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Characterization of an antibody scFv that recognizes fibrillar insulin and β-amyloid using atomic force microscopy

Warren D. Marcus PhD,[a,b,*,1] Hongda Wang PhD,[a] Stuart M. Lindsay PhD,[a,c] Michael R. Sierks PhD[b]

[a]Department of Physics and Astronomy, Arizona State University, Tempe, Arizona, USA
[b]Department of Chemical and Materials Engineering, Arizona State University, Tempe, Arizona, USA
[c]The Biodesign Institute at Arizona State University, Arizona State University, Tempe, Arizona, USA

Abstract Fibrillar amyloid is the hallmark feature of many protein aggregation diseases, such as Alzheimer's and Parkinson's diseases. A monoclonal single-chain variable fragment (scFv) targeting insulin fibrils was isolated using phage display technology and an atomic force microscopy (AFM) mica substrate. Specific targeting of the scFv to insulin fibrils but not monomers or other small oligomeric forms, under similar conditions, was demonstrated both by enzyme-linked immunosorbent assays and AFM recognition imaging. The scFv also recognizes β-amyloid fibrils, a hallmark feature of Alzheimer's disease. The results suggest that the isolated scFv possibly targets a shared fibrillar motif—probably the cross-β-sheet characteristic of amyloid fibrils. The techniques outlined here provide additional tools to further study the process of fibril formation. The scFvs isolated can have potential use as diagnostic or therapeutic reagents for protein aggregation diseases.

Key words Amyloid fibrils; Single-chain variable fragment; Atomic force microscopy; Phage display; Cross-β-sheet

There are approximately 20 known diseases (such as Alzheimer's disease, Parkinson's disease, and type II diabetes) that are characterized by the aggregation of benign, physiological monomeric peptides/proteins into toxic oligomeric and/or fibrillar aggregates[1]. There is evidence that both oligomeric[2,3] and fibrillar[4] aggregates cause the characteristic cytotoxicity leading to some of these diseases. Therefore, there is considerable interest in understanding these aggregates and developing sensitive diagnostics and therapeutics that can target them for removal, either proteolytically or immunologically.

Insulin is a polypeptide hormone that regulates the metabolism of carbohydrates. A significant reduction in (or resistance to) the amount of insulin that the body produces is a hallmark of type II diabetes. The structure of insulin consists of two polypeptide chains (chain A consists of 21 amino acids, chain B has 30) linked by two disulfide bridges. The ability of insulin to readily aggregate into amyloid fibrils at acidic pH[5], as well as its affordability relative to other amyloidogenic proteins/peptides, made it an ideal target for this work.

Antibody phage display is a powerful technique for isolating antibodies against protein antigens[6]. Here, a synthetic antibody phage display library was used to isolate monoclonal single-chain variable fragments (scFvs) targeting insulin fibrils using mica, an atomic force microscopy (AFM) substrate, during the biopanning process. AFM recognition imaging[7,8] was used to verify specific binding of the scFv to the fibrillar form of insulin. The scFv also bound fibrillar β-amyloid, a protein correlated with Alzheimer's disease[9]. Although antibodies have been isolated against amyloid fibrils using conventional immunization protocols[10], the scFvs isolated

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*Corresponding author. Altor Bioscience Corporation, 2810 N Commerce Parkway, Miramar, Florida 33025, USA.
E-mail address: wdmarcus@alum.mit.edu (W.D. Marcus).
[1]No longer at Arizona State University but all work performed while there.

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here by phage display techniques provide several significant advantages, including these: (1) They can be readily affinity-matured to yield scFvs with high affinity and specificity[11]; (2) bispecific scFvs or diabodies[12] can be constructed that can modify, label, or clear the targeted amyloid fibrils; and (3) the scFvs can be expressed intracellularly as intrabodies[13], allowing the targeting of intracellular fibrillar aggregates.

Methods

Materials

The human single-fold scFv libraries I + J (Tomlinson I + J), *Escherichia coli* TG1 and HB2151, and KM13 helper phage were obtained from the Medical Research Council (MRC). Insulin (from bovine pancreas) was obtained from Sigma-Aldrich (St. Louis, Missouri).

Biopanning

Selection of phage against the fibrillar morphology of insulin was performed by incubating a 10-μL aliquot of 4×10^{12} plaque-forming units (pfu)/mL of phage obtained from the Tomlinson (I + J) library with a 10-μL sample of fibrillar insulin for 2 minutes. Presence of fibrillar insulin was verified by AFM. A 10-μL aliquot of the incubated solution was deposited on freshly cleaved mica and allowed to adsorb onto the mica surface for 5 minutes. The sample was subsequently washed 10 times with 1 mL phosphate-buffered saline (PBS)–Tween 0.1% and 15 times with PBS to eliminate the nonspecifically bound phage. The bound phage were eluted from the mica surface by incubation with triethylamine (7.18 M, pH 11) for 10 minutes, followed by neutralization with 1 M Tris-HCl, pH 7.4, and then hydrolysis with trypsin (1 mg/mL) and calcium chloride (1 mM) for 30 minutes. To determine the number of eluted phage, 1.2 mL of TG1 at $OD_{600} = 0.4$ was added to 500 μL of eluted phage and incubated for 30 minutes at 37 °C. The infected *E. coli* cells were plated in serial dilution on agar containing ampicillin (100 μg/mL) plates. Eluted phage were amplified by infection of fresh *E. coli* TG1 cells in the presence of helper phage KM13 (5×10^{10} pfu/mL) following the protocol provided on the MRC website (http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf). The phage were purified from the culture supernatant by polyethylene glycol (PEG) precipitation and resuspended in PBS before use in the next round of panning as described. We have successfully used this biopanning protocol to isolate scFvs specific to both fibrillar[14] and oligomeric[15] protein morphologies.

Monoclonal phage–scFv enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) were performed as described on the MRC website (http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf). Briefly, after three rounds of panning, individual clones in TG1 were grown in microtiter wells, and phage were produced by addition of KM13 helper phage. Antibody-coated phage were then added to corresponding wells of a coated (10 μg of target protein) plate and detected by an anti-M13 (horseradish peroxidase, HRP) antibody (GE Healthcare, Piscataway, New Jersey). For scFv ELISAs, the periplasmic fraction of induced HB2151 clones were added to the wells instead of phage. The Myc-tag site on the scFv was used for detection by the anti-Myc antibody (9E10) and a secondary goat anti-mouse IgG (HRP) antibody was used to label the anti-Myc antibody. Both antibodies are from Santa Cruz Biotechnology (Santa Cruz, California).

Preparation of periplasmic fraction

The periplasmic fraction of an induced overnight culture was prepared by resuspending the bacterial pellet in a 1:20 (v/v) amount of TSE (50 mM Tris, 20% sucrose, 1 mM EDTA, pH 7.5) for 30 minutes at 4°C. This mixture was then spun down for 30 minutes at 10,000 *g*, and the periplasmic fraction was collected in the supernatant.

Site-directed mutagenesis

Mutagenesis was performed essentially as described[7]. Briefly, primers were designed to change the spurious amber codon (TAG) to CAG (glutamine) at amino acid position His53 (http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf) by using the 21 nucleotides flanking the amber codon in the heavy chain: forward (5′-TGG GTC TCA GGT ATT GAT CCT **C**AG GGT CTG AGG ACA GTG TAC GCA-3′) and reverse (5′-TGC GTA CAC TGT CCT CAG ACC CTG AGG ATC AAT ACC TGA GAC CCA-3′). The following mixture was added to a polymerase chain reaction tube on ice: 33 μL of water, 2 μL of 50 mM $MgSO_4$, 5 μL of 10× *Pfx* amplification buffer, 4 μL dNTPs, 1 μL of each mutagenesis primer, 5 μL of isolated plasmid, and 2 μL platinum *Pfx* polymerase. The following thermocycler conditions were used: initial cycle for 60 seconds and 94°C, then 12 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and an extension time of 10 minutes at 68°C (extension time is based on 2 minutes per kilobase pair of plasmid). A 1-μL aliquot of *Dpn*I and 5.7 μ L of NEBuffer 4 were added to the polymerase chain reaction mixture and incubated at 37°C for 1 hour. A 200-μL aliquot of chemically competent HB2151 cells were added to the mixture. The mixture was placed on ice for 30 minutes, at 42°C for 3 minutes to heat-shock the culture, and then placed in ice for 3 minutes. A 100-μL aliquot of the mixture was plated onto Luria-Bertani agar plates (supplemented with 100 μg/mL of ampicillin) and grown overnight at 37°C.

Dot blot

A 2-μL aliquot of sample was placed on a nitrocellulose membrane. The membrane was blocked with 2% milk in PBS pH 7.4 for 1 hour at room temperature (~22°C). The membrane was washed once with PBS and then stained with

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anti-Myc-tag mouse antibody (Santa Cruz Biotechnology, Santa Cruz, California) at 1:500 dilution in PBS for 1 hour at room temperature. The membrane was washed three times with PBS and then stained with a goat anti-mouse IgG HRP conjugate antibody at 1:1000 dilution in 2% Milk PBS (MPBS) for 1 hour at room temperature. The membrane was then washed three times with PBS and developed using diaminobenzidine substrate (Sigma).

Purification of G5 scFv

A 1-L culture of *E. coli* HB2151 containing the G5 (anti–insulin fibril) scFv phagemid was grown (shaking at 250 rpm at 37°C) in 2× TY and 100 μg/mL ampicillin to $OD_{600} = 0.5$. A 1-mL aliquot of 1 M IPTG was added to the culture to induce scFv expression. The culture was grown overnight (shaking at 250 rpm) at 30°C. The following day the culture was spun down at 10,000 *g* for 10 minutes. The pellet was resuspended in 50 mL of the osmotic shock buffer TSE (50 mM Tris, 20% sucrose, 1 mM EDTA, pH 7.5) for 30 minutes at 4°C. The buffer was spun down at 10,000 *g* for 30 minutes. The supernatant was added to the sample loop of the ÄKTA FPLC system (Amersham Pharmacia Biotech, Piscataway, New Jersey), and the scFv was purified using a HiTrap Protein A (5 mL) column. Glycine (0.2 M, pH 3.0) was used to elute the scFv from the column. After collection of eluted peaks, the pH was immediately neutralized by addition of NaOH. The purified sample was then dialyzed overnight in PBS and purity-checked by sodium dodecyl sulfate gel electrophoresis. The scFv concentration was determined using a BCA protein assay kit (Pierce, Rockford, Illinois).

Sample preparation

Fibrillar insulin was prepared by dissolving 1 mg of lyophilized insulin into a 1-mL solution of 40 mM HCl for 24 hours at 60°C. Human Swi/Snf, a chromatin-regulating complex, was prepared as described[16]. The human Swi/Snf was deposited on glutaraldehyde aminopropyltriethoxysilane (GD-AP TES)–treated mica, derivatized at 1 μM levels with GD as described[17], and allowed to adsorb for 40 minutes. Fibrillar β-amyloid samples were prepared as described[18].

AFM recognition imaging

The anti–fibrillar insulin scFv, G5, and the control anti-BRG1 scFv were tethered to silicon nitride cantilevers and used to generate recognition images essentially as described[8]. Briefly, antibodies were thiolated and attached to a PEG tether on the end of the AFM probe. Amination of the probe was carried out by exposing an ultraviolet-cleaned silicone nitride probe to aminopropyltriethoxysilane vapor for 1 hour. The recognition signal was obtained by PicoTREC (Molecular Imaging, Tempe, Arizona). Magnetized cantilevers are driven by a MacMode dynamic-force microscope (Molecular Imaging). Images were taken in 10 mM NaCl–5 mM phosphate buffer, pH 7.5, with 3-nm peak-to-peak amplitude oscillation at 8 kHz, imaging at 70% setpoint, and scanning at 1 Hz. The recognition and topographical images were obtained simultaneously.

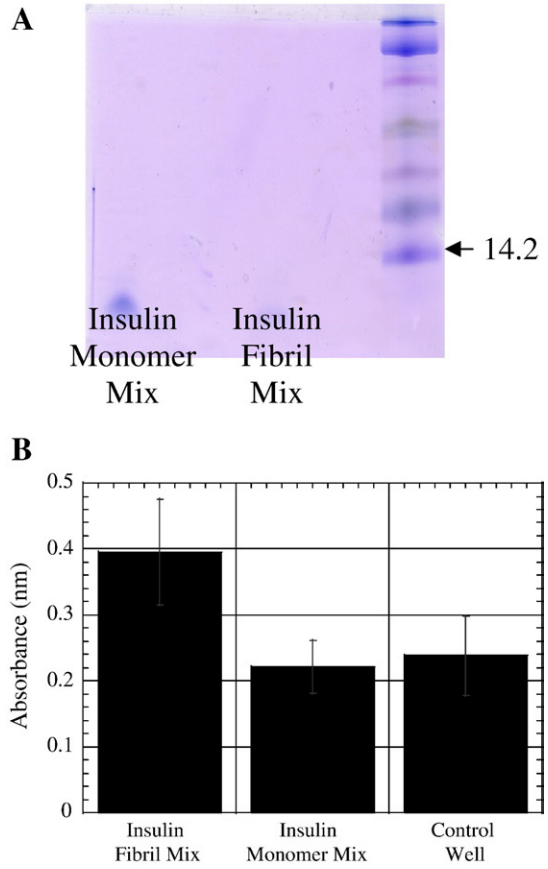

Figure 1. Protein and antibody characterization. **A,** Monomeric fraction and fibrillar fraction of insulin, used to coat ELISA wells, separated on a tricine gel. **B,** An scFv ELISA result using the G5 scFv in wells coated as labeled above. The control wells are coated with 2% MPBS. The results are an average of five replicates, with $P < .005$ for the fibrillar and monomeric mix values.

Results

Insulin sample characterizations

The prepared "monomeric" and "fibrillar" insulin samples were characterized by electrophoresis on a 10% acrylamide gel in a tricine buffer. The monomeric sample showed a dominant broad band below 14.2 kDa, whereas the fibrillar sample showed only a faint band at a similar size with the bulk of the sample remaining in the loading well (Figure 1, *A*). A more sensitive silver stain development protocol indicated that the broad band seen with the "monomeric" sample contained four bands corresponding to monomer, dimer, trimer, and tetramer forms of insulin (data not shown), indicating some oligomerization of the sample immediately after dissolving insulin powder in 40 mM HCl. Fibrillar samples were also verified by AFM imaging.

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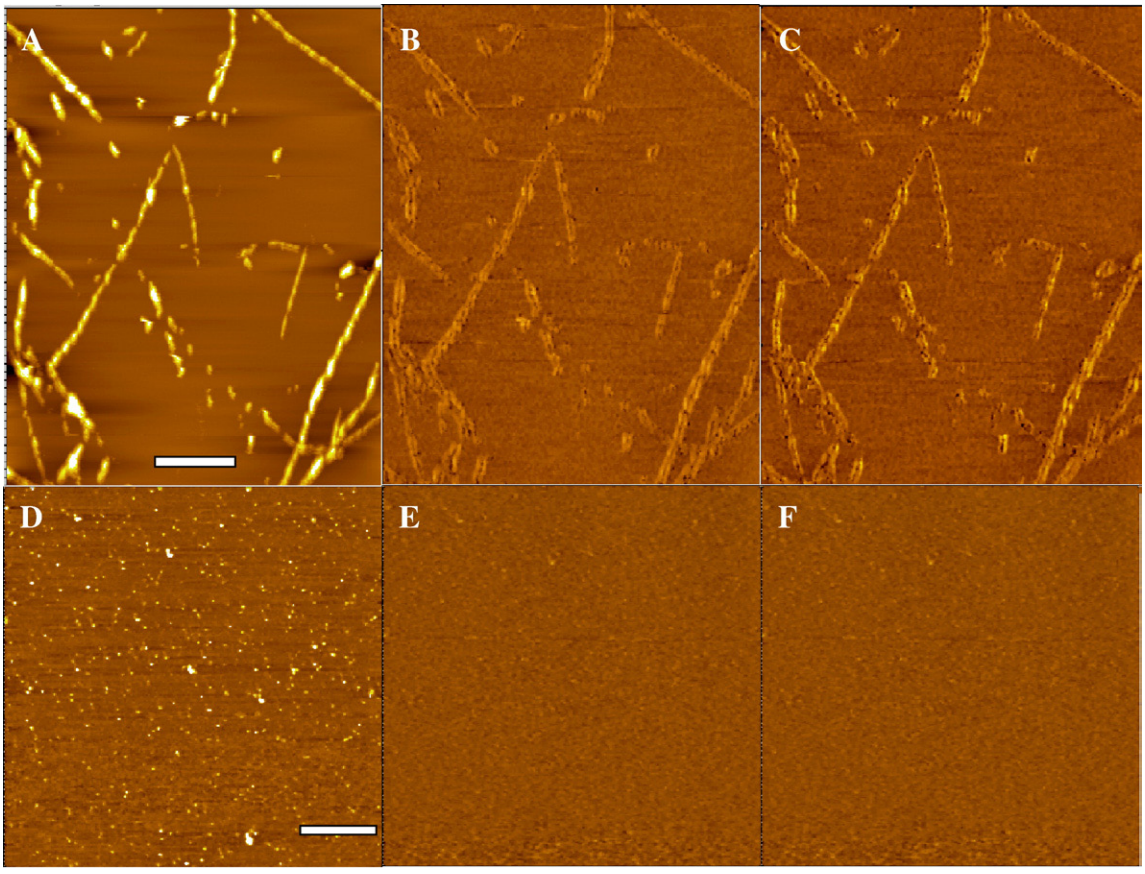

Figure 2. AFM recognition imaging of insulin by the G5 scFv. **A,** A representative topographical image of insulin fibrils, with the recognition by the G5 scFv shown in black on scan **B**, representing the recognition image scanned from left to right, and on scan **C** representing the recognition image scanned from right to left. **D,** A representative topographical image of monomeric/small aggregates of insulin, with the recognition scans shown in **E** and **F**. Scale bars on topographical scans = 400 nm.

Isolation of scFvs targeting insulin fibrils

After three rounds of biopanning with the Tomlinson (I + J) scFv library against insulin fibrils that were deposited on freshly cleaved mica, individual clones were selected and binding specificity for insulin fibrils determined by monoclonal phage ELISA (data not shown). The clone having the highest ELISA signal, G5, was selected for further characterization.

The DNA sequence of the G5 scFv indicated the presence of a spurious amber stop codon (TAG) at amino acid position His53, one of the randomized residues of the heavy chain. Site-directed mutagenesis was used to correct the gene sequence, changing the amber codon to glutamine (CAG). The corrected gene was then transformed into *E. coli* bacteria HB2151 for induction and soluble secretion of scFvs. Soluble secretion of the G5 scFvs was verified by dot blot analysis. Binding specificity of the soluble G5 scFvs for the different forms of insulin was determined by soluble ELISA using the periplasmic fraction of the induced corrected G5 clone. Similar amounts of fibrillar and monomeric insulin were coated to the ELISA plates (verified by BCA analysis of wells; data not shown). There is significant binding of the G5 scFvs to the fibrillar sample when compared with the monomeric sample ($P < 0.005$). However, there was no significant binding of the monomeric insulin sample when compared to the control well, which contains no insulin (Figure 1, *B*).

AFM recognition imaging of insulin fibrils

To demonstrate that the scFv binds insulin fibrils, we used AFM recognition imaging as previously described[7,8]. Insulin fibrils were deposited on freshly cleaved mica and imaged topographically (Figure 2, *A*). The G5 scFv was immobilized on the AFM tip and was used for recognition imaging with directional scans going from left to right (Figure 2, *B*) and right to left (Figure 2, *C*). The black areas seen on the recognition images represent specific antibody-antigen interactions.

We then tested whether the scFv would recognize nonfibrillar forms of insulin. A fresh sample of insulin dissolved in 40 mM HCl was placed on freshly cleaved mica and imaged both topographically (Figure 2, *D*) and with recognition imaging (Figure 2, *E* and *F*) using the same AFM tip used in the fibrillar studies above. AFM recognition imaging did not show any interaction between the immobilized scFv and the monomeric, dimeric, trimeric, and tetrameric insulin forms

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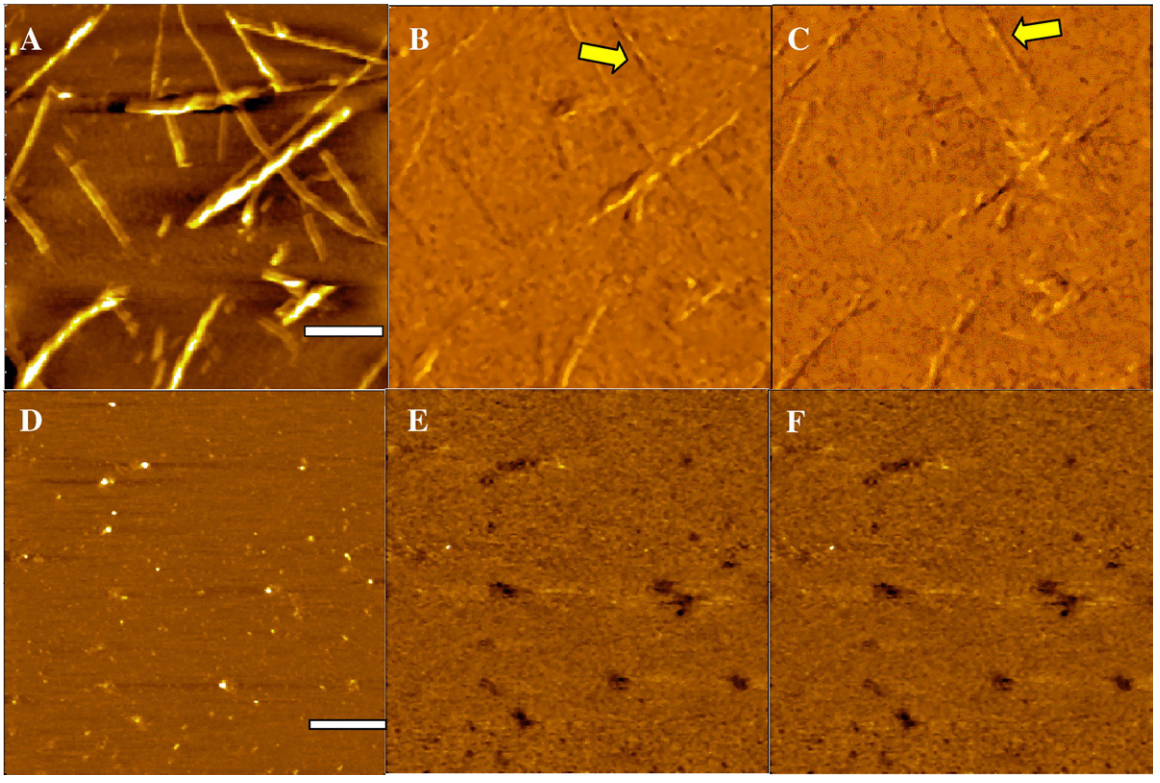

Figure 3. Control recognition images. **A,** A representative topographical image of insulin fibrils, with apparent recognition by the irrelevant anti-BRG1 scFv shown in black on scan **B** representing the recognition image scanned from left to right and on scan **C** representing the recognition image scanned from right to left. The yellow arrows point out the shifting "recognition" for a particular fibril that occurs depending on the direction of the scan. The anti-BRG1 scFv was then used for (**D**) topographical scans and for (**E** and **F**) recognition imaging of Swi/Snf. Scale bar = 400 nm.

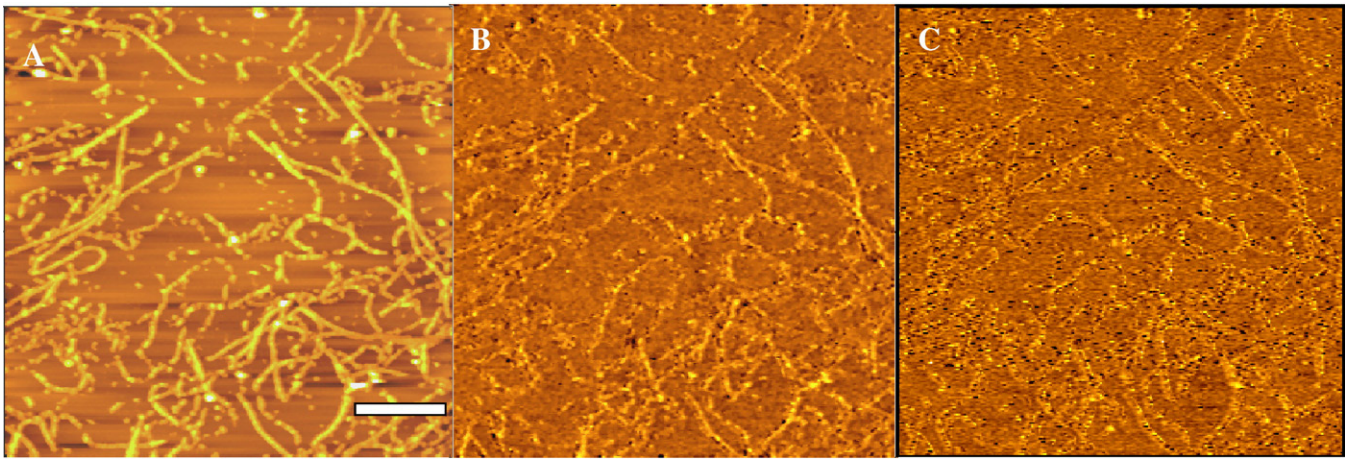

Figure 4. AFM recognition imaging of β-amyloid fibrils by the G5 scFv. This representative scan shows β-amyloid imaged (**A**) topographically, with recognition shown as black spots, (**B**) scanned from left to right, and (**C**) scanned from right to left. Scale bar = 400 nm.

(as determined by tricine gel separation of the "monomeric" sample in Figure 1, *A* and silver staining; not shown).

Control recognition imaging data

To verify that a control scFv not specific to fibrils did not also bind to fibrillar insulin, we again deposited insulin fibrils on freshly cleaved mica and imaged them both topographically (Figure 3, *A*) and by recognition imaging using an AFM tip with an anti-BRG1 scFv immobilized, scanning from left to right (Figure 3, *B*) and from right to left (Figure 3, *C*). The Brahma-related gene 1 (*BRG1*) protein is a subunit of Swi/Snf. Recognition areas shown in

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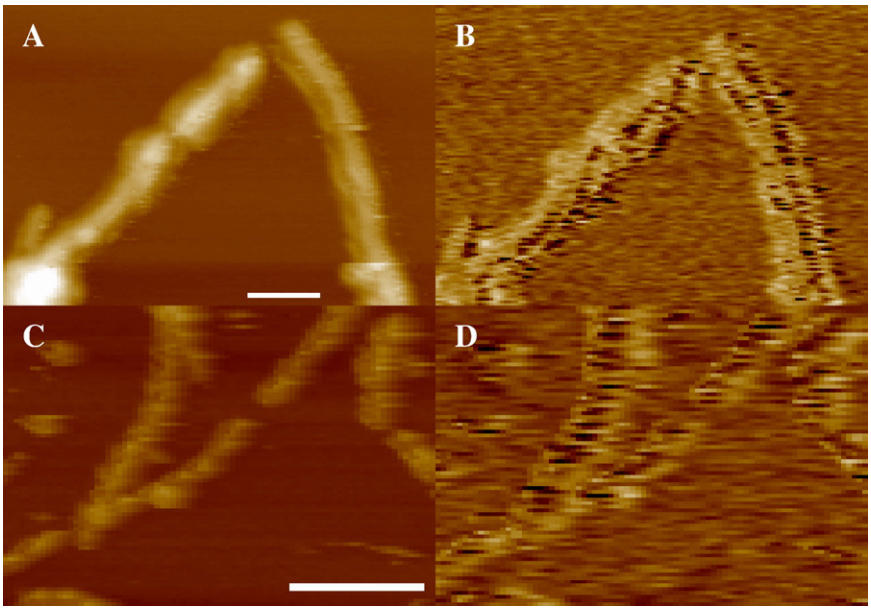

Figure 5. Higher magnification images of an insulin fibril imaged (**A**) topographically or by (**B**) recognition imaging; and β-amyloid fibrils imaged (**C**) topographically and by (**D**) recognition imaging. Periodicity is determined by the average distance between near pairs of black dots. Scale bar = 50 nm.

black are observed in each directional scan, however these areas shift substantially according to the direction of the scan (as demonstrated by the yellow arrows). The shift in recognition area to the leading edge of the fibrils seen with the control but not the G5 scFv indicates that this interaction with the control scFv is an artifact of the recognition imaging process. To ensure that the lack of specific recognition patterns observed with the control tip was not due to an absence of functional antibody immobilized on the AFM tip, we replaced the mica substrate containing insulin fibrils with a GD-AP TES–treated mica substrate containing deposited human Swi/Snf, a demonstrated target antigen for the anti-BRG1 scFv[7]. The anti-BRG1-coated control tip showed substantial specific recognition to its target Swi/Snf antigen, as shown by the images in Figure 3, *E* and *F*.

AFM recognition imaging of β-amyloid fibrils

To test whether the G5 scFv would also recognize amyloid fibrils formed by an unrelated protein, we studied the force interactions between the scFv and β-amyloid, a protein implicated in the etiology of Alzheimer's disease[9]. β-amyloid (1-42) fibrils were deposited on freshly cleaved mica and imaged topographically (Figure 4, *A*). Recognition imaging of the fibrils was performed by scanning from left to right (Figure 4, *B*) and from right to left (Figure 4, *C*). Periodicity in the recognition spots obtained with both the insulin and β-amyloid fibrils can be observed. The average spacing between recognition spots as calculated by measuring the distances between over 100 different pairs of spots is 22 ± 7 nm and 11 ± 4 nm for insulin and β-amyloid fibrils, respectively (Figure 5). The periodicity of the black recognition spots was similar to those seen on insulin fibrils (Figure 2, *B* and *C*), demonstrating specific interactions.

Discussion

Aggregation of physiological, monomeric proteins into amyloid fibrils has been correlated with a number of different diseases including Alzheimer's disease, Parkinson's disease, and type II diabetes[1]. Understanding and preventing the formation of amyloid fibrils is an important goal in developing therapeutics for many of these diseases. Here we describe the isolation and characterization of a monoclonal scFv raised against fibrillar insulin that also targets β-amyloid fibrils.

A novel aspect of this research was the use of mica. Mica is well suited for phage biopanning, because phage do not adhere to a freshly cleaved mica surface, whereas many other proteins and protein aggregates, including amyloid fibrils, do. We could thus image the mica surface and verify that fibrils were on the surface, as opposed to assuming a fibrillar sample when using ELISA plates for biopanning. One could also monitor the biopanning process by using amplified phage from successive rounds of biopanning. Presumably, one would see an increase in the amount of bound phage after each round as specific clones are being isolated. However, we chose to do a phage titer instead of imaging as a time-saving procedure.

A control scFv that targets BRG1, instead of insulin, was used to verify that fibrillar insulin does not nonspecifically interact with the scFv/linker/AFM tip. Although several black areas (which typically represent antibody-antigen interaction) were observed (Figure 3, *B* and *C*), these areas only occurred

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along the leading edges of the fibrils, depending on the direction of the scan. Furthermore, no periodicity of binding was observed as seen with the G5 scFv. Therefore, the recognition areas observed with the control scFv represent an artifact arising from the topography of the fibrils. Presence of functional control anti-BRG1 scFv on the AFM tip was verified by recognition imaging with its target antigen, the BRG1 subunit of human Swi/Snf[7,19] (Figure 3, *E* and *F*). We postulate that the nonspecific recognition events observed at the leading edge of the fibrils during the scanning process is the result of the sharp change in height the AFM tip and the tethered scFv experience in going from scanning the mica surface to scanning the top surface of the fibrils. This artifactual recognition event is not observed when specific antibody-antigen interactions are present, as shown by the scans made of the fibrils with the G5 scFv-coated tip. Actual recognition events between the tip and fibrils may prevent the generation of artifactual recognition events, in that any height change–induced fluctuations of the tethered scFv on the tip are damped by binding to the immobilized fibrils.

Because antibodies against fibrils were previously shown to target a variety of fibrils regardless of protein composition[10], we tested the specificity of G5 scFvs for recognition to a different type of fibril formed from the protein β-amyloid (Figure 4, *B* and *C*). The G5 scFv shows periodicity in binding to both insulin and β-amyloid fibrils, indicating that the G5 scFv recognizes a shared motif between insulin and β-amyloid fibrils, probably the cross-β-sheet motif characteristic of amyloid fibrils[20]. Periodicity in amyloid fibril structure has been well documented as a result of packing of the 0.47-nm β-sheet structures, with reported periodicity measurements for islet amyloid propeptide and β-amyloid of 25 nm and 40 nm, respectively[21,22]. The measured periodicity values obtained here of 22 and 11 nm (Figure 5) for insulin and β-amyloid, respectively, are in the same range as reported before, indicating that recognition imaging can be a valuable tool for probing amyloid structure.

This work has outlined a protocol that researchers can adopt for studying protein aggregation. Because the recognition imaging can detect periodic binding to a particular site, antifibrillar antibodies and recognition imaging can be used to further probe the structure of amyloid fibrils. The scFvs can also serve as potential therapeutics aimed at targeting fibrils in vivo, because the scFv is based on a human antibody scaffold.

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