Rational design of antibodies targeting specific epitopes within intrinsically disordered proteins

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Antibodies are powerful tools in life sciences research, as well as in diagnostic and therapeutic applications, because of their ability to bind given molecules with high affinity and specificity. Using current methods, however, it is laborious and sometimes difficult to generate antibodies to target specific epitopes within a protein, in particular if these epitopes are not effective antigens. Here we present a method to rationally design antibodies to enable them to bind virtually any chosen disordered epitope in a protein. The procedure consists in the sequence-based design of one or more complementary peptides targeting a selected disordered epitope and the subsequent grafting of such peptides on an antibody scaffold. We illustrate the method by designing six single-domain antibodies to bind different epitopes within three disease-related intrinsically disordered proteins and peptides (α-synuclein, Aβ42, and IAPP). Our results show that all these designed antibodies bind their targets with good affinity and specificity. As an example of an application, we show that one of these antibodies inhibits the aggregation of α-synuclein at substoichiometric concentrations and that binding occurs at the selected epitope. Taken together, these results indicate that the design strategy that we propose makes it possible to obtain antibodies targeting given epitopes in disordered proteins or protein regions.

protein design | protein aggregation | complementary peptides

Antibodies are versatile molecules that are increasingly used in therapeutic and diagnostic applications, as they can be used to treat a wide range of diseases, including cancer and autoimmune disorders (1–5). These molecules can be obtained with well-established methods, such as immunization or phage and associated display methods, against a wide variety of targets (6–11). In some cases, however, these procedures may require significant amounts of time and resources, in particular if one is interested in targeting weakly immunogenic epitopes in protein molecules. In this work, we introduce a computational method of rational design of complementarity determining regions (CDRs) that makes it possible to obtain antibody against virtually any target epitope within intrinsically disordered peptides and proteins or within disordered regions in structured proteins.

Intrinsically disordered proteins, in particular, play major roles in a wide range of biochemical processes in living organisms. A range of recent studies has revealed that the functional diversity provided by disordered regions complements that of ordered regions of proteins, in particular in terms of key cellular functions such as signaling and regulation (12–18). The high flexibility and lack of stable secondary and tertiary structures allow intrinsically disordered proteins to have multiple interactions with multiple partners, often placing them at the hubs of protein–protein interaction networks (19–21). It has also been realized that the failure of the regulatory processes responsible for the correct behavior of intrinsically disordered proteins is associated with a variety of different pathological conditions (22–24). Indeed, intrinsic disorder is often observed in peptides and proteins implicated in a series of human conditions, including cancer, cardiovascular diseases, and neurodegenerative disorders (22–24). It would therefore be very helpful to develop methods to facilitate the generation of antibodies against disordered proteins, a goal that has a great therapeutic potential (25, 26).

Here, we address this problem by introducing a rational design procedure that enables one to obtain antibodies that bind specifically target disordered regions. This procedure is based on the identification of a peptide complementary to a target region and on its grafting on to the CDR of an antibody scaffold. Related methods of altering rationally antibodies have been discussed in the literature, which include the exploration of specificity-enhancing mutations (27, 28), the design of CDRs to bind structured epitopes (28, 29), and the grafting of peptides extracted from aggregation prone proteins (30–32) or from other antibodies (33) in the CDR of an antibody scaffold. Here we show that designed antibodies can be obtained by the method that we present for essentially any disordered epitope. We illustrate the method for the Aβ peptide, α-synuclein, and the islet amyloid polypeptide (IAPP, or amylin peptide), which are respectively involved in Alzheimer's and Parkinson's diseases and type II diabetes (24).

Results

In this work, we present a method of rational design of antibodies targeting chosen epitopes within disordered regions of peptides and proteins. We first describe the method and then present the results obtained to test it, which show that the designed antibodies bind with good affinity and specificity their target proteins.

Rational Design of Complementary Peptides. The first step in the rational design of antibodies involves the identification of peptides, called here complementary peptides, that bind with good specificity and affinity target regions of a protein molecule

Significance

Although antibodies can normally be obtained against a wide variety of antigens, there are still hard targets, including weakly immunogenic epitopes, which are not readily amenable to existing production techniques. In addition, such techniques can be relatively time-consuming and costly, especially if the screening for a specific epitope is required. In this work we describe a rational design method that enables one to obtain antibodies targeting any specific epitope within a disordered protein or disordered region. We show that this method can be used to target three disordered proteins and peptides associated with neurodegenerative and systemic misfolding diseases.

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(Fig. 1). The identification of these complementary peptides is based on the analysis of the interactions between amino acid sequences in the Protein Data Bank (PDB). More specifically, we exploit the availability of a large number of protein structures in the PDB to identify potential interaction partners (i.e., the complementary peptides) for any given target sequence. With this choice, the affinity and the specificity of the interactions between the complementary peptides and their targets are already proven in a biological context. The complementary peptides are built through a fragment-and-join procedure (*SI Materials and Methods*), starting from short peptides found to interact in a β-strand with segments of the target sequence in at least one of the protein structures in the PDB database. The peptide design procedure consists in two steps. First, we collect from the PDB database all protein sequences that face in a β-strand any subsequence of at least three residues of a given target epitope. Second, complementary peptides to the target epitope are built by merging together some of these sequence fragments using a cascade method (Fig. 1*A* and *SI Materials and Methods*). In essence, this cascade method starts from one of these fragments and grows it to the length of the target epitope by joining it with some of the others following three rules: (*i*) all fragments generating the same complementary peptide must come from β-strands of the same type (i.e., parallel or antiparallel), (*ii*) all fragments must partly overlap with their neighboring fragments, and (*iii*) the overlapping regions must be identical both in the sequence and in the backbone hydrogen bond pattern (Fig. 1*A* and Fig. S1). Given this design strategy, the resulting complementary peptides are expected to bind the target epitope by enforcing a β-strand–like conformation. Therefore, such complementary peptides will be particularly effective in binding solvent-exposed regions of protein sequences that do not form persistent hydrogen bonds with other parts of the protein, such as in the case of disordered regions. Alternatively, this method may be used to design complementary peptides against any region of a target protein, including regions in the core of the native state. Such peptides could be used for example for a peptide-based detection in diagnostic, as recently proposed with naturally occurring peptides (34). Once a complementary peptide has been designed, it can be grafted in place of the CDR loop of an antibody scaffold (Fig. 1*B*). We also note that such a peptide could be used on its own as a drug candidate. However, the grafting on an antibody scaffold offers several advantages over the use of a peptide molecule by itself. As therapeutic molecules, with respect to peptides, antibodies have a longer half-life in vivo (35) and often lower immunogenicity, at least for human scaffolds. Moreover, in both research and diagnostics, antibodies can readily be used in a large number of biochemical and biophysical assays in vitro, including Western blotting, immunoprecipitation, and confocal imaging.

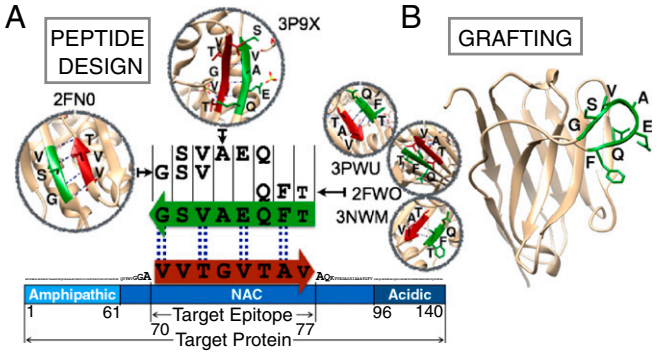

Fig. 1. Illustration of the method of designing antibodies targeting specific epitopes within disordered proteins. (*A*) Sequence-based design of complementary peptides. Sequence fragments in β-strand conformations are extracted from the PDB and combined using the cascade method to generate a peptide complementary to the target epitope (*SI Materials and Methods*). The example shows an antiparallel peptide for an epitope (residues 70–77) in the NAC region of α-synuclein. Dashed lines connect the amino acids predicted to form backbone-backbone hydrogen bonds. (*B*) The designed peptide is then grafted in place of the CDR loop of an antibody. In this example it is grafted in place of the CDR3 of a human single domain antibody scaffold (*SI Materials and Methods*). This example corresponds to DesAb-F in Table 1.

Generality of the Design Strategy of Complementary Peptides. Because the design of complementary peptides depends on the availability of specific sequences facing each other in a β-strand in the protein structures in the PDB, it may not always be possible to construct a complementary peptide for a given epitope. We thus asked how generally applicable our method is by investigating systematically how many complementary peptides can be found for all possible epitopes in a target protein. We ran the cascade method on each possible epitope of eight amino acids for three well-characterized and disease-related intrinsically disordered peptides and proteins: α-synuclein, Aβ42, and IAPP (24). Although other choices are possible, we used eight-residue epitopes because we reasoned that such complementary peptide size should be amenable for grafting in most antibody scaffolds, at least for the longer CDR loops. Consequently, it represents a good epitope size to assess the generality of the cascade method. Furthermore, naturally occurring amyloidogenic eight-residue peptides were found to be specific in recognizing their targets (34), suggesting that this is a convenient length for specific β-strand-like recognition. Our results show that more than 95% of the residue positions in these three proteins can be targeted with at least one peptide. Moreover, typically, the number of different complementary peptides covering one position is much larger than 1. We found that the median number is 200, and the mean is 570 (Fig. 2 *A*–*C*). Thus, at least in these three cases, our method can produce several complementary peptides to choose from for most target epitopes. Given these results, one can ask whether a given complementary peptide may have multiple possible target sequences, thus undermining the specificity of the interaction. We investigated this possibility by blasting all of the 15,587 eight-residue peptides shown in Fig. 2 *A*–*C* against the human proteome (*SI Materials and Methods*). The results show that only 0.2% of the designed peptides are actually found in the proteome, suggesting that the great majority of the complementary peptides will specifically interact with their targets, as also shown by the experimental tests below. To estimate the coverage at a proteomic scale, we ran the design method on two databases of disordered proteins. The first consists of all regions annotated as disordered in the DisProt database (36), whereas the second has been constructed by identifying disordered regions from measured NMR chemical shifts (37, 38). The dataset derived from DisProt included 980 different gapless disordered regions, whereas the one derived from the NMR chemical shifts 710. We found that 90% of the residue positions in the DisProt dataset and 85% in the chemical shift dataset are covered by at least one complementary peptide (Fig. 2*D*). Antiparallel peptides are more frequent than parallel peptides, reflecting the fact that parallel β-strands are less abundant than antiparallel ones in the PDB. An amino acid composition analysis (Fig. 2*E*) revealed that those positions that are not covered by any complementary peptide are highly enriched in proline residues ($\Delta f = 17\%$), in agreement with the observation that prolines disfavor secondary structure formation (37). Other amino acids preferentially found in regions not covered by complementary peptides, but to a much weaker extent, are aspartic acid ($\Delta f = 1.3\%$) methionine ($\Delta f = 1\%$), and glutamine ($\Delta f = 0.9\%$). Taken together, these results suggest that our

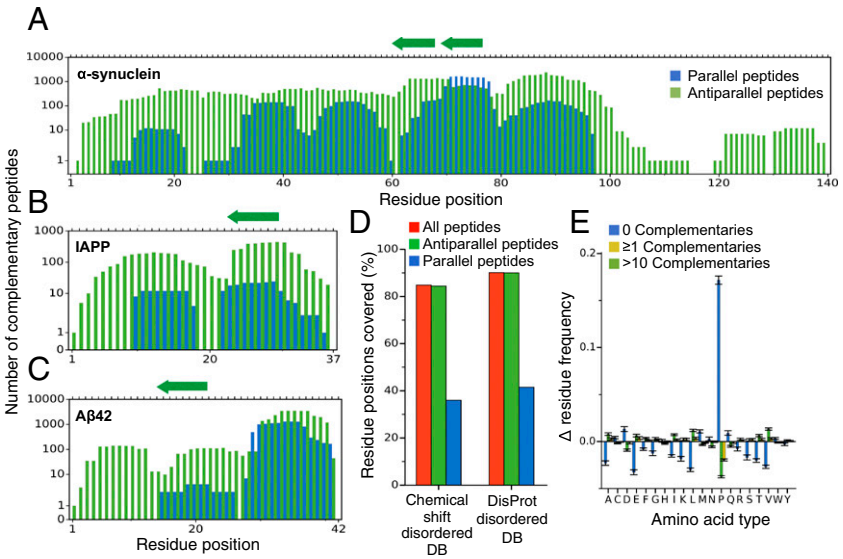

Fig. 2. Generality of the cascade method. (*A*–*C*) Coverage of α-synuclein (*A*), Aβ42 (*B*), and IAPP (*C*). For each residue in the sequence (*x* axis) we report the number of different complementary 8-residue peptides predicted to bind an epitope containing it. Peptides built from parallel β-strands are in blue and from antiparallel ones in green. The arrows on the top axis mark the positions of the peptides selected for experimental validation (Table 1). (*D*) Percentage of residues in the disordered regions of the δ2D database (37, 38) (*Left*) and of the DisProt database (36) (*Right*) covered by at least one complementary peptide. (*E*) Difference between the residue frequencies (*y* axis) observed in three classes of sequence regions within the two databases considered in *D* and those of the databases themselves. The classes are regions not covered by any complementary peptide (blue), by at least 1 complementary peptide (yellow) and by more than 10 complementary peptides (green).

design strategy is general and provides multiple candidates to choose from for most target epitopes (*SI Materials and Methods* and Fig. S2).

A Single Domain Antibody Scaffold for the Grafting of the Complementary Peptides. To assess the viability of the design method described above, we rationally designed antibodies targeting disordered proteins. First, we identified a stable antibody scaffold, tolerant to the grafting of peptide segments into one of the CDR loops. We selected a human heavy chain variable (VH) domain that is soluble and stable in the absence of a light chain partner, and whose folding is insensitive to mutations in its third CDR (CDR3) loop (39). Previous studies showed that this single domain antibody scaffold is relatively unaffected by insertions in its CDR3 (31). We found that this antibody is well expressed in bacteria (>5 mg/L), highly pure after a single chromatography step (>95% purity; *SI Materials and Methods*), and stable in its folded state (40).

Structural Integrity and Binding Capability of the Designed Antibody Variants. We designed complementary peptides for α-synuclein, Aβ42, and IAPP. The selected epitopes and the corresponding complementary peptides that we grafted in the CDR3 of the single domain antibody scaffolds (Fig. S3) are listed in Table 1. The purity of all of the designed antibodies (DesAb) was characterized by NuPAGE analysis (Fig. S4*A*) and their structural integrity by far-UV circular dichroism (CD) spectroscopy at 25 °C (*SI Materials and Methods* and Fig. S4*B*). All of the grafted variants showed high purity (>95%) and CD spectra compatible with the native-like structure of the single domain antibody scaffold. Therefore, we assessed the viability of the DesAb variants in binding their targets. To this end we used an ELISA test, which uses the basic immunology concept of an antigen binding to its specific antibody (41). We coated the wells with increasing amount of the designed antibodies, and then we incubated in the presence of a fixed amount of target protein (*SI Materials and Methods* and Fig. S5). All of the designed antibody variants showed a characteristic concentration-dependent curve, which is evidence of antibody–antigen binding (Fig. 3 *A*–*C*).

Specificity of the Designed Antibodies. The specificity of the DesAbs was assessed with a dot blot test by spotting different amounts of proteins from *Escherichia coli* cell lysates on a nitrocellulose membrane (*SI Materials and Methods*). The binding of three DesAb variants (DesAb-F, DesAb-Aβ, and DesAb-IAPP; Table 1) to lysates from cell lines where the expression of the antigen protein had been induced was compared with that to lysates where the expression had not been induced (Fig. 3 *D*–*F*). Because an *E. coli* cell line expressing IAPP was not available, 100 μM of synthetic IAPP was mixed to the *E. coli* lysate (+IAPP) before performing the experiment with DesAb-IAPP. The total protein amount of the lysate without IAPP (−IAPP) was adjusted accordingly. The results show that for all tested DesAb variants the intensity of the dots corresponding to cell lysates containing the target protein is always significantly greater than that of dots from lysates not containing it. Moreover, a control experiment performed with commercially available antibodies (C+ in Fig. 3 *D*–*F*; *SI Materials and Methods*) suggests that for α-synuclein and Aβ42, there may be a degree of basal expression of the antigen protein even without induced expression. As an additional control, we tested the cross-reactivity of the DesAb variants by probing with each designed antibody blots prepared with *E. coli* lysate mixed with equal concentrations of α-synuclein, Aβ, and IAPP, respectively (*SI Materials and Methods*). A clear trend is observed in this case as well, whereby each DesAb preferentially binds to its target (Fig. S6).

Detailed Characterization of DesAb-F. To obtain a more comprehensive characterization on the interaction of the designed antibody variants, we selected one (DesAb-F, with grafted sequence FQEAVSG; Table 1), for which we quantitatively assessed affinity, specificity, and effect on protein aggregation. To characterize the specificity of binding, in addition to the dot-blot test presented in Fig. 3 and Fig. S6, we quantified the

Table 1. List of target proteins, target epitopes and their sequences, designed complementary peptides, and designed antibodies (DesAb) used in this work for experimental validation

Target protein	Target epitope	Complementary peptide	DesAb
IAPP	23FGAILSS29	RLGVYQR	DesAb-IAPP
Aβ42	15QKLVFFA21	FKLSVIT	DesAb-Aβ
α-Synuclein	70VVTGVTA76	FQEAVSG	DesAb-F
α-Synuclein	61EQVTNVG67	DILVSYQ	DesAb-D
α-Synuclein	61EQNTNVG67	EILVSYQ	DesAb-E
α-Synuclein	65NVGGAVV TGVTAVA79	QEFVAAFSHTE +EVFQEAVSGS	Two-loop DesAb

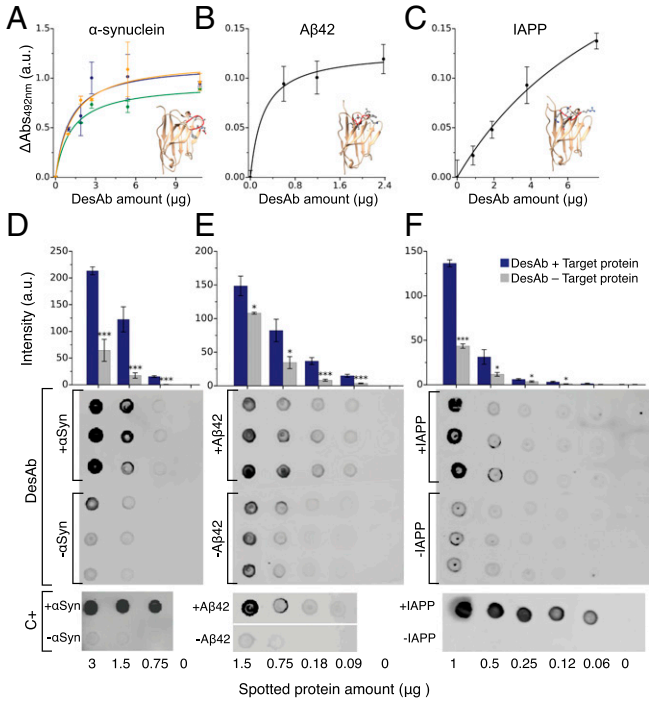

Fig. 3. Binding and specificity of the designed antibodies (DesAb). (*A–C*) ELISA test of the DesAbs in Table 1 with one complementary peptide grafted in the CDR3 that specifically target α-synuclein (*A*) (DesAb-D in green, DesAb-E in blue, and DesAb-F in orange), Aβ42 (*B*) (DesAb-Aβ), and IAPP (*C*) (DesAb-IAPP); the lines are a guide for the eye. Homology models of the structures of the designed antibodies are represented with the grafted complementary peptide in red. (*D–F*) Dot blot assay performed with three DesAb variants: DesAb-F (*D*), DesAb-Aβ (*E*), and DesAb-IAPP (*F*) and three commercially available antibodies used as a positive control (C+) for the binding to *E. coli* lysates from cell lines expressing the target protein (dots labeled with +, blue columns) and not expressing it (–, gray column). In the case of DesAb-IAPP, synthetic amylin peptide was mixed to the *E. coli* lysate (+IAPP) before performing the experiment, as a cell line expressing IAPP was not available. Protein amount is the micrograms of total protein (lysate) spotted on the membrane. The bar plot is a quantification of the intensities of the DesAb dot blots (*SI Materials and Methods*). Intensities are *>2 σ_{eq} away, ** > 3 σ_{eq}, and *** > 4 σ_{eq}, with $\sigma_{eq} = \sqrt{SE_+^2 + SE_-^2}$ and SE being the standard error from the intensities of the three dots.

reactivity against α-synuclein, Aβ42 peptide, and IAPP. Thus, we performed an ELISA in which we coated the wells of the ELISA plates with a given amount of DesAb-F and then we incubated in the presence of the same amount of the three different antigens (*SI Materials and Methods*). The amount of α-synuclein, Aβ42, and IAPP bound to DesAb-F was estimated measuring the absorbance at 492 nm after verifying that the primary antibodies exhibited similar reactivity against an equal amount of antigen absorbed to the ELISA well (Fig. S5). We found that DesAb-F clearly shows a preferential binding for α-synuclein than for Aβ42 peptide or IAPP (Fig. 4*A*). We then characterized in a more quantitative manner the binding constant of the antibody for monomeric α-synuclein. To do so, we assessed the ability of the antibody to bind a labeled variant of α-synuclein carrying the fluorophore dansyl (dansyl-α-synuclein) at position 90. Following a strategy already used for other systems (42, 43), the formation of the complex was studied by titrating increasing quantities of DesAb-F into solutions containing dansyl-α-synuclein and following the fluorescence properties of the dansyl moiety (Fig. 4*B*). The results of the titration experiments reveal that DesAb-F was able to bind α-synuclein with a K_d of 18 μM, derived assuming a single-site binding model (*SI Methods*). As the K_d value is highly sensitive to small displacements of the data points, we calculated the 95% CI on the fitting parameters with the bootstrap method (*SI Materials and Methods*), which placed the K_d between 11 and 27 μM. We note that this affinity, which is within a biologically relevant range but smaller than that of typical antibodies, has been reached by engineering only one loop of the antibody scaffold, whereas standard antibodies generally have more than two loops involved in antigen binding. Furthermore, a relatively high K_d can be effective in affecting protein aggregation (see below), because the antibody can actively interfere with the aggregation process rather than sequestering individual antigen monomers. Finally, to verify that DesAb-F binds specifically the chosen target epitope of α-synuclein, we generated one α-synuclein variant (α-synuclein-P73) with a proline residue inserted in the middle of the target epitope sequence (VVTGPVTA). The reason for this choice is that, if binding indeed occurs at this site, we expect such insertion to cause a significant inhibition of the interaction between the complementary peptide of DesAb-F and α-synuclein. Thus, we performed a florescence competition assay in the presence of 2 μM dansyl-α-synuclein and equimolar concentrations of nonlabeled α-synuclein WT or α-synuclein-P73 (*SI Materials and Methods*). In the presence of α-synuclein, the percentage of the complex DesAb-F:dansyl-α-synuclein decreased more than 50% in agreement with a competitive reversible inhibition (Fig. 4*C*). On the contrary, when the mutant variant α-synuclein-P73 was present in solution, no significant decrease was observed (Fig. 4*C*). The fact that α-synuclein-P73 was not able to compete with dansyl-α-synuclein for the binding to DesAb-F indicates that the proline insertion was able to disrupt the interaction between the designed antibody and α-synuclein, and, therefore, that the complementary peptide of DesAb-F is specifically binding to the region of α-synuclein containing the target epitope.

Antiaggregation Activity of DesAb-F. A general feature of amyloid-like aggregates is that they preferentially contain parallel β-sheet conformations (24), which, differently from β-sheets typically found within globular proteins, have one or more β-strands exposed to the solvent (i.e., the fibril elongation sites). Because the designed antibodies contain complementary peptides that enforce a β-strand conformation on their target sequence, we

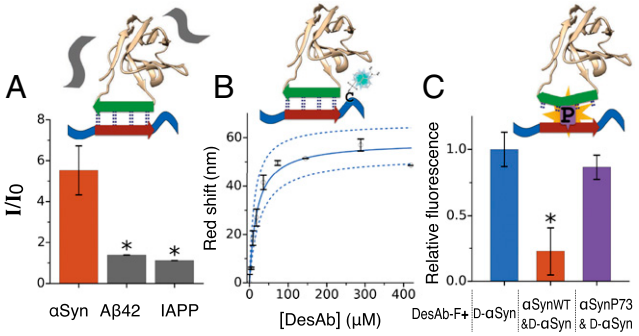

Fig. 4. Comprehensive characterization of the designed antibody DesAb-F. (*A*) The binding of DesAb-F to its target α-synuclein is much stronger than that for Aβ42 and IAPP; in the ELISA, we report the increase in the Abs_{490nm} in the three cases. (*B*) Fluorescence titration with dansylated α-synuclein in the presence of increasing concentrations of DesAb-F (following the red shift of λ_{max}). The solid blue line represents the best fit (K_d = 18 μM) using a single-binding model, and the broken lines the 95% CI on the fitting parameters (K_d between 11 and 27 μM). (*C*) Fluorescence competition assay; the *y* axis report the fraction of complex dansyl-α-synuclein:DesAb-F in the absence (blue) and presence of nonlabeled α-synuclein (red) or α-synuclein-P73 (purple). In *A* and *C*, the statistical significance of the difference with the first column was assessed with a Welch's *t* test (*$P < 0.05$).

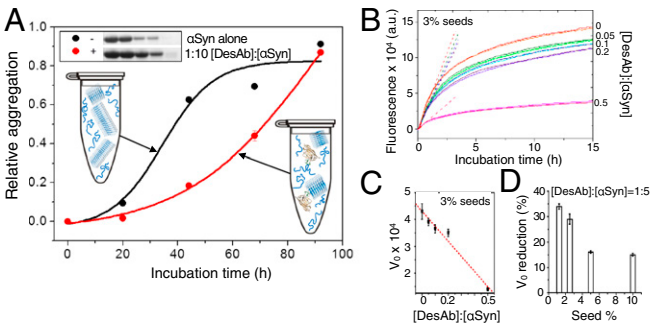

Fig. 5. The designed antibody DesAb-F inhibits α-synuclein aggregation. (*A*) Analysis on the soluble fraction of α-synuclein during its aggregation in the absence (black line) and presence (red line) of 1:10 molar ratio of DesAb-F:α-synuclein. (*B*) Seeded aggregation assay (3% seeds) at increasing molar ratios of DesAb-F (reported on the right axis). Different replicates for each condition are reported. (*C*) Initial growth rates over the molar ratios of single domain antibody scaffold. (*D*) Initial growth rate over the percentage of seeds in the presence of a fixed ratio of antibody (1:5).

expect that the affinity toward target proteins should be higher when these are found in aggregated species rather than as free monomers in solution, as the entropic cost of binding should be smaller in this case. By monitoring soluble α-synuclein over four-day aggregation (*SI Materials and Methods*), we found that DesAb-F has a strong inhibitory effect, even at a substoichiometric concentration (1:10) (Fig. 5*A*). This result suggests that this designed antibody preferentially binds aggregated species rather than to monomeric forms of α-synuclein. To support this conclusion, we performed seeded aggregation assays at increasing concentrations of DesAb-F (*SI Materials and Methods*). We found a specific concentration-dependent effect of the antibody on the elongation phase of α-synuclein aggregation (Fig. 5 *B* and *C*), and we also detected a strong dependence on the concentration of α-synuclein seeds (Fig. 5*D*). Besides, the fact that DesAb-IAPP only shows a negligible effect on the aggregation of α-synuclein, even at a 1:2 DesAb-monomer ratio (Fig. S7), suggests that the observed inhibition specifically comes from the grafted complementary peptide. Taken together, these data show that DesAb-F is able to reduce α-synuclein aggregation.

Affinity Increase by Grafting Two Complementary Peptides. Although the affinity of DesAb-F for monomeric α-synuclein ($K_d \sim 20$ μM) is probably ideal for inhibiting protein aggregation (see previous sections), it is still far from that of typical antibodies obtained with standard techniques. Because antibodies usually bind their antigens with more than one CDR loop, we decided to design an additional DesAb variant targeting α-synuclein with two loops engineered (two-loop DesAb in Table 1). These loops contain two complementary peptides predicted to cooperatively bind to the target epitope (*SI Materials and Methods* and Fig. S8). To add a second loop to our scaffold, we replaced 6 amino acids in the region of the CDR2 with 12 amino acids containing a complementary peptide (modeled structure in Fig. 6 and *SI Materials and Methods*). Thus, in the attempt of compensating for the impact on the domain stability, we changed the expression system to an *E. coli* strain that enables the formation of the intrachain disulphide bond (*SI Materials and Methods*), and we changed the purification protocol by eluting the protein with imidazole rather than at low pH. With this strategy, we were able to successfully purify the protein and confirm its structural integrity with far-UV CD (Fig. S8*E*). However, as we envisaged, this human single V_H domain with two extended loops is quite unstable, and for instance, it starts to precipitate at about pH 6. An advantage of this construct is that the binding site is now located between the CDR3 and CDR2, which is in close vicinity of residue Trp-47 on the scaffold (Fig. 6). This feature allows the binding to be measured in a label-free way by monitoring the change in the intensity of the intrinsic fluorescence of the DesAb at 348 nm, with varying concentration of WT α-synuclein, which does not contain Trp residues (*SI Materials and Methods*). The titration curve in Fig. 6*A* is best fitted with a K_d of 45 nM, and the 95% CI analysis places its upper value at 185 nM. For comparison, we performed the same type of experiment with the one-loop DesAb-F. In this case, the change in fluorescence is much weaker, probably because the binding site is further away from the fluorescent Trp on the DesAb (Fig. S9). The fitting of the titration curve gives a K_d of 5 μM, in agreement with the more accurate dansyl fluorescence estimate (Fig. 4*B*). In addition, we assessed the specificity of the two-loop DesAb with a dot-blot experiment as performed for the one-loop DesAb variants (*SI Materials and Methods*). The preferential binding for the cell lysate containing α-synuclein is apparent at a qualitative level (Fig. 6*B* and Fig. S6). Finally, we successfully employed the two-loop DesAb in the Western blot detection of its antigen protein (*SI Materials and Methods* and Fig. S10). No signal, however, was observed when probing the Western blot with the one-loop DesAb variants, probably because of the relatively low affinity of these variants and the fact that in a SDS/PAGE, the monomers preferentially populate elongated conformations, which may further weaken the interactions with the grafted complementary peptide. Because of its instability, the two-loop DesAb cannot be considered a viable antibody for most applications, but it represents a proof of principle that it is possible to greatly improve the affinity (by two or three orders of magnitude) in a rational way using our design method, by engineering two binding loops.

Conclusions

In this work, we have presented a method of rational design of antibodies, which works through a complementary peptide design and grafting procedure, to target specific epitopes within intrinsically disordered proteins. We have shown that this method generates antibodies that can bind with good specificity and affinity target regions in three disordered peptides and proteins associated with protein misfolding diseases and that they can be effective in reducing their aggregation. Compared with

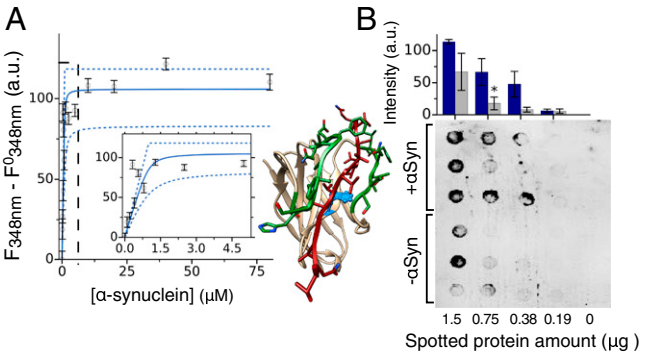

Fig. 6. Binding and specificity of the 2-loop DesAb. (*A*) Intrinsic fluorescence (Trp) titration assay performed at a constant concentration of two-loop DesAb (1 μM) and increasing concentration of α-synuclein (*x* axis). The solid blue line represents the best fit ($K_d = 45$ nM) and the broken lines the 95% CI on the fitting parameters (K_d up to 185 nM). (*Inset*) Zoom of the region highlighted by the dashed-black line. (*B*) Dot blot assay for the binding of the two-loop DesAb variant to an *E. coli* lysate from a cell line expressing α-synuclein (top three rows, blue column) and not expressing it (bottom three rows, gray columns). The structure shown is a coarse-grained model to illustrate the concept of two loops grafted into the sdAb scaffold, the complementary peptides are in green and the α-synuclein epitope (residues 64–80) in red, and Trp-47 is shown in light blue.

antibodies obtained with standard experimental techniques, however, our designed antibodies exhibit some limitations. The one-loop DesAb variants have relatively low affinity and selectivity, which may undermine their usefulness for some applications (e.g., Western blot detection). To improve on these aspects, we have shown that the simultaneous grafting of two complementary peptides can bring the affinity in the range of that of standard antibodies (Fig. S11) and lead to antigen detection in a Western blot, although this procedure also reduced the stability of the domain scaffold that we used here. We anticipate that our design strategy, and in particular the grafting of multiple loops, will be applicable to scaffolds that are intrinsically more stable than the human V_H domain that we used and can better tolerate loop insertions. Also, this rational design approach can be combined with existing in vitro affinity maturation techniques, such as error-prone PCR and phage display. We also suggest that the complementary peptide design strategy that we presented may be applied to rationally engineer interactions of other classes of proteins of biomedical and biotechnological interest.

Materials and Methods

The method of identifying complementary peptides and of grafting them on a single domain antibody scaffold is described in *SI Materials and Methods*. The method of protein expression is also described *SI Materials and Methods*. The details of all experimental assays are reported in *SI Materials and Methods*.

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