Published in final edited form as:

Proteomics. 2008 January ; 8(1): 100–105. doi:10.1002/pmic.200700772.

Use of 2-D DIGE analysis reveals altered phosphorylation in a tropomyosin mutant (Glu54Lys) linked to dilated cardiomyopathy

Chad M. Warren¹, Grace M. Arteaga¹, Sudarsan Rajan², Rafeeq P. H. Ahmed², David F. Wieczorek², and R. John Solaro¹

¹ Department of Physiology and Biophysics, Center for Cardiovascular Research, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA

² Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati Medical Center, Cincinnati, OH, USA

Abstract

Current electrophoretic methods have not been optimized to fully separate post-translationally modified mutant forms of tropomyosin (Tm) from wild-type cardiac samples. We describe here a method employing a modified 2-D PAGE/2-D DIGE protocol, to fully separate native, mutant (E54K), and phosphorylated forms of Tm. Our data demonstrate the first evidence of a significant (~40%) decrease in Tm phosphorylation in transgenic compared to non-transgenic mouse hearts, and indicate that altered phosphorylation may be a significant factor in the linkage of the E54K mutation to dilated cardiomyopathy.

Keywords

2-D DIGE; Familial cardiomyopathies; Kinases; Phosphatases; Sarcomere

We report here an adapted approach for separating charged forms of tropomyosin (Tm) existing in hearts of non-transgenic (NTG) mice and transgenic (TG) mice expressing a Tm mutant (E54K), which is linked to dilated cardiomyopathy (DCM). Linkage of mutations in sarcomeric proteins to hypertrophic (HCM) and dilated cardiomyopathies (DCM) is well established. However, the mechanisms by which a modification of contractile or regulatory proteins leads to remodeling, altered function and, in many cases sudden death, remains poorly understood. Among the various theories, an attractive hypothesis is that a primary trigger for these changes is an altered response of the sarcomeres to Ca^{2+} . For example, there are data demonstrating that DCM is linked to sarcomeric mutations inducing a depressed sarcomeric response to Ca^{2+} , whereas HCM are linked to an enhanced sarcomeric response to Ca^{2+} [1]. Our studies, which focused on Tm mutants linked to HCM, agree with this hypothesis [2,3]. More recently, we reported the first evidence that detergent extracted fiber bundles from hearts expressing the DCM-linked mutant α -Tm(E54K) demonstrate significantly depressed cardiac function and sarcomeric Ca^{2+} -sensitivity [4]. These data support the concept that structural modifications in Tm induce an altered sarcomeric response to Ca^{2+} that may be a significant primary cause of the altered function leading to remodeling of the myocardium.

Correspondence: Dr. R. John Solaro, Department of Physiology and Biophysics (M/C901), College of Medicine, 835 S. Wolcott Ave, University of Illinois at Chicago, Chicago, IL 60612, USA, E-mail: SolaroRJ@uic.edu, Fax: +1-312-996-1414.

The authors have declared no conflict of interest.

In considering the mechanisms by which mutations in sarcomeric proteins lead to an altered response to Ca^{2+} , it is now apparent that one must include the potential for associated PTM that may either exacerbate or ameliorate the effects of the mutation. We have reported that a mutation in cardiac troponin-C (TnC-G159D) linked to DCM blunts the Ca^{2+} -desensitization induced by PKA-dependent phosphorylation of cardiac troponin I (cTnI) [5]. We have also reported that PKC-phosphorylation of cTnI(R146G), an HCM-linked mutation, had a significantly smaller effect on cTnC structure, Ca^{2+} -binding and sarcomeric Ca^{2+} -sensitivity than phosphorylation of cTnI [6]. These findings indicated that tropomyosin phosphorylation (Tm-P) in hearts of TG mice expressing Tm(E54K) may be different from wild-type Tm. We therefore set out to adapt procedures for separating various charged forms of Tm along with their respective phosphorylated forms.

The high copy DCM TG $\alpha\alpha$ -Tm(E54K) and NTG lines used for this study were previously characterized [4]. The Institutional Animal Care and Use Committee approved the handling and maintenance of animals. The high copy DCM TG $\alpha\alpha$ -Tm(E54K) mutation is lethal shortly after 1 month of age. The hearts were extracted from animals of about 1 month of age due to the lethality of the mutation and the separated ventricles were immediately placed in liquid nitrogen. The liquid nitrogen frozen mouse ventricles were then used to prepare either myofibrils or ventricular homogenate samples.

Ventricular homogenates were prepared by cutting a small piece of ventricle tissue (~50 mg) and placing it in a 2-mL dounce homogenizer with universal sample buffer (8 M urea, 2 M thiourea, 4% CHAPS, and 10 mM EDTA pH 8.0) at a 1:20 w:v ratio. The tissues were homogenized first with pestle A (loose clearance) once and then pestle B (tighter clearance) twice with at least 15 strokes *per* homogenization cycle. The samples were clarified by microfuge centrifugation $18\,000 \times g$ for 10 min. Myofibrils were purified from the TG and NTG samples as previously described [7]. Myofibrils were also solubilized in universal sample buffer at a 1:20 w:v ratio using the dounce homogenizers similarly to ventricular homogenates. After clarification by microfuge centrifugation ($18\,000 \times g$ for 10 min), both myofibril and ventricular homogenate sample concentrations were determined using the RC-DC assay kit (Bio-Rad, Hercules, CA). Samples were diluted in universal sample buffer to 5 $\mu\text{g}/\mu\text{L}$ in a total volume of 50 μL . To insure a pH above pH 8.0, we added 1 μL of 1.5 M Tris-HCl pH 8.8 to the samples.

Samples used for 2-D DIGE were labeled by adding 100 pmoles of CyDye to 50 μg of total protein. The protein was labeled at room temperature for 2 h protected from light. After labeling, the reaction was quenched by adding 0.2 μL of 10 mM lysine. The samples were stored in the -80°C freezer until running the gels. The samples were randomly labeled with Cy3 and Cy5 in order to correct for any differences. NTG and TG samples were mixed with 340 μL of IEF buffer [8 M urea, 2 M thiourea, 4% w/v CHAPS, 10 mM EDTA pH 8.0, 250 mM DTT, 0.25% v/v 3–11 NL, 0.25% v/v 4.5–5.5 IPG buffer (GE Healthcare) and 2 mM tributylphosphine (TBP)] to give 10 μg total protein from each sample. Mixed sample was added to 18-cm IPG strips pH 4.5–5.5 (GE Healthcare) and rehydrated in the Bio-Rad Protean IEF cell, after which, the focusing method was initiated as follows: step 1, 250 V rapid ramp for 15 min; step 2, 10 000 V slow ramp for 2 h; step 3, 10 000 V rapid ramp for 45 000 Vh; and step 4, a hold at 500 V.

After focusing, both ends of the strip were cut to fit in the 2-D gel. Strips were equilibrated in 6 M urea, 2% SDS, 50 mM Tris-HCl, pH 8.8, 20% v/v glycerol, and 2% DTT for 15 min and then for additional 15 min placed in the same buffer except DTT was replaced with 2.5% iodoacetamide. Strips were placed onto the stacking gel without an agarose plug. The resolving gel was 12% total acrylamide, 0.5% bisacrylamide, 10% v/v glycerol [8,9], pH 8.8; the stacking gel was 2.95% acrylamide, 15% N,N'-diallyltartardiamide (DATD) [8,10], 10% v/v glycerol

[8,9], pH 6.8 and 0.01% bromophenol blue. The stacking gel composition used a relatively high concentration of DATD (15%) [10] for the special purpose of allowing for a lower total acrylamide concentration (2.95%) and retaining mechanical strength. The DATD crosslinker allows for a large pore size thus facilitating the movement of the protein from the IPG strip into the second dimension gel, while still allowing efficient stacking of the proteins. The inclusion of 10% glycerol into both the stacking and resolving gels helps with resolution of the spots due to the increased viscosity of the gel matrix and subsequent lower diffusion rate during the run [8,9].

A critical aspect was pouring the gels into 18 × 8-cm glass plates with custom-made 8 mm × 1 mm × 8 cm spacers. The spacers can be made easily by using Bio-Rad Protean II XL spacers and then cutting them to length for the 8-cm height of the plates. The importance of the gel size was to allow the use of a narrow pH range and an 18-cm strip with only cutting the ends of the strip allowing it to still fit on top of the second dimension gel. The separation of the NTG Tm from the Tm-P TG was not possible without the combination of the narrow range and length of the strip. We felt it was important not to cut the strip in the middle due to possible migration differences between the pieces allowing for additional inconsistencies between gels. To separate TnT and Tm species we had to utilize 16 cm of the strip, which will just fit the width of the gel. Due to the full utilization of the gel there was no space available to run an external size standard in a separate lane. However, identified spots within the gel can serve as an internal size standard. The gel was run at 16 mA with constant cooling (8°C) in a Hoefer SE600 unit until the dye front reached the bottom of the gel.

The gels were washed in ddH₂O twice for 30 min and then imaged using a Typhoon 9410 Imager (GE Healthcare) with Cy3 (532-nm laser) and Cy5 (633-nm laser) at a resolution of 100 μm. Gels were stained with Pro-Q Diamond stain [7] (Molecular Probes, Eugene, OR) and imaged with the Typhoon 9410. After staining with Pro-Q diamond stain, the gels were silver-stained as previously described [11].

SDS-PAGE gels and transfer onto PVDF membranes were performed as described [7,8]. The primary antibodies and dilutions used were tropomyosin-CH1 1:500 (Iowa Hybridoma Bank), TnI-pan M46 1:2500 (Fitzgerald), MYBP-C-pan 1:80 000 (a generous gift of Dr. Richard Moss), phospho-cTnI 1:500 (Cell Signaling), phospho-serine PKC-substrate 1:500 (Cell Signaling), phospho-PKA-substrate 1:500 (Cell Signaling), and a rabbit phospho-serine-283-specific Tm (custom produced by 21st Century Biochemicals) 1:100 000. The secondary antibodies and dilutions used were antimouse-IgG (FAB) specific 1:80 000 Sigma and anti-rabbit-IgG 1:30 000 (GE Healthcare). The immunoblots were processed and developed as previously described [7].

All data are represented as means ± SEM with a level of significance set at $p < 0.05$. The 2-D DIGE gel spots were analyzed using PDQuest (Bio-Rad), which determined the quantity of the spots and Excel (Microsoft) was used to ratio the quantities and perform *t*-tests. The ratio of Tm-P to total Tm was used to get the percent of Tm that was phosphorylated. The percent reduction from NTG was calculated by $(\%NTG - \%TG)/\%NTG$. The immunoblots were analyzed with Imagequant v5.2 (GE Healthcare) to obtain the band intensities and Excel (Microsoft) was used to ratio and perform *t*-tests.

TG and NTG samples were labeled separately and equally mixed and separated on a modified 2-D PAGE gel cropped to only show region of interest as shown in Fig. 1A. The samples were equally mixed in order to have all species represented for subsequent Pro-Q Diamond staining and immunoblots to show which spots are phosphorylated and identify the Tm spots. The phosphorylated proteins were identified using Pro-Q Diamond stain, which specifically detects phosphorylated proteins (Fig. 1B). The 2-D immunoblot of a TG sample (Fig. 1C) identified

all four spots as Tm. In Fig. 2A an NTG sample labeled with Cy3 had two spots, and in (Fig. 2B), a TG sample labeled with Cy5 had four spots. TG samples had four spots (Fig. 2B) because there was approximately 50% replacement of the endogenous Tm with the high copy mutant E54K in the myofilaments [4]. The E54K mutation induced a strong positive charge, which causes the phosphorylated and non-phosphorylated mutated protein to migrate at a different isoelectric position on the gels. Due to only one additional P-Tm spot in the TG and one in the NTG that was identified as phosphorylated (Fig. 2B), there is likely only one phosphorylated site at position 283 in both NTG and TG [12]. Moreover, the phospho-specific Tm antibody (epitope site is phospho-serine-283) reacted with both spots, indicating that the TG and NTG are probably the same (Fig. 2D).

Using 2-D DIGE, we were able to clearly distinguish which spots were NTG and TG Tm when two channels were merged as demonstrated in Fig. 2C. For quantification, the channels were separated and analyzed in order to differentiate the NTG from the TG overlay (TG samples have about 50% NTG Tm). The benefit of using DIGE over other methods is that the gel-to-gel variation is significantly reduced due to the separation of both NTG and TG samples in the same gel.

Immunoblot analysis using a phospho-specific cTnI and MYBP-C (myosin binding protein C) antibody in conjunction with a pan TnI M46 and MYBP-C pan antibody did not indicate any significant differences between NTG and TG (Supporting Information Table 1). Based on our 2-D DIGE analysis, there were no significant differences in expression or phosphorylation of MLC-2 and TnT (troponin T) (Supporting Information Table 2). Data from the 2-D DIGE analysis of Tm from ventricular homogenates ($n = 8$) (Table 1) indicated that compared to the NTG Tm, there was a significant (~40%) decrease in mutant Tm(E54K) phosphorylation. Interestingly, the myofibrillar preparations ($n = 3$) analyzed in the same way as the ventricular homogenates had similar reductions in Tm-P (Table 1). Thus, it is apparent that Tm-P was similar in sarcomeric myofilaments and the ventricular homogenates (containing 50% exogenous Tm). We therefore conclude that the unincorporated cytoplasmic Tm-P was altered similarly to the sarcomeric Tm-P.

Our data are the first to demonstrate that altered phosphorylation of Tm may be an important aspect of the mechanisms linking the Tm(E54K) mutation to DCM. In our previous study, we reported that, when compared to NTG controls, detergent-extracted (skinned) fibers regulated by Tm(E54K) demonstrated a depression in maximum tension and Ca^{2+} -sensitivity [4]. The present data indicate that an altered PTM of Tm must be taken into account in the determination of the mechanism of the effect of Tm-E54K to depress the response of the myofilaments to Ca^{2+} .

Evidence of modifications in Tm-P and other charge modifications associated with altered cardiac function offers some insights into the significance of our data. Tm-P significantly increases in myocytes with development to the adult [13]. Compared to adults, tension development of neonatal heart myofilaments is more sensitive to Ca^{2+} [13]. There is also evidence that specific charge modifications associated with switching from alpha to beta Tm, also alters myofilament sensitivity to Ca^{2+} [14,15]. The charge differences in beta-Tm compared to alpha-Tm are a Ser for Glu at Tm-229 and a His for Asn at Tm-276. These amino acid substitutions make beta-Tm more negative than alpha-Tm. Thus, in both cases (phosphorylation and isoform switching), the more negatively charged Tm induces an increase in myofilament sensitivity to Ca^{2+} . The E54K mutation results in more positively charged Tm and an associated depression in myofilaments response to Ca^{2+} [4]. Our hypothesis is that the depression in Tm-P documented in the present study contributes to this depression in myofilament response to Ca^{2+} and to the overall pathology leading to DCM. We think it is significant that we found a similar depression in myofilament response to Ca^{2+} and a correlated

depression in Tm-P occurring in skinned fibers prepared from hearts of TG mice expressing active MKK6be, the upstream activator of p38 MAPK [16]. Activation of p38 MAPK also leads to DCM.

Although there is a lack of detailed knowledge regarding the molecular mechanisms by which phosphorylation affects the structure-function relations of Tm, there are some *in vitro* data relevant to our findings. The interpretation of these findings is couched in current knowledge regarding molecular mechanisms by which Ca^{2+} activates tension in cardiac myofilaments, which are reviewed in Kobayashi and Solaro [17]. Ca^{2+} binding to troponin C (TnC) releases a functional unit (actin:Tn:Tm in a 7:1:1 ratio) of the thin filament from a prevailing inhibition by promoting altered interactions of TnI (the inhibitory unit) with actin and TnT (the Tm-binding unit). These protein-protein interactions trigger a release of Tm from a position on the thin filament that hinders the actin-cross-bridge reaction. End-to-end interactions of Tm along the thin filament are also important in the spread of the activation of a functional unit to a near neighbor. N-terminal structural changes, which may alter the end-to-end interactions have been determined in Tm(E54K) [18]. Moreover, S283 the penultimate amino terminal residue is located in this region, which is not only important in end-to-end interactions of Tm, but also interacts with actin and TnT in sustaining activation. There is *in vitro* evidence from studies using reconstituted thin filament preparations that phosphorylation of Tm affects its affinity for TnT, and affects end-to-end interactions as determined by studies of Tm polymerization [19,20]. Moreover, Sano *et al.* [21], who employed an S283E mutation to mimic Tm-P, reported that α -TmS283E had a lower affinity for actin, while retaining an enhanced polymerization reported earlier [22]. Enhanced end-to-end interactions by phosphorylation of Tm as well the other effects noted above would be expected to ease activation of the myofilaments by Ca^{2+} . These effects of Tm-P fit with our hypothesis that Tm-P increases myofilament activity and that the fall in Tm-P associated with expression of the E54K mutant contributes to the depression in cardiac function that progresses to DCM.

Acknowledgements

Special thanks to Chao Yuan for many discussions pertaining to 2-D DIGE. This work was supported by National Institutes of Health Grants R37 HL22231 and P01-HL62426 (RJS), HL-71952 (DFW), and K01 HL-67709 (GMA).

References

1. Ashrafian H, Watkins H. Reviews of translational medicine and genomics in cardiovascular disease: new disease taxonomy and therapeutic implications cardiomyopathies: therapeutics based on molecular phenotype. *J Am Coll Cardiol* 2007;49:1251–1264. [PubMed: 17394955]
2. Muthuchamy M, Pieples K, Rethinasamy P, Hoit B, et al. Mouse model of a familial hypertrophic cardiomyopathy mutation in alpha-tropomyosin manifests cardiac dysfunction. *Circ Res* 1999;85:47–56. [PubMed: 10400910]
3. Prabhakar R, Boivin GP, Grupp IL, Hoit B, et al. A familial hypertrophic cardiomyopathy alpha-tropomyosin mutation causes severe cardiac hypertrophy and death in mice. *J Mol Cell Cardiol* 2001;33:1815–1828. [PubMed: 11603924]
4. Rajan S, Ahmed RP, Jagatheesan G, Petrashevskaya N, et al. Dilated cardiomyopathy mutant tropomyosin mice develop cardiac dysfunction with significantly decreased fractional shortening and myofilament calcium sensitivity. *Circ Res*. 2007
5. Biesiadecki BJ, Kobayashi T, Walker JS, Solaro RJ, de Tombe PP. The troponin C G159D mutation blunts myofilament desensitization induced by troponin I Ser23/24 phosphorylation. *Circ Res* 2007;100:1486–1493. [PubMed: 17446435]
6. Kobayashi T, Dong WJ, Burkart EM, Cheung HC, Solaro RJ. Effects of protein kinase C dependent phosphorylation and a familial hypertrophic cardiomyopathy-related mutation of cardiac troponin I on structural transition of troponin C and myofilament activation. *Biochemistry* 2004;43:5996–6004. [PubMed: 15147183]

7. Layland J, Cave AC, Warren C, Grieve DJ, et al. Protection against endotoxemia-induced contractile dysfunction in mice with cardiac-specific expression of slow skeletal troponin I. *FASEB J* 2005;19:1137–1139. [PubMed: 15855227]
8. Fritz JD, Swartz DR, Greaser ML. Factors affecting polyacrylamide gel electrophoresis and electroblotting of high-molecular-weight myofibrillar proteins. *Anal Biochem* 1989;180:205–210. [PubMed: 2817350]
9. Porzio MA, Pearson AM. Improved resolution of myofibrillar proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochim Biophys Acta* 1977;490:27–34. [PubMed: 836873]
10. Baumann G, Chrambach A. A highly crosslinked, transparent polyacrylamide gel with improved mechanical stability for use in isoelectric focusing and isotachopheresis. *Anal Biochem* 1976;70:32–38. [PubMed: 1259153]
11. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996;68:850–858. [PubMed: 8779443]
12. Mak A, Smillie LB, Barany M. Specific phosphorylation at serine-283 of alpha tropomyosin from frog skeletal and rabbit skeletal and cardiac muscle. *Proc Natl Acad Sci USA* 1978;75:3588–3592. [PubMed: 278975]
13. Heeley DA, Moir AJ, Perry SV. Phosphorylation of tropomyosin during development in mammalian striated muscle. *FEBS Lett* 1982;146:115–118. [PubMed: 7140972]
14. Gaffin RD, Gokulan K, Sacchetti JC, Hewett T, et al. Charged residue changes in the carboxy-terminus of alphanotropomyosin alter mouse cardiac muscle contractility. *J Physiol* 2004;556:531–543. [PubMed: 14766940]
15. Palmiter KA, Kitada Y, Muthuchamy M, Wieczorek DF, Solaro RJ. Exchange of beta- for alpha-tropomyosin in hearts of transgenic mice induces changes in thin filament response to Ca^{2+} , strong cross-bridge binding, and protein phosphorylation. *J Biol Chem* 1996;271:11611–11614. [PubMed: 8662805]
16. Vahebi S, Ota A, Li M, Warren CM, et al. p38-MAPK induced dephosphorylation of alpha-tropomyosin is associated with depression of myocardial sarcomeric tension and ATPase activity. *Circ Res* 2007;100:408–415. [PubMed: 17234967]
17. Kobayashi T, Solaro RJ. Calcium, thin filaments, and the integrative biology of cardiac contractility. *Annu Rev Physiol* 2005;67:39–67. [PubMed: 15709952]
18. Mirza M, Robinson P, Kremneva E, Copeland O, et al. The effect of mutations in alpha-tropomyosin (E40K and E54K) that cause familial dilated cardiomyopathy on the regulatory mechanism of cardiac muscle thin filaments. *J Biol Chem* 2007;282:13487–13497. [PubMed: 17360712]
19. Heeley DH. Investigation of the effects of phosphorylation of rabbit striated muscle alpha alpha-tropomyosin and rabbit skeletal muscle troponin-T. *Eur J Biochem* 1994;221:129–137. [PubMed: 8168502]
20. Heeley DH, Watson MH, Mak AS, Dubord P, Smillie LB. Effect of phosphorylation on the interaction and functional properties of rabbit striated muscle alpha alpha-tropomyosin. *J Biol Chem* 1989;264:2424–2430. [PubMed: 2521628]
21. Sano K, Maeda K, Oda T, Maeda Y. The effect of single residue substitutions of serine-283 on the strength of head-to-tail interaction and actin binding properties of rabbit skeletal muscle alpha-tropomyosin. *J Biochem (Tokyo)* 2000;127:1095–1102. [PubMed: 10833280]
22. Rao V, La Bonte LR, Xu Y, Yang Z, et al. Alterations to myofibrillar protein function in non-ischemic regions of the heart early after myocardial infarction. *Am J Physiol Heart Circ Physiol* 2007;293:H654–H659. [PubMed: 17400716]

Abbreviations

cTnI

cardiac troponin I

DATD

N,N'-diallyltartardiamide

DCM

	dilated cardiomyopathy
HCM	hypertrophic cardiomyopathy
MYBP-C	myosin binding protein C
NTG	nontransgenic
TG	transgenic
Tm	tropomyosin
Tm-P	tropomyosin phosphorylation
TnT	troponin T

Figure 1.

The 2-DE and immunoblot. (A) Silverstained 2-D gel of NTG and TG ventricular homogenates labeled separately and equally mixed. (B) Pro-Q-stained 2-D gel from panel (A), which stains phosphorylated proteins. (C) The 2-D immunoblot of a transgenic myofibrillar sample using a specific tropomyosin antibody CH1 to identify tropomyosin spots. (D) The 2-D immunoblot of a transgenic myofibrillar sample using a specific Tm phospho-serine 283 antibody to identify phosphorylated spots. TnT₃ or ₄, troponin T (isoform 3 or 4); p, phosphorylated; MLC-2, regulatory myosin light-chain.

Figure 2. The 2-D DIGE of ventricular homogenates. (A) Non-transgenic sample labeled with Cy3. (B) Transgenic sample labeled with Cy5. (C) Merged images from panels (A) and (B). P, phosphorylated. Only the Tm area of the gel is shown. The NTG and TG samples were labeled separately and equally mixed.

Table 1

Quantification of Tm-P from 2-D DIGE

	NTG	TG	% reduction from NTG
VH	39 ± 3.0% *	24 ± 4.0% *	38.5
MF	42 ± 1.2% **	27 ± 1.9% **	35.7

Values are means ± SEM. VH, ventricular homogenate; MF, myofibril preparation.

* $p = 0.014$ between NTG vs. TG ventricular homogenates;

** $p = 0.004$ between NTG vs. TG myofibrils.