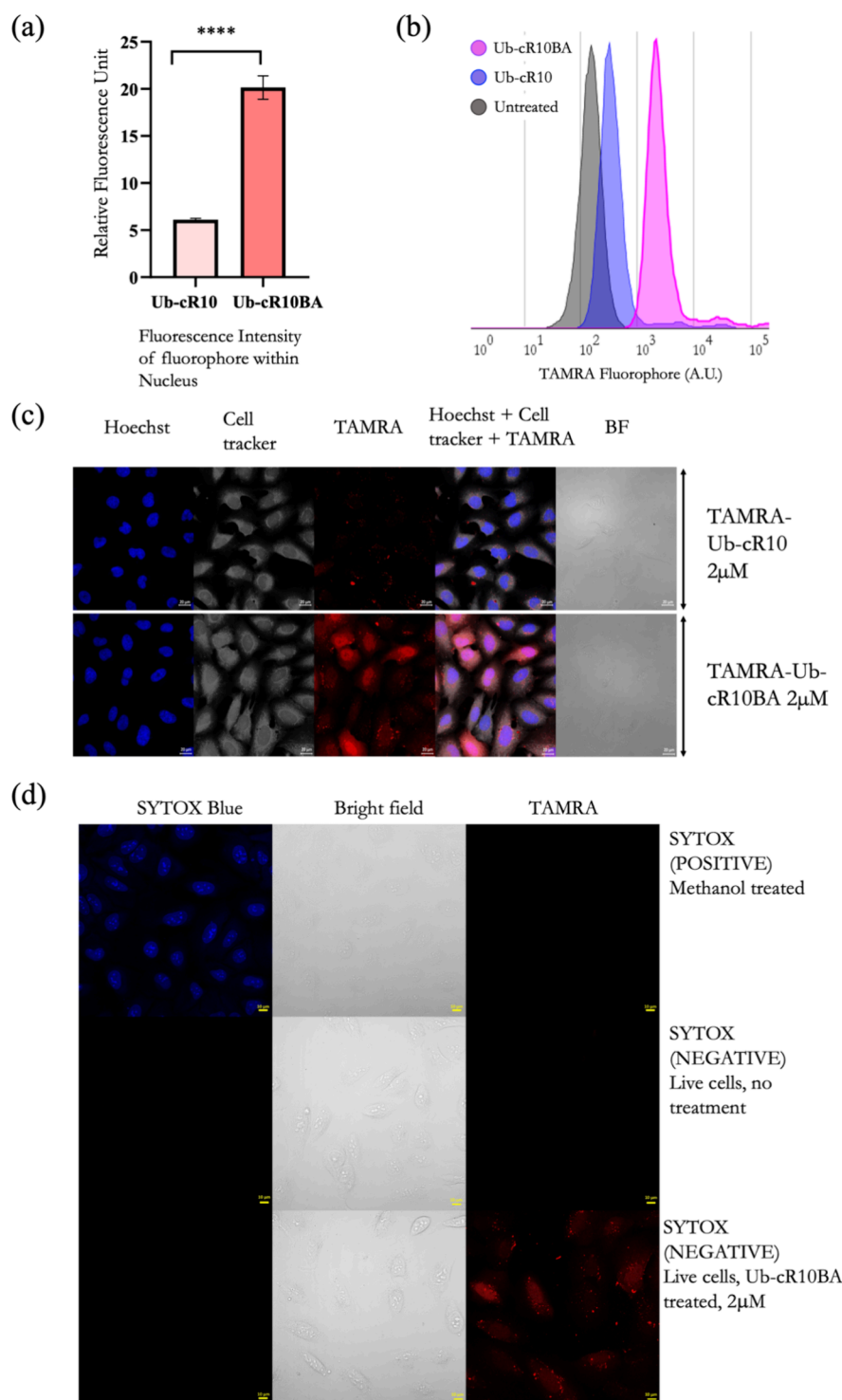
Figure 3. (a) Measurement of fluorescence intensity of the fluorophore present in the sequence within the nucleus (TAMRA in the present case) to measure the delivery efficiency; data are plotted as mean ± standard error of the mean of three independent biological experiments, and statistical significance was determined by the unpaired t test (****$P < 0.0001$)[16]; (b) flow cytometry experiment between Ub-cR10 and Ub-cR10BA with U2OS cells; (c) colocalization experiment between cell-tracker deep red and Ub-cR10/Ub-cR10BA, scale bars: 20 μm; (d) cellular delivery of Ub-cR10BA with SYTOX Blue stain for the live U2OS cell line. Scale bars, 10 μm.

quantified using FiJi software by a nuclear masking algorithm for similar analysis. The analysis revealed more than 3-fold better delivery of Ub-cR10BA than Ub-cR10. The cR10BA concentration used in this study was lower than the limit reported previously.[10] The statistical distribution of Ub-cR10 and Ub-cR10BA was validated by flow cytometry, where the Ub-

cR10BA showed higher shifting in comparison to Ub-cR10, ensuring its higher efficacy and corroborating the nuclear TAMRA intensity measurement (Figure 3a). To show the cytosolic distribution of the Ub delivered by cR10BA, a cell-tracker deep red (500 nM) marker was used to stain the whole cell. The resulting colocalization experiment between cell-

tracker deep red and the TAMRA from Ub-cR10BA confirms the cytosolic distribution of the delivered Ub. It is interesting to observe that there are no puncta-type signals in the cytoplasm of the delivered cells, ruling out the endolysosomal signals from the trapped Ub-cR10BA analogues (Figure 3c). The designed CPP did not damage the cell membrane of the treated cells (as clearly visible from the bright field images of the analysis). Moreover, to confirm the membrane integrity of live U2OS cells in the presence of Ub-cR10BA, upon treatment, the cells were cross-verified using SYTOX blue. The outcome showed no SYTOX blue-stained cells (Figure 3d).

Outlook for Connecting AI-ML with CPP-Based Research. Modified cR10 has been used by several researchers, including those currently working toward delivering proteins. In every case, with cR10 kept as a base material, small additives are connected to the cR10 scaffold, which helps in cellular uptake, e.g., boronic acid, in the present case. Looking precisely, the only difference between cR10 and modified cR10 is the additive that controls the delivery. The enhanced cellular delivery could be governed by different parameters of the additive, such as the (i) structure, (ii) dipole moment, (iii) influence on folding, and (iv) efficiency in cellular delivery compared with the unmodified CPP. As only a handful of reports about cR10 are known, artificial intelligence machine learning-based methods may not be helpful appropriately at the current stage. Thus, this discussion aims to show the path and vision of how AI-ML methods can be useful for CPP-based research.

An AI-ML model could read the data received from the reported probes, and multiple ML algorithms such as support vector machines (SVM), convolutional neural networks (CNN), or random forest classifiers could be used for numerous purposes. The structure of the additive could be directly used as an input for the mode to understand the dipole moment by theoretical calculation[31−34] would be useful, the influence of folding could be established by the circular dichroism (CD) data, and efficiency can be used as a readout coming from multiple analysis. Several researchers currently use AI-ML for multiple applications in chemosensing and/or protein-based research.[35−37] Other than investigating individually with varying additives or mere alternation of additive substitution, which leads to exhaustive experimentation, the AI-ML method could be a predictor similarly to suggest future probes (Figure S11).[38,39]

CONCLUSIONS

Delivering functional proteins as cargo to cells is essential due to the emerging application of protein therapeutics. Among all of the delivery methods, the CPP can be more advantageous due to the ease of operation, efficient delivery, better on-site applicability, etc., compared to physical methods. cR10 is known for its competence in delivering proteins to live cells, and it works through positive $_{(CPP-protein)}$−negative $_{(cell\ membrane)}$ electrostatic interactions for cellular uptake. However, cR10 could be toxic to the cell due to its high positive charges. Thus, next-generation CPPs can be formed based on boronic acid as boronic acid is susceptible to a nucleophilic attack followed by rapid hydrolysis of alcohol and boric acid.[40] Boric acid is not genotoxic, and long-term analysis of mice showed no tumor increase.[41] As per the U.S. Environmental Protection Agency, boric acid has low toxicity as oral LD50 for male rats is 3450 mg/kg, whereas 4080 mg/kg is for female rats.[42] Moreover, boronic acid is well documented for its interaction with carbohydrates and so are the cell surface glycans, and no CPPs have been

known to combine with boronic acid. This work demonstrates the designed synthesis of boronic acid-linked CPPs, i.e., cR10BA, and compares it with control cR10 for tuning the intracellular delivery of chemically synthesized Ub. The crucial role of this protein has been observed in many cellular metabolisms, including cancer. Thus, to explore Ub with better insight, its delivery has become a next-level target. This work uses the boronic acid-based CPP to exhibit a 3-fold greater delivery of Ub than its control CPP in live U2OS cells. This result opens an avenue for CPP-associated protein delivery. It helps to understand that boronic acid-based CPPs could be useful in the future for enhanced delivery of the protein of interest.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09689.

> Details of synthetic procedures, experimental details, HPLCs, MS traces, and biological experimental information (PDF)

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Notes

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