

Using CRISPR-X for Optimization of Antibodies Towards A30P α -synuclein Oligomers in Immunotherapy of Parkinson's Disease



URNCST Journal
"Research in Earnest"

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Abstract

Introduction: Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive cell death in the substantia nigra portion of the midbrain, which results in severe dopamine imbalance and a subsequent loss of refined motor control. Pathogenicity arises from mutations driving the misfolding of alpha synuclein (α -syn), thus leading to oligomerization and formation of Lewy body aggregates in neurons. Previous research has identified the A30P mutated oligomers, which are connected to familial early-onset PD, as particularly toxic intermediates. However, there are insufficient methods of targeting them in isolation of other forms of the α -syn protein. We aim to modify existing approaches to immunotherapy of PD, by use of CRISPR-X and phage display, to design antibodies that are highly specific to the A30P oligomer.

Methods: We will use CRISPR-X to produce a diverse library of antibodies by introducing point mutations in the sequences encoding their variable light (VL) and heavy (VH) chains. Then, via insertion into bacteriophages for multiple rounds of phage display selection, we should obtain a polyclonal selection of antibodies that exclusively recognize epitopes on A30P α -syn. Each variant then produces a distinct monoclonal phage colony to be incubated with relevant antigens for affinity testing via enzyme-linked immunosorbent assay (ELISA).

Expected Results: We expect that measurements from ELISA would indicate low dissociation constants (K_D) within the low micromolar (10^{-6}) to nanomolar (10^{-7} to 10^{-9}) range, when incubated with A30P oligomers. We predict that the resultant antibodies would have a higher affinity for the A30P oligomeric α -syn than other forms of the protein. Furthermore, the novel antibodies are expected to have a higher specificity to this target antigen than the original antibodies that were modified.

Discussion: By determining binding affinity, we verify the effectiveness of this method for improving specificity of in vitro antibody design. Upon further investigation using mice models, we would anticipate these novel antibodies could produce a specific reversal of the toxic effects of A30P in neuronal tissue.

Conclusion: This study aims to establish a potential for CRISPR-X and phage selection to be used in antibody optimization for developing an immunotherapeutic treatment for PD.

Keywords

Parkinson's disease; alpha synuclein; annular protofibrils; oligomerization; phage display; CRISPR-X antibody design; immunotherapy

Introduction

More than 10 million people around the world are affected by PD, a neurological disorder that progresses most rapidly in those over the age of 60 years old [1]. It manifests itself through uncoordinated movements, tremors, difficulty walking, and poor balance due to a dopamine deficiency which hinders the communication between neurons regulating voluntary movement [2]. While the exact cause remains unknown, evidence suggests that its onset can be explained by a combination of environmental influences, genetic predisposition and individual susceptibility [3]. PD disproportionately affects males and has a delayed onset in females due to the neuroprotective effects of estrogen on dopaminergic neurons and pathways

in the brain [4]. Although there are various treatments available to control and alleviate the symptoms of the disease, there is currently no known solution to prevent or reverse the pathophysiological state.

In healthy individuals, dopamine synthesis is regulated by the α -syn protein found in the presynaptic terminals of neurons in the central nervous system. Although its function is not entirely understood, it is thought to mediate the normal functioning of dopamine release and synaptic plasticity by assisting in assembly of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes for vesicle fusion [5]. Several point mutations in α -syn confer pathogenicity in neurodegenerative disorders called synucleinopathies, including PD, where protein misfolding

leads to the deposition of filamentous aggregates called Lewy Bodies [6]. Evidence from in vitro work supports the fact that monomeric α -syn first aggregates into small

oligomers stabilized by beta sheet-like interactions before aggregating into larger fibrils [7-8] (Figure 1).

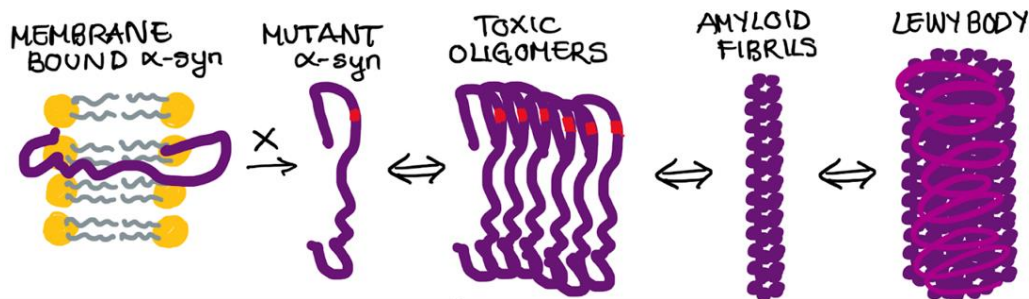


Figure 1. Equilibrium of mutant monomeric α -syn and aggregates

Research shows that while these structures exist in heterogeneous equilibrium, fibrils formed at a faster rate do not elicit toxicity [9] while oligomers that are conformationally trapped and unable to fibrillize are related to accelerated cell death [10]. Furthermore, some PD-linked mutations are known to inhibit fibrillization and allow nonfibrillar oligomers to accumulate [11].

Our study focuses on the A30P mutation in particular, which has been identified as particularly disruptive to regular brain functioning in PD [12]. It occurs when proline replaces alanine at position 30 in the N-terminal sequence, disrupting the formation of alpha helices and resulting in beta-sheet rich oligomers of α -syn. This forms annular pore-like structures that rupture synaptic vesicles, promote calcium influx, and cause widespread neuronal cell death [13-14]. Importantly, it has been shown that A30P monomers fibrillize more slowly than both wild-type (WT) α -syn and the A53T monomer, which is another known PD-linked mutation [15]. This reinforces the link between oligomerization and neurodegeneration, supporting the need for methods of targeting A30P oligomers in isolation of other forms.

While immunotherapy has been previously studied as a potential treatment for PD, the antibodies are often obtained through monoclonal selection in rodent hybridomas. This method has not allowed for a high level of specificity to a particular protein sequence but rather has created antibodies that broadly target either monomeric, oligomeric or fibril forms. Of those currently identified, mAb38F and mAb38E2 are of particular interest to us because they are known to bind to large α -syn oligomers in the context of A30P mutations in transgenic mice modelling PD [16]. Our aim is to adapt existing cost-efficient gene editing technologies to refine their VL and VH domains and obtain a product with higher specificity for A30P oligomers in particular. However, instead of conventional mechanisms for mutagenesis like error-prone PCR or site-specific homology directed repair [17], we use a modified version

of CRISPR known as CRISPR-X to generate this library of antibody variants. This technique makes use of a catalytically inactive Cas-9 protein that recruits an enzyme for somatic hypermutation at specific sites in the antibody encoding gene of a hybridomal cell [18]. This allows for the introduction of random point mutations to create a library of distinct antibody varieties that can be selected for affinity towards A30P [19]. We use CRISPR-X as an alternative to PCR-based mutagenesis, which is much less desirable due to the codon bias associated with introducing single nucleotide mutations [20]. This occurs because changing only one nucleotide in a codon limits the identity of the resulting amino acid. There is also a significant error bias as the polymerase is more likely to perform certain misincorporations than others. Any library established by error-prone PCR will therefore lead certain amino acid replacements to be more common and not allow for true randomization in mutagenesis [21]. Using CRISPR-X therefore produces an antibody library of significantly greater quality as it is not subject to the same biases.

We aim to then identify the best variants in this library using antibody phage display (APD), a well-known, indispensable technique that can quickly and reliably select antibodies for specificity and affinity [22]. Due to the physical connection between the genetic information within the phage and the antibody fragment, APD serves as a powerful tool in understanding the genetic component of antibodies designed against specific antigens [23]. Previous studies have been successful in using phage display to isolate single chain antibody fragments that bind specifically to oligomeric α -syn and inhibit its aggregation and toxicity in vitro to combat PD [24]. This makes it a promising tool when paired with CRISPR-X for time- and cost-efficient affinity maturation. In this protocol, we will therefore apply these techniques together to further improve antibody specificity to target only A30P oligomers for use in immunotherapy for PD.

Methods

Antibody-library preparation

Hybridomas expressing known mAb38F and mAb38E2 antibodies are isolated and distributed into several wells of a microtiter plate. Plasmids for mutagenesis are then obtained, containing a gene drive with dead Cas9 (dCas9) fused to the cooperating mutating enzyme called Activation induced cytidine deaminase (AID) for CRISPR-X [19]. Several guide RNAs (gRNAs) are also designed which are each complementary to a sequence downstream from a different PAM site in the VL and VH encoding regions of the antibody gene. These are also included in the plasmids, which are added to the hybridomal cells to be mutated (one gRNA type per well). The gRNAs will then direct the dCas9 to relevant sites where the AID enzyme acts by cytosine deamination to induce point mutations, insertions or deletions in the VL and VH domains [19]. After mutagenesis, the cells from each well are further diluted into a separate microtiter plate (one cell per well) and a clonal population is produced for each well.

Identification of mutated products

For each well individually, the total RNA is isolated from the hybridoma, purified and reverse transcribed to cDNA. Polymerase chain reaction (PCR) is then used to amplify the VH and VL chain regions of the antibody gene using primers corresponding to known flanking immunoglobulin gene sequences [25]. This introduces possible amplification bias due to the exponential nature of the PCR process, meaning that extra copies of certain reads may appear in the final library that would cause it to over-represent the sequences copied early in the amplification process [26]. Performing separate PCR reactions with a lower number of cycles is done to minimize this bias. Robust Adjustment of Sequence Tag Abundance (RASTA) is then used to further rectify the errors by distinguishing natural read duplicates from any incorrect reads using hierarchical clustering and distributional approximation via the zero-truncated Poisson distribution approach for appropriate purging of duplicates [26]. Next, the PCR products are cloned into a standard sequencing vector for massively parallel Next-Generation sequencing (NGS) to determine which have successfully obtained a diversified amino acid sequence [27].

Bacteriophage insertion

After identifying any successfully mutated products, the relevant sequences are ligated into phage display vectors (pComb3X) and used to transform *Escherichia Coli*. pComb3X allows expression of the engineered VH and VL chains as single-chain variable fragments (scFv) fused with pIII minor capsid proteins on the phage surface. Helper phages must also be added to *E. Coli* which carry the rest of the genes necessary for the full M13 bacteriophage. Ultimately, this produces a diverse phage library where each one displays modified surface proteins corresponding to a

particular antibody variant as well as containing the identifying genetic code in the vector within [23].

Immobilizing target proteins on microtiter plate

To select for specificity to A30P oligomers, cells from the neuronal tissue of (Thy-1)-h[A30P] α -syn transgenic mice [28] are collected and the protein containing this particular mutation is purified in large quantities. This target protein is then placed in a hydrophilic microtiter plate for immobilization during phage display selection.

Bio-panning for high binding specificity

The phage library is added to the A30P-bound microtiter plate and incubated for binding to occur. Following interaction with the target protein, unbound phages are washed away. Bound phages remain attached to the dish to be selectively eluted and reproduced in a bacterium (Figure 2). This cyclic panning process is repeated several times (3-4 rounds) to improve the specificity of the phages to the A30P oligomers [23]. Eluted binding phages must then undergo sequencing to identify all the unique varieties based on the genetic code corresponding to their VH and VL chains.

Sequencing of novel antibody genes

To do so, the relevant antibody DNA sequence is excised from the phage display vector and PCR amplified for sequencing of the region of interest. The individual colonies can then be isolated from each other for the amplification of monoclonal phage pools for ELISA affinity testing.

Testing affinity using phage ELISA

To verify antigen binding affinity, we obtain measurements of K_D from various antibody-antigen complexes through a phage ELISA. This indirect assay involves incubating a monoclonal phage pool with an antigen of interest and then adding enzyme-bound antibodies that recognize and bind to the phage epitopes. This mixture is developed by addition of a substrate that induces a colorimetric change in the enzyme which can be quantified to determine binding rates of the phage to the antigen-coated plate [23]. This can be interpreted as the binding rate of the antibody fragments on the phage to that particular antigen (Figure 3). K_D values are calculated using the ratio of the antibody dissociation rate (K_{off}) and antibody association rates (K_{on}) to describe antibody affinity [29]. For our purposes, each monoclonal phage colony is divided into separate dishes to be tested with A30P oligomers, WT α -syn, A53T oligomers, and potentially other relevant fibrillar aggregates as needed. The original antibodies (mAb38F and mAb38E2) should also be expressed in monoclonal phage colonies to be tested under the same conditions as the control. There is an inverse relationship between K_D and antigen affinity, therefore the novel colonies are expected to display low K_D and therefore higher binding affinity for A30P oligomers as the antigen of interest [29].

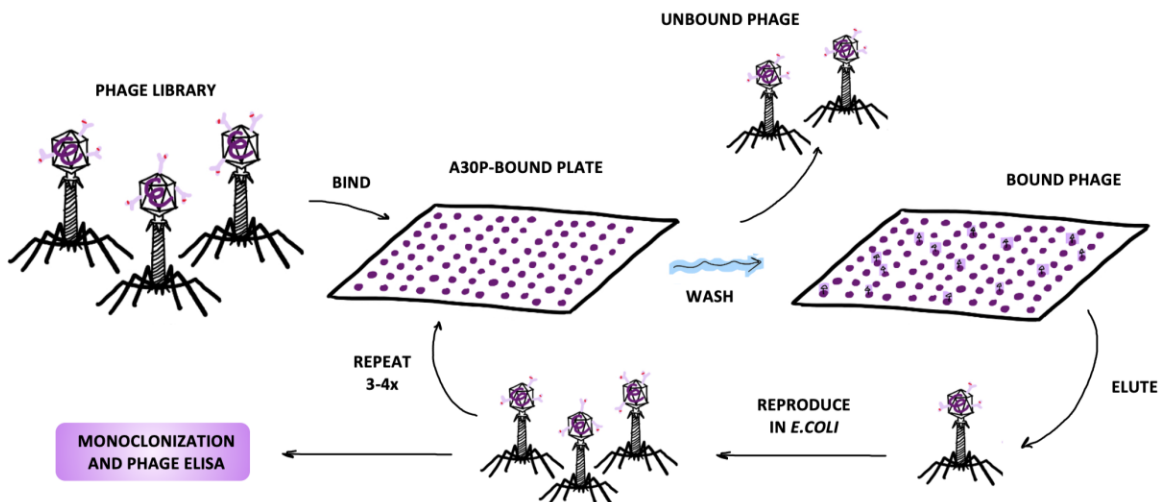


Figure 2. Bio-panning with an antibody library displayed on pIII

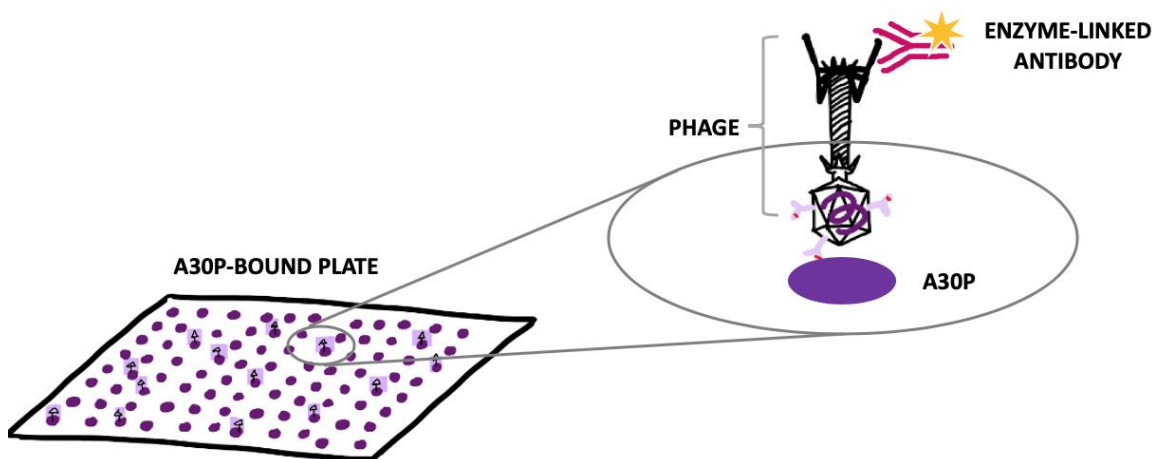


Figure 3. Graphic representation of a phage ELISA showing how antibody fragments expressed in phage surface proteins can bind to epitopes on A30P, which can be quantified using the enzymatic signal on the phage.

Analysis of results

As sequencing has already been performed before phage ELISA, we can refer to the corresponding genetic code and eventually use it to reproduce these A30P-specific recombinant antibodies in vitro.

Expected Results

Mutated Antibody Library Preparation

Analysis of DNA sequencing data from the CRISPR-X engineered hybridoma cells will allow us to identify which cells underwent mutagenesis. Only those that have successfully obtained a mutation will qualify for PCR amplification and phage display selection.

ELISA Dissociation Constants

The ideal antibodies for our purposes would display low K_D values when incubated with A30P oligomers (the antigen

of interest), indicating high binding affinity. Generally, this would be a value in the low micromolar (10^{-6}) to nanomolar (10^{-7} to 10^{-9}) range, as with most effective antibodies [29]. This number is expected to be lower than the original antibodies when incubated with A30P oligomers, thereby indicating improvement in specificity. Once identified, their genetic code is valuable for future reproduction of these antibodies for other uses and experiments.

Discussion

The results reported in this study have several potential implications for antibody design, immunotherapy and PD in general. The K_D values obtained through ELISA of various antibody-antigen complexes allow for comparison of the new antibodies to each other as well as to those that have been previously identified. If the K_D values corresponding to any of the novel antibodies are significantly lower than

those of the original antibodies when incubated with A30P, these methods can be considered promising for antibody optimization. To establish that specificity has also been improved, their K_D values should be significantly lower when incubated with A30P than other fibrillar aggregates or non-threatening forms of α -syn. However, the indirect nature of ELISA yields some limitations in the technique as the signal can be misleading in some cases. For example, if a given antibody has weak specificity this will result in a high general background signal that can be mistaken for high affinity for that antigen. Furthermore, weak binding strength will falsely result in a low signal after washing despite potentially high specificity [30]. We reduce these potential errors by comparing the K_D of these antibodies during incubation with a variety of antigens, as well as having the original mAb38F and mAb38E2 antibodies as the control condition.

Bio-panning for phage display selection also presents its own caveats as some antibody varieties may be lost in the process of building the phage library. This can be a result of some phages being unable to express the scFv as surface proteins due to possible errors in assembly resulting from the foreign nature of the fragment. It is also possible to lose variants during purification if there is incomplete recovery of the corresponding hybridoma RNA [23].

Increasing antibody selectivity would boost recognition of target antigens through passive immunity and thereby allow existing biological mechanisms such as intracellular proteasomes to degrade them with greater efficiency [31]. We see additional value in these methods for research purposes as they decrease the time and money expenditure normally required for monoclonal antibody production. By selectively degrading oligomeric A30P, the resultant antibodies are also relevant to further studies of PD. They can be used to confirm whether or not disease progression is dependent on the toxicity of this form of α -syn, by leaving other forms intact and observing the neurological effects. Further experimentation would be required to determine whether these antibodies are applicable to large-scale models for PD treatment, as it is difficult to predict whether an immune response will be mounted *in vivo*, given these results alone [32].

Conclusions

The proposed study aims to create antibodies that bind with high selectivity to A30P oligomeric α -syn, which is known to confer pathogenicity in PD. The procedure makes use of CRISPR-X and phage display selection to improve the specificity of existent antibodies in order to effectively target this particular form of α -syn. Designing antibodies via this method allows for large-scale production of stable recombinant antibodies that cannot be made endogenously. This provides a high level of transferability to a variety of contexts and may substitute current techniques that have not been viable for producing antibodies that are sufficiently specific. They are likely applicable to targeting

other α -syn mutants including the A53T oligomers that are similarly linked to early-onset PD.

Future application in mice models would investigate the antibodies' ability to degrade A30P oligomers *in vivo* by observing changes in motor and cognitive function. This could subsequently open up potential avenues for treatment of PD and other neurodegenerative disorders through immunotherapy.

List of Abbreviations Used

α -syn: alpha synuclein
AID: Activation induced cytidine deaminase
APD: antibody phage display
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
ELISA: enzyme-linked immunosorbent assay
gRNA: guide ribonucleic acid
 K_D : dissociation constant from enzyme-linked immunosorbent assay
 K_{off} : antibody dissociation rate
 K_{on} : antibody association rate
PAM: protospacer adjacent motif site
PCR: polymerase chain reaction
PD: Parkinson's disease
RASTA: Robust Adjustment of Sequence Tag Abundance
SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor
VL: variable light
VH: variable heavy
WT: wild-type

Conflicts of Interest

The author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

As a research protocol, this study did not require ethics approval and/or participant consent. If carried out, we would specify humane considerations and seek approval from an ethics board.

Authors' Contributions

IP: Contributed substantially to the conception and design of the study, drafted and revised the manuscript critically, produced relevant graphics, and gave final approval of the version to be published.

CO: Contributed substantially to the conception and design of the study, drafted and revised the manuscript critically, produced relevant graphics, and gave final approval of the version to be published.

Funding

This study was not funded.

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Article Information

Managing Editor: Jeremy Y. Ng

Peer Reviewers: Jordan Donders, Madison Best

Article Dates: Received May 09 20; Accepted Jul 16 20; Published Aug 17 20

Citation

Please cite this article as follows:

Pitigoi IC, Ostromecki CE. Using CRISPR-X for Optimization of Antibodies Towards A30P α -synuclein Oligomers in Immunotherapy of Parkinson's Disease. *URNCST Journal*. 2020 Aug 17: 4(7).

<https://urncst.com/index.php/urncst/article/view/188>

DOI Link: <https://doi.org/10.26685/urncst.188>

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