Because the diagnostic performance is poor, monoclonality needs to be confirmed by IFE (14).

We conclude that monitoring of renal involvement and BJP in patients with FLC myeloma can be improved by measuring both TUP and FLC in urine. Monitoring of the TUP concentration should be performed with the same assay.

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References

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Human N-Terminal proBNP Is a Monomer, Dan L. Crimmins (Department of Pathology and Immunology, Division of Laboratory Medicine, Washington University School of Medicine, 660 South Euclid Ave., Box 8118, St. Louis, MO 63110; fax 314-454-5208, e-mail crimmins@pathology.wustl.edu)

The cardiac hormone B-type natriuretic peptide (BNP) is synthesized in myocytes as a propre 134-amino acid residue molecule. The 108-residue proBNP mature form of the hormone is proteolytically cleaved to a biologically active form of 32 amino acids (residues 77–108) and an N-terminal fragment (residues 1–76; NT-proBNP) with an as yet undefined biological function (1). Clinically, both BNP and NT-proBNP have shown great promise as secreted, bloodborne diagnostic markers of left ventricle dysfunction. Measurement of each is based on immunnoassays; it therefore is likely that changes in the molecular form, e.g., posttranslational modifications, further proteolytic processing, and an oligomeric state for either analyte, could affect their measurements (2–5). These types of confounding molecular issues are likely for many analytes, with the myocyte damage marker cardiac troponin I one of the better studied. In this case, the commercial assays use antibodies directed to different epitopes, making “universal” calibration and determination of absolute analyte concentration difficult (6).

A previous report has indicated that NT-proBNP exists as a coiled-coil trimer, based on size-exclusion HPLC (SE-HPLC) of human, plasma-extracted material and a computer algorithm that predicts coiled-coils (7). I reinvestigated this claim on synthetic NT-proBNP, using the physicochemical techniques of analytical sedimentation, equilibrium ultracentrifugation, and circular dichroism (CD), and demonstrate that NT-proBNP is a monomer and not a trimer.

NT-proBNP was produced by solid-phase peptide synthesis (AnaSpec, Inc.) and obtained as a gift from Dade-Behring (Newark, DE). I used N-terminal sequencing and mass spectrometry as quality assurance procedures. Edman sequencing (8) was performed by Midwest Analytical, Inc. on 2 Coomassie-stained Immobilon-PSQ (Sigma) NT-proBNP-containing membrane sections. The first 52 residues were positively identified, with no preview, before the signal was not discernable from background (data not shown). Matrix-assisted laser desorption/ionization mass spectrometry (8) gave an experimental mass of 8457.0 compared with a calculated value of 8457.6 (data not shown). Lyophilized NT-proBNP was dissolved in and exhaustively dialyzed vs phosphate-buffered saline (pH 7.2) at 4°C. Analyte concentration was estimated gravimetrically and based on a molar absorptivity at 280 nm (ε280) of 0.82 L·g⁻¹·cm⁻¹; the 2 different techniques yielded better than 95% agreement.

The synthetic peptide in a neutral pH physiologic salt solution was run at 0.5 mL/min on SE-HPLC, and the resulting elution profile is plotted in Fig. 1A as A214nm vs time in minutes. The chromatographic process is monitored at 214 nm, which measures “peptide bond” absorbance; there thus is no bias in analyte detection under these analysis conditions. This is in contrast to the antibody-based, postcolumn analysis of plasma-extracted peptide (7), where detection is strictly a function of antibody reactivity. Furthermore, it is unclear what effect, if any, the C18 solid-phase plasma extraction procedure used in that study has on the molecular state of NT-proBNP before chromatography. Fig. 1A shows NT-proBNP eluting well before cytochrome C (12.4 kDa) and
just after myoglobin (17 kDa). One might be tempted to interpret this result to imply that NT-proBNP is a dimer, i.e., 8.5 kDa × 2 = 17 kDa; this is incorrect, however, as discussed below. The analyte profile observed here is not identical to that of the previous data (7), and is likely a result of combined use of a different SE-HPLC column packing material, different detection procedures, different protein markers, and different sample preparation methods. Nonetheless, one common attribute is that the elution of NT-proBNP is earlier than what would be expected of a typical globular protein of ~8.5 kDa. It is invalid here, and in general, on SE-HPLC performed under benign conditions to use the elution position as a surrogate for analyte molecular mass. The elution position on SE-HPLC is dictated by hydrodynamic volume, which is a function of the degree of hydration, molecular asymmetry, and the polar/nonpolar nature of the analyte and not on molecular mass (9). It becomes possible to estimate molecular mass for single-chain species only when the protein calibrators and analytes possess the same tertiary structure, as occurs when denaturing/disulfide-reducing solvents are used, for example (10). In the neutral-pH phosphate-buffered saline solution used here, all one can conclude is that NT-proBNP elutes unexpectedly with a larger molecular volume than the corresponding globular protein of ~8.5 kDa.

Sedimentation equilibrium ultracentrifugation was used to assess the oligomeric state of synthetic NT-proBNP. This analysis was performed at Iowa State University Protein Facility (ISUPF) on a Beckman Optima XL-A rotor at 4 °C. A concentration of 0.26 g/L was used at rotor speeds of 20 000, 30 000, and 40 000 rpm for various run

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Fig. 1. SE-HPLC (A), sedimentation equilibrium (B), and CD (C) of NT-proBNP. 
(A), SE-HPLC on a TosoHaas G2000SW column (600 × 7.5 mm (i.d)). (Top), Bio-Rad Gel Filtration Protein Mixture (left to right: thyroglobulin, IgG, ovalbumin, myoglobin, and vitamin B12) with indicated molecular masses; (middle), horse heart cytochrome C (12.4 kDa); (bottom), NT-proBNP. (B), analytical sedimentation equilibrium ultracentrifugation. (Top), residuals; (bottom), A_{280} vs radius. (C), CD analysis in a 1-mm pathlength cell at room temperature. Curve 1, 0.04375 g/L; curve 2, 0.0875 g/L; curve 3, 0.175 g/L. CD units are millidegrees.
times with optical scanning at 280 nm, which monitors aromatic residues in proteins/peptides. Data were plotted as $A_{280\text{nm}}$ vs radius in centimeters. This plot is shown in Fig. 1B, with the corresponding residuals for the 40 000 rpm rotor speed. For a single sedimenting ideal species, the instrument software (Ver. 2.01) transforms the absorbance vs radius data into a molecular weight of 8351, i.e., a monomer. The residuals, which are a measure of the goodness-of-fit of the curved line through the data points, are randomly yet narrowly distributed around 0. This attests to the high quality and lack of bias of the data. I attempted to fit the 40 000 rotor speed data to a 2-ideal-species monomer–dimer and a monomer–trimer equilibrium. After 11–13 computer program iterations, the results, given as concentrations of each species, were as follows: 0.262 g/L monomer with $1.4 \times 10^{-4}$ g/L dimer, and 0.262 g/L monomer with $5.7 \times 10^{-7}$ g/L trimer. Clearly, the only species present during ultracentrifugation was a monomer. It would not be possible to analyze the serum-generated sample (7) by this physicochemical technique because of the extremely low (ng/L) sample concentration.

NT-proBNP was reported to be a trimer containing a coiled-coil motif of repeating heptad units (7). Specifically, residues 17–38 were predicted to form a trimeric coiled-coil in a pattern represented as a-b-c-d-e-f-g. Positions a and d in this 7-residue repeat are almost invariably hydrophobic residues, e and g are usually charged residues of opposite sign, and the remaining 3 residues are usually hydrophilic. The molecular forces, including sequence position and specific amino acids along the 7-residue motif, that determine coiled-coil formation have been studied extensively (11). Typically a 4- or 5-heptad repeat or greater is necessary to produce stable coiled-coils in benign neutral-pH buffer depending on the exact amino acid sequence. Thus, the predicted 3-heptad coiled-coil would have to be extraordinarily stable to exist as a trimer in benign medium, a point also discussed by Seidler et al. (7). This 22-residue stretch represents ~30% of the sequence; it therefore is reasonable to expect that the helix content of this putative trimer would be at least ~30%. CD provides an excellent physicochemical measurement of protein helix content because the helix spectrum has a large diagnostically distinct negative doublet minima pair at 222/208 nm (12). The CD run performed at ISUPF on a Jasco J-710 instrument (Fig. 1C) showed no such minima pair for 3 different NT-proBNP concentrations. However, the spectra did show a minimum at <200 nm, which is indicative of a random coil (12), i.e., an unordered, possibly extended-like tertiary structure. The CD results showing no helix implies the absence of coiled-coils because such a quaternary structure requires association of slightly left-hand-twisted helices of 3.5 residues per turn.

The solution structure of NT-proBNP inferred from the respective results of the 3 experimental techniques is summarized in Table 1. The experimental techniques so chosen allow for solution structural assignment of the peptide. Collectively, these data convincingly indicate that NT-proBNP is not a coiled-coil trimer and in fact is a monomer. This is a consequence of essentially no helix as assessed by CD, which implies no coiled-coil and therefore no quaternary association, i.e., oligomerization, of individual molecules. Finally, the ultracentrifuge data conclusively show that at moderate concentrations and in a benign medium, synthetic human NT-proBNP is monomeric.

It is not intuitively obvious how to reconcile the results from this study and previous work (1, 7, 13) regarding the oligomeric nature of human NT-proBNP. In the earlier work, the sample was prepared by hydrophobic solid-phase extraction and elution with organic solvent. It is unclear how this procedure could affect either association or disassociation of the analyte. The extractant was then chromatographed by SE-HPLC in benign buffer, and the column eluate was measured by immunoreactivity. The identified fraction was of “high molecular weight”, the inference being oligomeric NT-proBNP. Another possibility involves a putative non-NT-proBNP binding component partner in serum, stable to SE-HPLC, that would produce an immunoreactive high–molecular-weight complex that collapses to “normal-eluting” (1, 7, 12) NT-proBNP after SE-HPLC run under denaturing conditions. This would be expected because the putative non-NT-proBNP–binding component partner is silent, i.e., unobservable, by immunodetection.

The actual solution structure of NT-proBNP must await high-resolution nuclear magnetic resonance or x-ray studies. One can speculate from the data presented here, however, that synthetic NT-proBNP is likely an unordered random coil with an extended-like structure. Whatever the case, human synthetic NT-proBNP is a monomer, and the potential confounding issue of analyte oligomerization is not a problem for this analyte.

### Table 1. Structural assignment of synthetic NT-proBNP from results of the study.

<table>
<thead>
<tr>
<th>Structural assignment*</th>
<th>SE-HPLC</th>
<th>Sedimentation equilibrium ultracentrifugation</th>
<th>CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2° structure</td>
<td>NA</td>
<td>Extended, non-globular</td>
<td>NA</td>
</tr>
<tr>
<td>3° structure</td>
<td>Extended, non-globular</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>4° structure</td>
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<td></td>
</tr>
<tr>
<td>Coiled-coil</td>
<td>NA</td>
<td>Extended, non-globular</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Random coil, no helix</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

*a 2° structure refers to helix, β-sheet, or random coil content; 3° structure refers to the overall three-dimensional shape of the molecule; and 4° structure refers to the putative state of association of individual molecules.

b NA, not available from experimental technique.
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References
11. Lau SYM, Taneja AK, Hodges RS. Synthesis of a model protein of defined carbohydrate antigen 15-3 (CA15-3) is frequently measured as a breast cancer marker test. Here we describe a novel type of optical biosensor system, the optical protein chip (OPC), to detect CA15-3 in serum.

The complex formed by interaction between an antibody molecule and its corresponding antigen can be detected on a silicon substrate by an optical sensor, as described in previous reports (1, 2). For processing and modification of the silicon substrate surface, silicon wafers were cut into ~2 × 0.7 cm rectangles and made hydrophilic by immersion in an acidic peroxide solution (300 mL/L H2O2–980 mL/L H2SO4, 1:3 by volume) and light shaking in a shaker for 30 min. The solution not only removed contaminants from the silicon surface but also increased the number of silanol groups on the surface. The hydrophilic surfaces were rinsed in distilled water 3 times and in absolute ethanol 3 times, then incubated in a mixture of 3-aminopropyltriethoxysilane and ethanol (1:15 by volume) and shaken lightly in a shaker for 2 h. The liquid was then removed, and the silicon wafers were rinsed in absolute ethanol 3 times and in phosphate-buffered saline (PBS) buffer 3 times. The wafers were then placed in a mixture of glutaraldehyde and PBS (1:10 by volume), shaken lightly in a shaker for 1 h, and finally, washed in PBS buffer 3 times and left in a beaker with PBS buffer until use. Through the reaction of glutaraldehyde with 3-aminopropyltriethoxysilane, Fc regions of the antibody molecules were covalently immobilized on the chip surfaces.

Protein chip preparation and detection included the following steps: (a) CA15-3-specific monoclonal antibody (Biodezign) was concentrated to 0.1 g/L, and then 20 μL of CA15-3 solution was delivered individually to each analytical spot on the chip by a microfluidics system (MFS) at a flow rate of 2 μL/min for 10 min. (b) After the entire volume of solution flowed onto each analytical spot on the silicon surface, 40 μL of distilled water was delivered individually to each spot on the chip by the MFS at a flow rate of 8 μL/min for 5 min to remove all nonadsorbed CA15-3 monoclonal antibody molecules on the analytical spot surface. (c) After the entire volume of distilled water flowed onto the analytical spots, 20 μL of a 1 g/L bovine serum albumin solution was delivered in the same way at a flow rate of 2 μL/min for 10 min to block nonspecific binding. (d) The chip was rinsed with 50 μL of distilled water in the same way at a flow rate of 10 μL/min for 5 min. (e) Serum samples were diluted with equal volumes of Tween 20 (20 mL/L) to a final volume of 50 μL, then the diluted samples were delivered individually to each analytical spot on the chip by the MFS at a flow rate of 2 μL/min for 25 min until the entire serum solution had flowed onto the analytical areas. (f) The chip was rinsed with 100 μL of distilled water in the same way at a flow rate of 20 μL/min for more than 5 min. (g) The chip was removed from the MFS and dried under a stream of nitrogen. The thicknesses of layers in the analytical areas were measured with biosensor imaging ellipsometry, which produced an ellipsometric image of a surface of each chip with a lateral resolution of 2 μm. The biosensor system used here was developed to visualize

Evaluation of a New CA15-3 Protein Assay Method: Optical Protein-Chip System for Clinical Application, Hong-Gang Zhang,1* Cai Qi,2 Zhan-Hui Wang,3 Gang Jin,2 and Rui-Juan Xiu1 (1 Institute of Microcirculation, Peking Union Medical College & Chinese Academy of Medical Science, Beijing, Peoples Republic of China; 2 Institute of Mechanics, Chinese Academy of Sciences, Beijing, China; * address correspondence to this author at: Institute of Microcirculation, Peking Union Medical College & Chinese Academy of Medical Science, 5 DongDanSanTiao, Beijing 100005, China; e-mail Zanghng1966126@yahoo.com.cn)

Carbohydrate antigen 15-3 (CA15-3) is frequently measured as a breast cancer marker test. Here we describe a