Fullerenolates: metallated polyhydroxylated fullerenes with potent anti-amyloid activity†

A. G. Bobylev,^a A. B. Kornev,^b L. G. Bobyleva,^a M. D. Shpagina,^a I. S. Fadeeva,^a R. S. Fadeev,^a D. G. Deryabin,^c J. Balzarini,^d P. A. Troshin^{*b} and Z. A. Podlubnaya^{a,e}

Received 12th January 2011, Accepted 26th April 2011

DOI: 10.1039/c1ob05067b

It has been revealed for the first time that sodium fullerenolate Na₄[C₆₀(OH)₃₀] (NaFL), a water soluble polyhydroxylated [60]fullerene derivative, destroys amyloid fibrils of the Aβ(1–42) peptide in the brain and prevents their formation in *in vitro* experiments. The cytotoxicity of NaFL was found to be negligibly low with respect to nine different culture cell lines. At the same time, NaFL showed a very low acute toxicity *in vivo*. The maximal tolerable dose (MTD) and LD50 for NaFL correspond to 1000 mg kg^{−1} and 1800 mg kg^{−1}, respectively, as revealed by *in vivo* tests in mice using intraperitoneal drug injection. The observed pronounced anti-amyloid activity and low toxicity of NaFL make it a very promising lead drug for the development of potent fullerene-based therapeutic approaches for the treatment of amyloidoses, such as Alzheimer's disease and others.

Introduction

It is known that more than 40 human diseases are related to the erroneous folding of proteins. Among these are amyloidoses, in particular Alzheimer's disease, Parkinson's disease, cataract, type II diabetes and others.^{1–2} Alzheimer's disease (AD) is one of the most widespread and severe neurological disorders.³ The deposition of amyloid β-peptide (Aβ-peptide) in the brain is a pathological hallmark of AD. This peptide is a product of the proteolysis of the transmembrane amyloid precursor protein and the major component of amyloid plaques.⁴ There is substantial evidence indicating that the aggregation and accumulation of neurotoxic Aβ-peptides in the brain can trigger a cascade of pathological reactions leading to neuronal dysfunction and neurodegeneration.^{5–7} Therefore, the therapy of AD is primarily aimed at preventing the aggregation of Aβ-peptides or destroying the aggregates that are already formed.^{8–9}

A possible approach for the destruction of amyloid fibrils is to use fullerenes. The results of fluorescence analysis showed that the derivative of fullerene C₆₀ 1,2-dimethoxymethanofullerene

suppresses the aggregation of the Aβ(1–40) peptide.¹⁰ Previously, we have shown for the first time that hydrated fullerene C₆₀ (C₆₀HyFn) has anti-amyloid activity toward the fibrils of the Aβ(25–35) and Aβ(1–42) peptides.¹¹ The experiments also showed that C₆₀HyFn injected into a rat brain has a beneficial effect on normal cognitive processes, indicating the absence of its neurotoxicity. There is a correlation between the above-described anti-amyloid action of C₆₀HyFn on the fibrils of Aβ-peptides *in vitro* and its ability to prevent the disturbance of performing cognitive probabilistic tasks in experimental animals induced by aggregated Aβ(25–35) peptide.¹¹

Here, we describe the results of an electron microscopic and fluorescence analysis of the effects of sodium fullerenolate (NaFL) on fibrils of the Aβ(1–42) peptide of the brain, examine its cytotoxicity in a number of cell cultures and its acute toxicity after intraperitoneal injection in mice.

Experimental

Materials and cell cultures

All commercially available solvents and reagents were purchased from Acros or Aldrich and were used as received. NaFL was prepared by the hydrolysis of nitrofullerenes with aqueous sodium hydroxide, as described previously.¹² Chemical analysis data for NaFL: C, 54.10; H, 2.71; Na, 6.81; O, 35.6; calculated for average composition Na₄[C₆₀(OH)₃₀]: C, 54.48; H, 2.29; Na, 6.95; O, 36.28. ESI-MS: broad distribution of negative ion signals with *m/z* = 1000 ± 1400 amu; maximum intensity peaks were observed in the 1200–1250 amu range. FTIR: ν = 521, 584, 776, 836, 850, 1065, 1341, 1377, 1610, 2937 and 3418 cm^{−1}.

^aInstitute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow region, 142290, Russia

^bInstitute of Problems of Chemical Physics, Russian Academy of Sciences, Chernogolovka, Moscow region, 142432, Russia. E-mail: troshin@cat.icp.ac.ru; Fax: +7 496-522-3507

^cOrenburg State University, Prospect Pobedy 13, GSP, Orenburg, 460018, Russia

^dRega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Minderbroedersstraat 10, B-3000, Leuven, Belgium

^ePushchino State University, Pushchino, Moscow region, 142290, Russia

† Electronic supplementary information (ESI) available: Enlarged electron microscopy images, fluorescence spectra obtained using the thioflavine T assay and the FTIR spectrum of NaFL. See DOI: 10.1039/c1ob05067b

Human epidermoid larynx carcinoma (HEp-2) cells were obtained from the Russian Cell Culture Collection (Institute of Cytology, Russian Academy of Sciences, St. Petersburg). HEp-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (w/v) fetal bovine serum, 40 mg l⁻¹ gentamycin, 35 mM sodium bicarbonate and 20 mM HEPES at 37 °C in an atmosphere of 5% CO₂. The fetal bovine serum was from HyClone; culture medium DMEM, crystal violet and other chemicals for cytotoxicity tests were purchased from the Sigma Chemical Co.

Preparation of the Aβ(1–42) peptide amyloid fibrils

The amyloid fibrils were formed from the Aβ(1–42) peptide (Sigma-Aldrich) in a solution containing 30 mM KCl and 10 mM imidazole, pH 7.0, for 24 h at 37 °C. The amyloid nature of the fibrils was tested from their binding to Congo red and thioflavin T by a Cary 100 spectrophotometer (Varian, USA) and a Cary Eclipse fluorescence spectrophotometer (Varian, USA). NaFL was added to the Aβ(1–42) peptide in the ratio 1 : 1 (w/w) before and after the formation of the amyloid fibrils. The peptide was incubated with fullereneol in a solution containing 30 mM KCl and 10 mM imidazole, pH 7.0, for 24 h at 37 °C.

Fluorescence analysis

The anti-amyloid effect of the fullereneolate was estimated by the intensity of thioflavin T fluorescence in samples containing 30 mM KCl, 10 mM imidazole, pH 7.0, 5 μM thioflavin T and 5 μg ml⁻¹ of the Aβ(1–42) peptide prior to and after the formation of the amyloid fibrils. The fluorescence was measured at λ_{ex} = 440 nm and λ_{em} = 482 nm by a Cary Eclipse fluorescence spectrophotometer (Varian, USA).

Electron microscopy

A drop of a suspension of the protein at a concentration of 0.1 mg ml⁻¹ was applied to a carbon-coated collodion film on a copper grid and negatively stained with a 2% aqueous uranyl acetate solution.¹³ Samples were examined on JEM-100B and LIBRA 120 electron microscopes.

Cytotoxicity assay

For the cytotoxicity assay, HEp-2 cells were seeded in 96-well microplates or culture dishes (Corning, USA) at a concentration of 5 × 10⁴ cells ml⁻¹ (5 × 10³ cells in 100 μl well⁻¹). Freshly prepared fullereneolate solutions were added to the culture medium 24 h after seeding the cells. The anti-proliferative activity was evaluated using the crystal violet assay from the ratio of the optical densities at 560 nm of treated and untreated (control) cultures 48 h after adding the toxic agent.¹⁴ The optical density value was in direct proportion to the number of viable cells. Each experiment was performed at least three times. All the values represent the mean ± SEM. The statistical significance of the results was analyzed using Student's *t*-test. Measurements were carried out using an Infinite F200 plate reader (Tecan, Austria).

The cytostatic or cytotoxic effects of NaFL on murine leukemia cells (L1210), murine mammary carcinoma cells (FM3A), human T-lymphocyte cells (CEM), human cervix carcinoma cells (HeLa),

human embryonic lung cells (HEL), Madin Darby canine kidney cell (MDCK), Crandell-Rees Feline Kidney cells (CRFK), human osteosarcoma cells (OST TK⁻) and monkey kidney (Vero) cells was evaluated as follows: an appropriate number of (L1210, FM3A, CEM, HeLa, OST TK⁻) cells suspended in growth medium were allowed to proliferate in 200 μL wells of 96-well microtiter plates in the presence of variable amounts of the test compounds at 37 °C in a humidified CO₂-controlled atmosphere. After 48 to 96 h, the number of cells was counted in a Coulter counter. The IC₅₀ value was defined as the compound concentration required to inhibit cell proliferation by 50%. The monolayer HEL, MDCK, CRFK and Vero cells were seeded in 200 μL wells of 96-well microtiter plates and grown until confluency. Then, variable amounts of the test compounds were added, and the cultures were further incubated at 37 °C. After 72 h, microscopic alteration of the cellular morphology was estimated, and the MCC (minimum cytotoxic concentration) determined.

Results and discussion

Chemical nature of the sodium fullereneolate NaFL

Neutral polyhydroxylated fullerenes called “fullerenols” (FL, Fig. 1) form one of the most studied families of water soluble fullerene derivatives. Typically, samples of fullerenols are represented by multicomponent mixtures of products with different numbers of hydroxyl groups, oxygen atoms and even ketone moieties, resulting from partial destruction of the fullerene cage.¹⁵ There are just a few fullerene derivatives bearing two hydroxyl groups that have been isolated in chemically pure forms and characterized using spectroscopic methods.¹⁶

Fig. 1 Schematic representations of the molecular structures of (a) fullereneol (FL) and (b) sodium fullereneolate (NaFL). Some cyclopentadienyl anion units and Na⁺ cations are located on the other side of the fullerene cage in NaFL and are not shown. (c) The molecular structure of NaPCF.

The existence of ionic forms of hydroxylated fullerenes such as Na⁺_n[C₆₀O_x(OH)_y]ⁿ⁻ (where *n* = 2 or 3, *x* = 7–9 and *y* = 12–15) has been reported previously.^{12,17} It was proposed that fullerenols are rather strong O–H acids capable of forming stable metal salts *via* the replacement of hydrogen atoms with alkali metal cations. The resulting fullerene adducts bearing ionic –O⁻M⁺ moieties were called “metal fullereneolates”.¹²

We have reported very recently the formation, isolation and spectroscopic characterization of a number of fullerene derivatives comprising of stable anions such as [C₆₀(CN)₅]⁻, [C₆₀(CN)₈(OH)₂]²⁻, [C₆₀(CN)₉(OH)]²⁻ and [C₆₀(CN)₁₀]²⁻.¹⁸ These anions are characterized by the presence of negatively-charged cyclopentadienyl fragments accommodated on the fullerene cage. The stability of such C-anions seems to be much greater compared to O-anions. Therefore, we propose similar structures for the ionic

forms of polyhydroxylated fullerenes. A detailed study addressing the origin of different forms of hydroxylated fullerenes will be reported elsewhere.¹⁹

In the present paper, we will focus on the material with an average composition $\text{Na}_4[\text{C}_{60}(\text{OH})_{30}]$ that was revealed by chemical analysis and supported by electrospray mass spectrometry (ESI-MS). The preparation and spectroscopic characterization of such products has been reported previously. The chemical nature of the $\text{Na}_4[\text{C}_{60}(\text{OH})_{30}]$ material is represented by the model formula NaFL shown in Fig. 1. Even though the term “sodium fullerenolates” was proposed for O-metallated products, we will further apply it to C-metallated forms such as NaFL.

The ionic nature of NaFL was proved unambiguously by gel electrophoresis. In our experiments, NaFL behaved similarly to the fullerene derivative NaPCF (Fig. 1) bearing five negatively-charged carboxylic groups (COO^-). The synthesis and characterization of NaPCF has been reported previously. Both compounds migrate towards the anode under an applied electric field, which is a clear illustration of their anionic nature (Fig. 2). It was surprising that NaFL represents a quite well-defined spot on the electrophoretic plate. This suggests that all polyhydroxylated fullerene species present in the NaFL sample have similar compositions and identical charges. This makes NaFL similar to the individual and chemically pure fullerene derivative NaPCF.

Fig. 2 Agarose gel electrophoresis of NaFL and NaPCF. Starting tracks are denoted with arrows.

Cytotoxicity and acute toxicity of NaFL

To answer the question as to whether hydroxylated fullerenes can be used as pharmaceutical preparations for the treatment of neurodegenerative diseases, we examined the cytotoxicity of NaFL against a number of different types of cell culture. The obtained results are presented in Table 1. It is seen from these data that NaFL shows no toxic effects in all the tested cell lines in concentrations of up to 0.1 mg ml^{-1} (the upper concentration

level used). The cytotoxicity of NaFL with respect to the HEp-2 cells was evaluated in an even wider concentration range. It can be seen from Fig. 3 that the increase in the concentration of NaFL from 0.016 to 2 mg ml^{-1} does not affect the survival of HEp-2 cells. These results are highly superior compared to the cytotoxicity data obtained for compound NaPCF. The experimental IC_{50} values for NaPCF were indeed in the range of 0.01 – 0.09 mg ml^{-1} , depending on the cell culture used in the tests (Table 1). Thus, NaFL has much lower cytotoxicity compared to NaPCF.

Fig. 3 Concentration dependence of cytotoxicity against human larynx carcinoma cells, evaluated for NaFL using the crystal violet assay.

We also determined the acute toxicity of NaFL in mice after intraperitoneal injection of an aqueous solution of the drug. The maximal tolerable dose for NaFL was found to be around 1000 mg kg^{-1} , while the median lethal dose LD_{50} was about 1800 mg kg^{-1} . To the best of our knowledge, NaFL exhibits the lowest acute toxicity among all water soluble fullerene derivatives, including fullerenols FL.²⁰ We emphasize also that the acute toxicity of NaFL is much lower in comparison with the acute toxicity of the polycarboxylic fullerene derivative NaPCF (Fig. 4).

The most probable reason for the decreased toxicity of NaFL is the shielding of the hydrophobic fullerene cage by numerous (~ 30) highly polar hydroxylic groups. On the contrary, NaPCF has only five hydrophilic carboxylic groups located on one hemisphere of the carbon cage. The non-functionalized hemisphere of NaPCF remains highly hydrophobic, which might induce the substantial accumulation of this compound in cellular lipid membranes and

Table 1 Cytotoxicity data for water soluble fullerene derivatives

Compound→	NaFL	NaPCF
Cell line↓	Cytostatic concentration (IC_{50})/ mg ml^{-1}	
L1210	>0.1	0.09 ± 0.004
FM3A	>0.1	—
CEM	>0.1	0.035 ± 0.004
HeLa	>0.1	0.052 ± 0.001
OST TK ⁻	—	0.013 ± 0.003
HEp-2	>2	—
	Minimal cytotoxic concentration (MCC)/ mg ml^{-1}	
HEL	>0.1	—
MDCK	>0.1	—
CRFK	>0.1	—
Vero	>0.1	—

Fig. 4 Graphical overview of the maximal tolerable dose (MTD) and the median lethal dose (LD50) for fullerene derivatives, illustrating a markedly lower acute toxicity of NaFL in comparison with NaPCF.

also enable inhibition of many enzymes possessing hydrophobic active centers. The inhibition of HIV-protease is a good illustration of such a fullerene derivative–protein interaction.²¹

Anti-amyloid activity of NaFL

We found recently that the water soluble fullerene derivative NaPCF shows *in vitro* a clear anti-amyloid activity on fibrils of

X-protein.²² However, this fullerene derivative was found to be relatively toxic (see Table 1 and Fig. 4) and therefore it suppresses the activity of pyramidal neurons from the hippocampus of rats.¹¹ Thus, the *in vivo* application of NaPCF for the treatment of neuronal diseases seems to be problematic.

On the contrary, the low cytotoxicity and low acute toxicity of NaFL make this drug much more promising for development of anti-amyloid therapeutics. Electron microscopy imaging in combination with fluorescence analysis form a rather powerful toolkit for the straightforward evaluation of the anti-amyloid properties of various substances. According to our electron microscopy data, NaFL in the fullerene/protein ratio 1 : 1 (w/w) destroyed mature amyloid fibrils of the A β (1–42) peptide (Fig. 5 and Fig. S1–S4, ESI†). Moreover, it was also found that the A β (1–42) peptide in the presence of NaFL (with the same component ratio) does not form amyloid fibrils, even after 24 h of incubation. Thus, according to the electron microscopy data, NaFL efficiently disaggregates amyloid fibrils *in vitro* and prevents their formation, making it therapeutically interesting.

The data on the anti-amyloid activity of the fullerenolate towards the A β (1–42) peptide were confirmed by independent fluorescence tests. It has been demonstrated previously that the anti-amyloid properties of various compounds can be examined by fluorescence analysis by using thioflavin T as an indicator

Fig. 5 Electron micrographs of negatively-stained samples of A β (1–42)-peptide in the presence and absence of NaFL. A: Pre-formed amyloid fibrils of A β (1–42)-peptide. B: Dispersion of NaFL. C: Co-incubated mixture of A β (1–42)-peptide and NaFL in a 1 : 1 (w/w) ratio. D: Co-incubated mixture of the preformed amyloid fibrils of A β (1–42)-peptide and NaFL in a 1 : 1 (w/w) ratio. Arrows indicate small fragments of the destroyed amyloid fibrils of A β (1–42)-peptide. Images A–D were obtained from samples incubated in a solution containing 30 mM KCl and 10 mM imidazole (pH 7.0) at 37 °C for 24 h. Negative staining was applied using a 2% aqueous solution of uranyl acetate. Scale bars are 100 nm.

that interacts specifically with amyloid fibrils.^{23,24} This interaction results in an increased dye fluorescence at 482 nm.²⁵ In our experiments, the intensity of thioflavin T fluorescence decreased after the addition of NaFL to amyloid fibrils of the A β (1–42) peptide. This decrease in the fluorescence intensity was quite evident from a comparison of the samples treated with NaFL with samples containing mature amyloid fibrils in the absence of NaFL (Fig. 6 and Fig. S5, ESI†). It should be also noted that the fluorescence of the thioflavin T dye remained relatively weak after 24 h of incubation of a A β (1–42) peptide sample with NaFL. These results support the electron microscopy data, suggesting that NaFL is capable of destroying mature fibrils of the A β (1–42) peptide and inhibiting its aggregation.

Fig. 6 The fluorescence intensity of thioflavine T added to different systems. A: Pre-formed amyloid fibrils of A β (1–42)-peptide. B: Dispersion of NaFL. C: Co-incubated mixture of A β (1–42)-peptide and NaFL in a 1 : 1 (w/w) ratio. D: Co-incubated 1 : 1 (w/w) mixture of pre-formed amyloid fibrils of A β (1–42)-peptide and NaFL. The “control” system was a buffer solution containing 30 mM KCl and 10 mM imidazole (pH 7.0) in the presence of thioflavin T. Excitation was performed at λ_{ex} = 440 nm; the fluorescence intensity was recorded at the emission maximum at λ_{max} = 482 nm).

It is possible that NaFL contributes to some extent to the fluorescence quenching of thioflavine T via a well-established electron transfer mechanism for C₆₀ and its simple derivatives.²⁶ We verified this possibility experimentally for the model thioflavine T + NaFL system. Thioflavine T exhibited a very weak but still recordable fluorescence in the absence of the amyloid fibrils. The addition of NaFL to thioflavine T did not change significantly the intensity of the fluorescence (Fig. S6, ESI†). This suggests that the contribution of NaFL to the fluorescence quenching of thioflavine T incorporated into the amyloid fibrils should be rather weak. Therefore, the fluorescent thioflavine T assay provides realistic results concerning the disaggregation of amyloid fibrils upon the action of NaFL, thus supporting the observations made above using electron microscopy.

Conclusions

We have demonstrated for the first time a strong anti-amyloid activity of sodium fullerenolate NaFL *in vitro*. A combination of fluorescence analysis and electron microscopy revealed that NaFL efficiently destroys mature amyloid fibrils. At the same time, the addition of NaFL to a medium containing the A β (1–42) peptide

of the brain prevents its aggregation and the formation of fibrils. The observed potent anti-amyloid activity of NaFL, together with its exceptionally low cytotoxicity and low acute toxicity, makes this drug very promising for pharmaceutical applications. Thus, NaFL can be recommended for pre-clinical *in vivo* trials to evaluate its applicability for the therapy of neurodegenerative disorders, in particular, Alzheimer's disease.

Acknowledgements

We gratefully acknowledge Prof. N. P. Konovalova of IPCP RAS and Drs. V. K. Balakin and A. S. Kinzirskiy of IPAC RAS for evaluating the acute toxicity of the fullerene derivatives. This work was supported by the Russian President Foundation within the Program “Leading scientific schools” and within contract no. 16.120.11.4916-MK, by the Presidium of the Russian Academy of Sciences (program “Fundamental sciences for medicine”), by the Russian Foundation for Basic Research (projects nos. 09-04-01161, 10-04-00141 and 10-03-00443), and also by the Federal Agency for Science and Innovations within the Program “Research and scientific-pedagogic personnel of innovative Russia” (State contracts nos. 02.740.11.0301, 02.740.11.0710 and 02.740.11.0749) and by the K. U. Leuven (GOA 10/15).

References

- 1 F. Chiti and C. M. Dobson, *Annu. Rev. Biochem.*, 2006, **75**, 333.
- 2 H. Ecroyd and J. A. Carver, *IUBMB Life*, 2008, **60**, 769.
- 3 D. B. Ascher, G. A. N. Crespi, H. L. Ng, C. J. Morton, M. W. Parker and L. A. Miles, *J. Proteomics Bioinf.*, 2008, **1**, 464.
- 4 J. Kang, H. G. Lemaire, A. Unterbeck, J. M. Salbaum, C. L. Masters, K. H. Grzeschik, G. Multhaup, K. Beyreuther and B. Müller-Hill, *Nature*, 1987, **325**, 733.
- 5 D. J. Selkoe, *Science*, 2002, **298**, 789.
- 6 C. Pereira, E. Ferreira, S. M. Cardoso and C. R. de Oliveira, *J. Mol. Neurosci.*, 2004, **23**, 97.
- 7 J. Hardy and D. J. Selkoe, *Science*, 2002, **297**, 353.
- 8 T. E. Golde, *J. Neurochem.*, 2006, **99**, 689; T. E. Golde and T. L. Kukar, *Science*, 2009, **324**, 603.
- 9 A. R. Salomon, K. J. Marcinowski, R. P. Friedland and M. G. Zagorski, *Biochemistry*, 1996, **35**, 13568.
- 10 J. E. Kim and M. Lee, *Biochem. Biophys. Res. Commun.*, 2003, **303**, 576.
- 11 I. Ya. Podolsky, Z. A. Podlubnaya, E. A. Kosenko, E. A. Mungantseva and E. G. Makarova, *J. Nanosci. Nanotechnol.*, 2007, **7**, 1479.
- 12 P. A. Troshin, A. S. Astahova and R. N. Lyubovskaya, *Fullerenes, Nanotubes, Carbon Nanostruct.*, 2005, **13**, 331.
- 13 H. E. Huxley, *J. Mol. Biol.*, 1963, **7**, 281.
- 14 M. Nomizu, W. H. Kim, K. Yamamura, A. Utani, S. Song, A. Otaka, P. P. Roller, H. K. Kleinman and Y. Yamada, *J. Biol. Chem.*, 1995, **270**, 20583.
- 15 L. Y. Chiang, R. B. Upasani, J. W. Swirczewski and S. Soled, *J. Am. Chem. Soc.*, 1993, **115**, 5453.
- 16 M. S. Meier and J. Kiegiel, *Org. Lett.*, 2001, **3**, 1717.
- 17 L. Husebo, B. Sitharaman, K. Furukawa, T. Kato and L. Wilson, *J. Am. Chem. Soc.*, 2004, **126**, 12055.
- 18 P. A. Troshin, E. A. Khakina, A. S. Peregudov, D. V. Konarev, I. V. Soulimenkov, S. M. Peregudova and R. N. Lyubovskaya, *Eur. J. Org. Chem.*, 2010, 3265.
- 19 P. A. Troshin, A. B. Kornev, A. V. Mumyatov, D. K. Susarova and D. G. Deryabin *et al.*, in preparation.
- 20 J. Kolosnjaj, H. Szwarc, F. Moussa, Toxicity Studies of Fullerenes and Derivatives, in *Bio-Applications of Nanoparticles*, ed. W. C. W. Chan,

- Landres Bioscience and Springer Science+Business Media, LLC, New York, 2007, ch. 13, pp. 168–180.
- 21 S. H. Friedman, D. L. DeCamp, R. P. Sijbesma, G. Srdanov, F. Wudl and G. L. Kenyon, *J. Am. Chem. Soc.*, 1993, **115**, 6506.
- 22 L. G. Marsagishvili, A. G. Bobylev, M. D. Shpagina, P. A. Troshin and Z. A. Podlubnaya, *Biophysics*, 2009, **54**, 135.
- 23 M. R. Krebs, E. H. Bromley and A. M. Donald, *J. Struct. Biol.*, 2005, **149**, 30.
- 24 S. A. Hudson, H. Ecroyd, T. W. Kee and J. A. Carver, *FEBS J.*, 2009, **276**, 5960.
- 25 H. Levine III, *Protein Sci.*, 2008, **2**, 404.
- 26 N. Martin, L. Sanchez, B. Illescas and I. Perez, *Chem. Rev.*, 1998, **98**, 2527.