Assembly and structure of neurofilaments isolated from bovine spinal cord*

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Abstract Neurofilaments (NFs) are neuron-specific intermediate filaments. The NFs were isolated from bovine spinal cord by differential centrifugation. The NFs were detected with electron microscopy and scanning tunneling microscopy (STM). Under STM, two kinds of sidearm of NFs were revealed: one was short, the other was long. They were arrayed along the 10-nm width core filaments one by one. The intervals between two adjacent long sidearms or two short sidearms were 20—22 nm, while those between two adjacent long and short sidearms were 10—11 nm. It was proposed that the rod domain of NF triplet proteins was 3/4-staggered. The assembly properties of NF triplet proteins were also studied. Immuno-colloidal-gold labeling assay showed that NF-M and NF-H are able to co-assemble into long filaments with NF-L. NF-M and NF-H can also co-constitute into winding filaments.

Keywords: neurofilaments (NFs), STM, sidearm, structure, assembly, immunogold.

Neurofilaments (NFs) are one type of intermediate filaments, which is typically expressed in neuronal cells. In axons, the NFs are arrayed in parallel[1]. Mammalian NFs are composed of three polypeptides: low molecular weight NF protein (NF-L), with M.W. of 68 ku; middle molecular weight NF protein (NF-M), with M.W. of 150—160 ku; high molecular weight NF protein (NF-H), with M.W. of 200 ku[2,3]. Like other intermediate filament proteins, NF triplet proteins consist of three domains in their secondary structures: an amino-terminal head, an α-helix-rich rod domain and a carboxy-terminal tail domain. NF-M and NF-H have much longer tail domains, so they have much higher molecular weight. Imaged by low angle rotary-shadowing, the neurofilaments were "test-tube-like" filaments composed of core filaments with many thin lateral projections protruding outside. The intervals between every two adjacent projections (sidearms) are about 20 nm[4]. It is proved that the sidearms represent the C-terminal tail domains of NF-M and NF-H[5].

NF triplet proteins are able to assemble *in vitro*. NF-L can assemble into 10-nm width filaments by itself[6]. NF-M and NF-H can co-assemble with NF-L, though they cannot assemble by themselves[4]. How the triplet proteins assemble into native NFs remains unknown yet.

We isolated NFs from bovine spinal cord, purified the triplet proteins, and studied their assembly *in vitro*. Immunogold assay showed that NF-M and NF-H were able to co-assemble with NF-L into 10 nm width filaments. STM assay showed that there were two kinds of sidearms along the core filaments, short ones and long ones.

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1 Materials and methods

1.1 Isolation of neurofilaments

NFs were isolated from bovine spinal cords by differential centrifugation[4]. Fresh bovine spinal cords were obtained from the Dahongmen Slaughterhouse (in Beijing). After the meninges were removed, the spinal cords were homogenized in an equal volume of PEM (100 mmol/L Pipes, pH 6.8, 1 mmol/L EGTA, 1 mmol/L $MgCl_2$) containing 1 mmol/L PMSF. The homogenate was centrifuged at 28 000 *g* at 2℃ for 50 min. The supernatant was made 20% (*v*/*v*) with glycerol and incubated for 20 min at 37℃. Then the NFs were isolated by centrifugation at 150 000 *g* for 90 min at room temperature. The supernatant was removed and the precipitate was stored at −80℃.

1.2 Purification of NFs triplet proteins

The triplet proteins were purified by methods of Hisanaga and Hirokawa[4]. After the precipitate was dissolved in buffer A (10 mmol/L NaH_2PO_4-Na_2HPO_4, pH 7.5, 1 mmol/L EGTA, 6 mol/L Urea, 0.5 mmol/L DTT, 1 mmol/L PMSF) and centrifuged at 18 000 *g* for 30 min, the supernatant was applied to a DEAE-cellulose column (Whatman DE-52) equilibrated with buffer A. The adsorbed proteins were eluted with NaCl gradient (0—300 mmol/L). The protein fractions were examined by SDS-PAGE.

1.3 Re-assembly of NFs proteins *in vitro*

Each purified NF protein (NF-L, NF-M and NF-H) at concentration of 0.2 mg/mL was dialyzed against assembly buffer (PEM, 0.15 mol/L NaCl, 2 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L PMSF) at 37℃ over night. For re-constitution, a 2∶1 (*w*/*w*) mixture of NF-Land NF-M, or a 2∶1 (*w*/*w*) mixture of NF-L and NF-H, or a 1∶1 (*w*/*w*) mixture of NF-M and NF-H was polymerized by dialysis against the same buffer under the same condition.

1.4 Electron microscopy

1.4.1 Negative staining. A 5 μL aliquot of the sample (0.2 mg/mL) was adsorbed for about 1 min to a glow-discharged carbon-coated formvar film on a copper grid. The grid was washed with a drop of distilled water, then stained with 2% uranyl acetate for 15—20 s. After removal of the excess liquid with filter paper, the grid was put into a grid box and air-dried.

1.4.2 Immuno-electron microscopy. A drop of sample was adsorbed on a glow-discharged, carbon-formvar-coated nickel grid for 1 min, then the excess liquid was removed with filter paper and blocked with 5% BSA at 37℃ for 15 min. The grid was incubated with primary monoclonal antibody (mAb) (for NF-L + NF-M, using mAb against NF-M; for NF-L + NF-H, using mAb against NF-H) at 37℃ for 1 h. Washed with PBS for three times (each for 10 min), the grid was blocked with 5% BSA for 15 min and incubated with second antibody that is goat anti-mouse IgG conjugated 5 nm gold. 1 h later, the grid was washed in PBS and negatively stained as above.

1.4.3 Rotary-shadowing. A small amount of NFs was diluted to adjust the protein concentration to about 0.1 mg/mL, then added with glycerol to 50% (*v*/*v*). About 50 μL sample was sprayed onto a newly cleaved mica surface and rotary shadowed with platinum (~2.5 nm) at an angle of 6° in a Balzers BAF 301. The replicas on mica were floated off in distilled water and picked up with naked

copper grids.

1.5 Scanning tunneling microscopy

The samples with protein concentration of 0.1 mg/mL were dropped onto a newly cleaved HOPG (highly oriented pyrolytic graphite) surface. 5 min later, the excess liquid was removed and the sample was air-dried. The surface was examined by CSPM-980a STM (made in the Institute of Chemistry of Chinese Academy of Sciences) under current-constant motif.

1.6 SDS-PAGE

SDS -PAGE was performed on 7.5 (w/v) % gels. And the gels were stained with Coomassie Brilliant Blue R-250.

2 Results

2.1 The structure of native NFs

The NFs isolated from bovine spinal cords were composed of three major components: NF-L, NF-M and NF-H, with molecular weight of 68, ~160 and 200 ku, respectively (fig.1(a)). Other contaminant proteins were much less than NFs. The rotary-shadowing image of NF showed that the NF was composed of a core filament and many thin filamentous projections protruding from the core filament (fig. 1(b)). We called the projections sidearms. The intervals of the sidearms were 20—22 nm. An axial repeat of 22 nm along the surface of the core filament was also observed. It may be due to the decoration of the roots of the sidearms by glycerol. Negative staining revealed that NFs were long and 10-nm width filaments (fig. 1(c)). Sidearms were invisible because they may shrink onto the surface of the core filament during the air-drying.

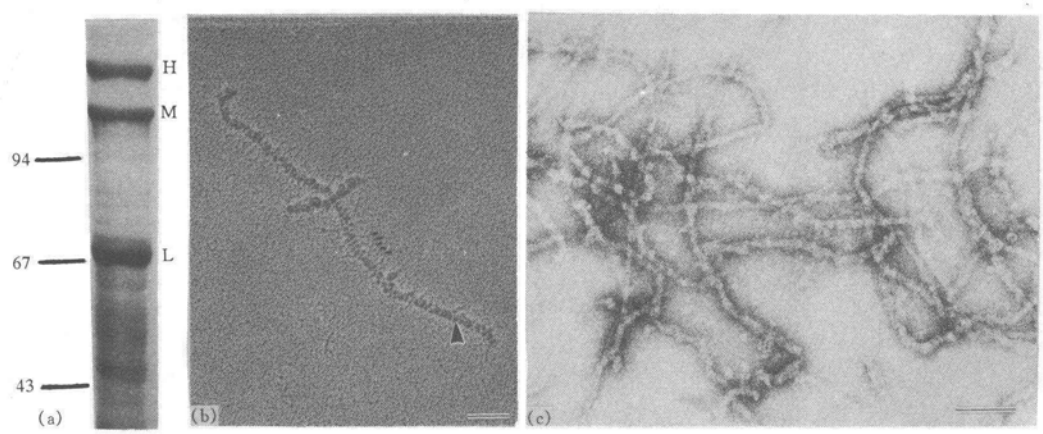

Fig. 1. The composition and view of native NFs. (a) 7.5% SDS-PAGE of NF fraction isolated from differential centrifugation. NF triplet proteins, NF-L, NF-M and NF-H, are indicated by H, M and L. NF was the predominant protein in the fraction; (b) rotary-shadowing view of NF. NF was composed of core filament and many thin projections——sidearms (indicated by small arrows) protruding from the core filament; (c) negative staining image of NFs. NFs were long and 10-nm width filaments (bar = 100 nm).

When observed with STM, NFs showed more details. In fig. 2, we can see three parallel filaments, each of them composed of a 10-nm width core filament and regularly arrayed projections protruding outside. These indicated that the filaments were NFs and the projections were sidearms. Dur-

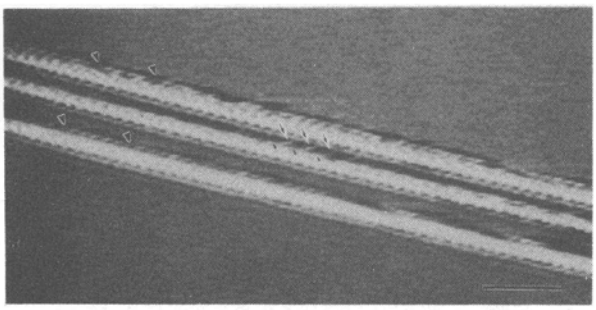

Fig. 2. NFs examined by STM. There were three parallel-arranging filaments. Each of them composed of a 10-nm width core filament and regularly arrayed projections (sidearms) projecting outside. Long sidearms with interval of 20—22 nm between the two adjacent filaments (indicated by arrows) and the projection tracks between the long sidearms (indicated by small arrow heads) were easily seen. Most short projections had spaces of 10—11 nm, while some had intervals of 20—22 nm and some were lower in height than their neighbors (indicated by big arrow heads) (bar = 50 nm).

ing the air-drying, the sidearms might shrink, so the projections appeared shorter and joined with one another in their terminals. The intervals of the sidearms were not 20—22 nm, but 10—11 nm, which was just the half length as that of the projections shown by rotary-shadowing. Some longer sidearms with space of 20—22 nm can also be detected between the two adjacent filaments (indicated by arrows). In the middle of the space between every two long sidearms, a track of the projection was visible (fig. 2, indicated by small arrow heads). It was due to the shorter sidearm which shrank more seriously during the air-drying.

Most intervals of the short sidearms were 10—11 nm, but some short sidearms with space of 20—22 nm were also detected often. Moreover, we noticed that some short sidearms appeared lower in height than their neighbors (indicated by arrow heads). The lower height indicated the shorter length, so the length of the "short" projections was not the same. Some were shorter than others.

The above suggested that there were two kinds of sidearms in the peripheral of the NFs, long ones and short ones. They were arrayed one by one along the core filaments. The space of two adjacent sidearms was 10—11 nm, while the interval of two adjacent long sidearms or two adjacent short ones was 20—22 nm.

2.2 The assembly of NFs triplet proteins *in vitro*

NFs triplet proteins can be purified by DE-52 anion exchange chromatography column (fig. 3(a)). While NF-M and NF-H were highly purified, NF-L was contaminated by a small amount of NF-M, which was too small to affect the structure of the re-constitute filaments. NF-L can assemble into 10-nm width filaments easily (fig.3(b)). NF-M can only form a few 10-nm width short filaments (fig.3(c), indicated by arrow head), and many NF-M proteins formed clusters or spherical structures. NF-H was not able to form filamentous structures, but form rod-like structures (figure 3(d)).

Both NF-M and NF-H were able to form 10-nm width filaments together with NF-L (fig.4(a), (b)). We can see many short filamentous fragments in fig. 4(b), which indicated that NF-H was more difficult in co-assembly with NF-L than NF-M.

Immunogold labeling assay showed that the filaments formed by NF-L and NF-M were recognized by mAbs against NF-M (fig.4(c)). The same results were obtained when the filaments co-assembled by NF-L and NF-H were labeled by mAbs against NF-H (fig.4(d)). That was to say that NF-M and NF-H really existed in the filaments. There are some clusters of gold particles in fig. 4(d) (indicated by small arrow heads). They might label the short filamentous fragments shown in figure 4(b).

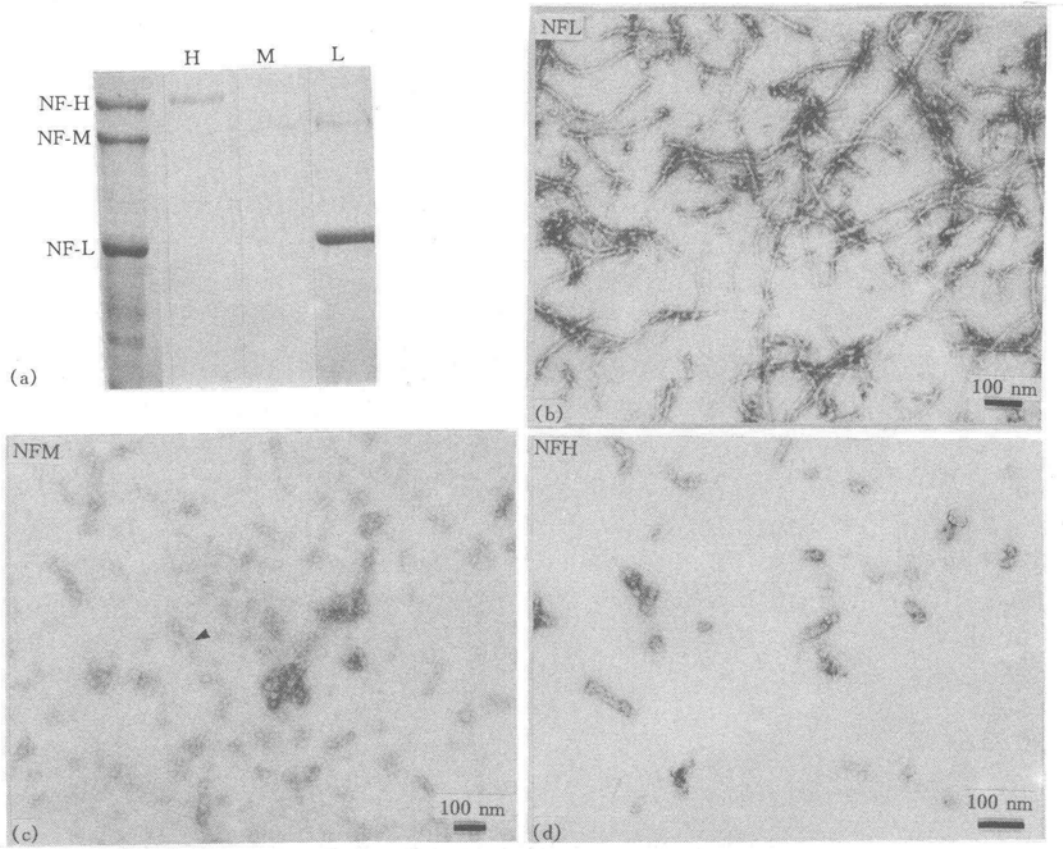

Fig. 3. The assembly properties of NF triplet proteins. (a) NF triplet proteins purified by DE-52 column; (b) NF-L re-assembled into 10-nm width long filaments; (c) NF-M formed a few 10-nm width short filaments (indicated by arrow head) and many clusters or globe-shaped structures; (d) NF-H can only form rod-like structures (bar = 100 nm).

As we know, NF-M or NF-H cannot form filaments by itself. When they assemble together, some long, 5—7 nm wide and winding filaments appeared (fig. 5). They are so different from typical intermediate filaments.

3 Discussion

Native NFs are hetero-polymers composed of NF-L, NF-M and NF-H. NF-L itself, NF-L and NF-H or NF-L and NF-M can assemble into 10-nm wide filaments. NF-M or NF-H cannot assemble, but they can co-assemble into some winding filaments of 5—7 nm wide. Though NF-M and NF-H are able to form 10-nm wide filaments with NF-L, the co-assembled filaments are shorter than that formed by NF-L itself. Moreover, there are many unassembled fragments even after being dialyzed for over 24 h. It suggests that NF-H and NF-M can postpone the formation of the filaments. So the formation of native NFs must be very complex.

From the two facts that the intervals of the projections shown by rotary-shadowing are 20—22 nm[4] and the filaments re-assembled by NF-L have 20—22 nm axial repeats[7], it is considered that there must be a half-staggered configuration between the molecules in NFs (the length of the rod do-

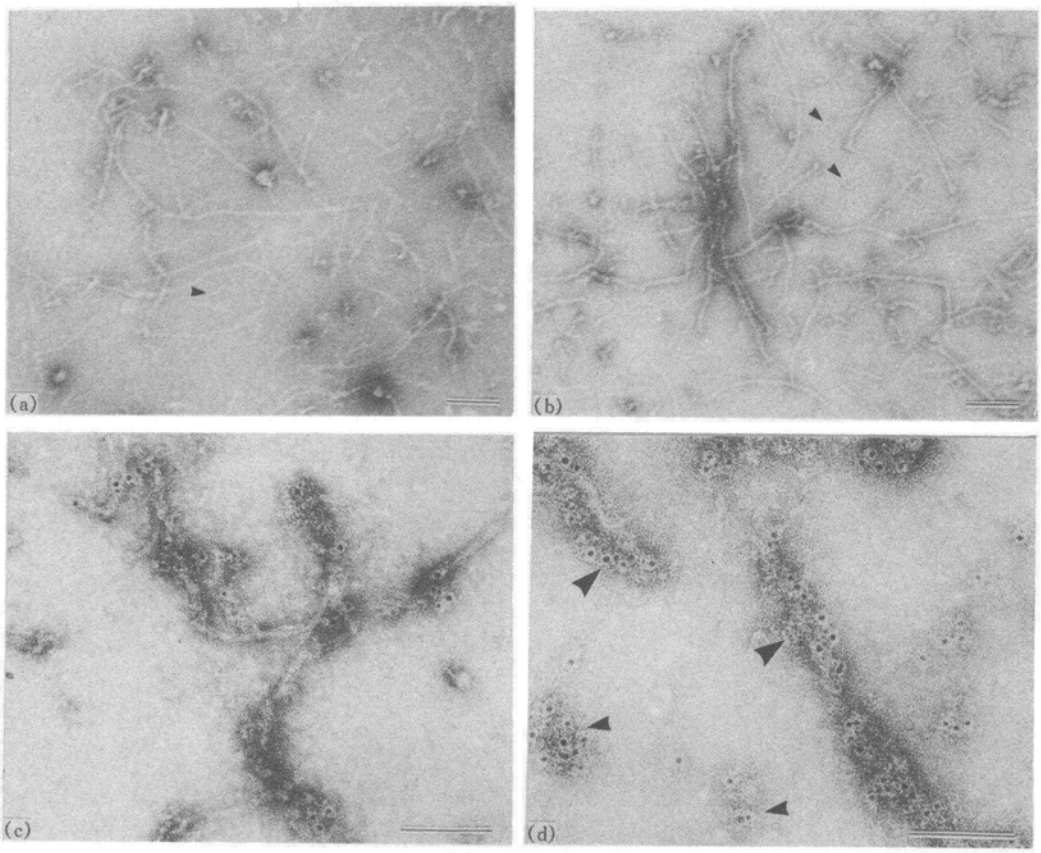

Fig. 4. Co-assembly of NF triplet proteins *in vitro*. (a) NF-L and NF-M co-assembled into 10-nm width filaments, a few short filamentous fragments were detected sometimes (indicated by arrow head); (b) NF-L and NF-H co-assembled into 10-nm width filaments, and many short filamentous fragments were detected (indicated by arrow heads); (c) immunogold-labeling of NFs re-assembled by NF-L and NF-M. The filaments were recognized by the mAbs against NF-M; (d) immunogold-labeling of NFs re-assembled by NF-L and NF-H. The filaments were recognized by the mAbs against NF-H. The short filaments (indicated by big arrow heads) and the terminal of the filaments were labeled strongly. The gold particle clusters (indicated by small arrow heads) might label the fragments as in fig. 4(b) (bar = 100 nm).

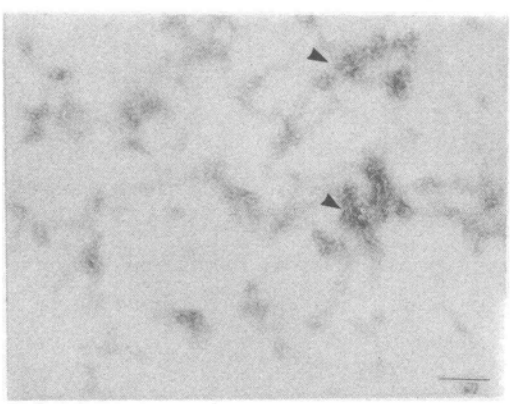

Fig. 5. NF-H and NF-M both were able to form winding filaments. The filaments were 5—7 nm wide and often twisted into clusters (indicated by arrow heads) (bar = 100 nm).

main of NF triplet proteins is 46—48 nm)[7,8]. The first fact indicates that the rod domain of NF-H (or NF-M) is half-staggered. The second fact indicates that the rod domain of NF-L is half-staggered. But what is the arrangement between the rod domain of NF-L and NF-H (or NF-M)? We can get no information just from the two facts.

The STM image of NFs gives us a insight into the molecular architecture of NF-L and NF-H (or NF-M) in native NFs. Besides the long sidearms well known, other short sidearms are detected by STM. Between every two adjacent long sidearms, there is a short one. The short arms may be due to the C-terminal tail domains of NF-

L. They cannot be detected by rotary-shadowing because the short C-terminals are covered by platinum. When the filaments formed by NF-L were viewed by rotary-shadowing, some short projections could be seen occasionally[5].

The interval of two adjacent long or short sidearms is 20—22 nm, so the rod domains of NF-H (or NF-M) or the rod domains of NF-L are half-staggered. It is the same as before. The long sidearm and the short sidearm are 10—11 nm spaced, therefore the rod domains of NF-H (or NF-M) and the rod domains of NF-L possibly have an axial stagger of 35—37 nm. That is to say that they are 3/4-staggered (figure 6).

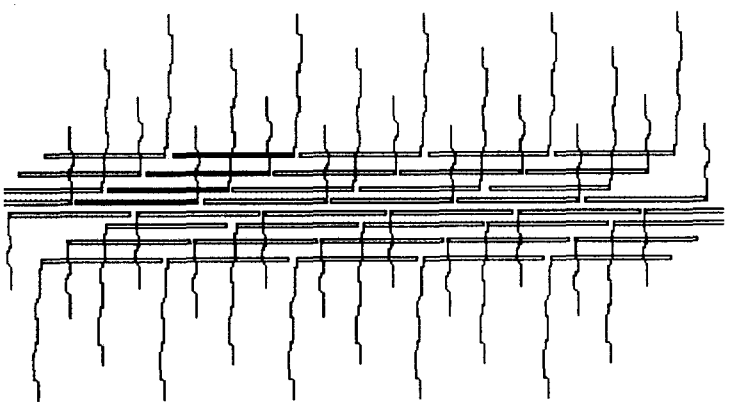

Fig. 6. Schematic representation of our tentative molecular model for NF architecture. Dimers or tetramers are represented by open bars. The long curves stand for the tails of NF-H or NF-M, while the short ones stand for those of NF-L. Open bars with long tails represent the dimers (or tetramers) formed by NF-H (or NF-M) and NF-L, and the bars with short tails represent the dimers (or tetramers) formed by NF-L alone. The same dimers or tetramers (open bars with long curves or open bars with short curves) were half-staggered, while the different or tetramers (open bars with long curves and with short ones) were 3/4-staggered.

NF-L-containing dimers are the elementary building blocks of NFs[9]. Dimers can form tetramers and octamers. We cannot determine whether the 3/4-staggered configuration takes place between the dimers or the tetramers. Further work must be done in the future.

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