

UC Irvine

UC Irvine Previously Published Works

Title

Shear-Stress-Mediated Refolding of Proteins from Aggregates and Inclusion Bodies

Permalink

<https://escholarship.org/uc/item/136688r5>

Journal

ChemBioChem, 16(3)

ISSN

1439-4227

Authors

Yuan, Tom Z
Ormonde, Callum FG
Kudlacek, Stephan T
et al.

Publication Date

2015-02-09

DOI

10.1002/cbic.201402427

Supplemental Material

<https://escholarship.org/uc/item/136688r5#supplemental>

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

Shear-Stress-Mediated Refolding of Proteins from Aggregates and Inclusion Bodies

Tom Z. Yuan,^[a] Callum F. G. Ormonde,^[b] Stephan T. Kudlacek,^[c] Sameeran Kunche,^[d] Joshua N. Smith,^[a] William A. Brown,^[c] Kaitlin M. Pugliese,^[c] Tivoli J. Olsen,^[c] Mariam Iftikhar,^[a] Colin L. Raston,^{*[e]} and Gregory A. Weiss^{*[a, c]}

Recombinant protein overexpression of large proteins in bacteria often results in insoluble and misfolded proteins directed to inclusion bodies. We report the application of shear stress in micrometer-wide, thin fluid films to refold boiled hen egg white lysozyme, recombinant hen egg white lysozyme, and recombinant caveolin-1. Furthermore, the approach allowed refolding of a much larger protein, cAMP-dependent protein kinase A (PKA). The reported methods require only minutes, which is more than 100 times faster than conventional overnight dialysis. This rapid refolding technique could significantly shorten times, lower costs, and reduce waste streams associated with protein expression for a wide range of industrial and research applications.

Overexpressed recombinant proteins for industrial, pharmaceutical, environmental, and agricultural applications annually represent a more than \$160 billion world market.^[1] Protein expression in yeast or *Escherichia coli* is highly preferred, due to the organism's rapid growth, low consumable costs, and high yields.^[2,3] However, large proteins overexpressed in bacteria often form aggregates and inclusion bodies.^[4–7] Recovery of the correctly folded protein then requires laborious and expensive processing of inclusion bodies by conventional methods.^[1,8] The most common method for refolding such proteins, for example, involves multi-day dialysis with large volumes (typically 1–10 liters for mg quantities of protein).^[9] Alternatively, expression of high-value proteins (e.g., therapeutic antibody

ies or GPCRs for structural biology) require extensively optimized mammalian or insect cell lines, media, and bioreactor conditions.^[10–12] Recovery of correctly folded proteins from aggregates is inefficient and challenging for large-scale industrial processes. Mechanical methods to solve this problem have been reported. One approach applies very high hydrostatic pressures (400 bar) to refold recombinant proteins from inclusion bodies.^[13–16] We report a novel method by applying finely controlled levels of shear stress to refold proteins trapped in inclusion bodies. This method might be capable of broadening the utility of bacterial overexpression, and could transform industrial and research production of proteins.

We report using a vortex fluid device (VFD) to apply shear forces for rapid equilibration of protein folding and isolation of intermediates during protein folding. In this method, a glass cylinder (10 mm by 16 cm) is spun rapidly (5 krpm) at a 45° angle. At high rotational speeds, the solution within the sample tube forms micrometer-thick, thin fluid films, which flow with the same speed and direction as the wall of the glass tube. The rotating glass tube generates a velocity gradient within the thin fluid film, which introduces shear stress into the solution (Figure 1A). We imagined applying the VFD with a similar range of input energies to the refolding of proteins: the device produces unusual shear within such films and has been shown to be effective in disassembling molecular capsules^[15] and reducing the size of micelles used in the fabrication of SBA-15 mesoporous silica with control over the pore size of the material.^[16] Importantly, the latter occurs at room

[a] Dr. T. Z. Yuan, J. N. Smith, M. Iftikhar, Prof. G. A. Weiss
University of California, Irvine
Department of Molecular Biology and Biochemistry
Irvine, CA, 92697-2025 (USA)
E-mail: gweiss@uci.edu

[b] C. F. G. Ormonde
The University of Western Australia, School of Chemistry and Biochemistry
Perth, Western Australia, 6009 (Australia)

[c] S. T. Kudlacek, W. A. Brown, K. M. Pugliese, Dr. T. J. Olsen, Prof. G. A. Weiss
University of California, Irvine, Department of Chemistry
Irvine, CA, 92697-2025 (USA)

[d] S. Kunche
University of California, Irvine, Center for Complex Biological Systems
Irvine, CA 92697-2280 (USA)

[e] Prof. C. L. Raston
Flinders University, Centre for NanoScale Science and Technology
Adelaide, South Australia, 5001 (Australia)
E-mail: colin.raston@flinders.edu.au

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.201402427>.

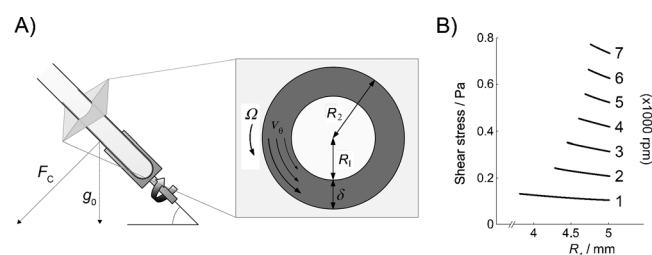


Figure 1. Protein refolding in vitro with the vortex fluid device (VFD), which generates shear flow inside thin fluid films (shaded). a) VFD schematic and parameters for modeling. The inset represents a two-dimensional slice of the tube through the axis of rotation. The inner diameter of the tube, R_2 , is 5 mm, and the thin fluid film thickness, δ , has been previously measured (not depicted to scale).^[17] The velocity of the solution, v_θ , increases from the inner surface of the film to match the velocity of the tube, Ω . b) Shear stress calculated as a function of rotational speed (rpm) and radius, R_1 , from the center of the tube.

temperature rather than requiring hydrothermal processing and suggests that the constant “soft” energy in the thin film has application in manipulating macromolecules, such as the refolding of proteins, without requiring heating. Other relevant applications and optimization of a similar VFD include exfoliating graphite and hexagonal boron nitride to generate mono- and multilayer structures,^[17,18] controlling the formation of different calcium carbonate polymorphs,^[19] and controlling chemical reactivity and selectivity.^[20,21]

Modeling the fluid behavior in the VFD allowed estimation of the shear forces experienced by proteins folding at various rotational speeds. Our analysis applies the solution for cylindrical Couette flow.^[20,21] The velocity of the solution, v_{θ} , is a function of the radius, r (Figure 1A). The boundary conditions for the liquid film interfaces are defined as follows. The inner air-liquid interface at $r=R_1$ slips, due to discontinuity in viscosity, and results in increasingly low shear stress ($dv_{\theta}/dr=0$). At the outer liquid-glass interface, the no-slip boundary dictates that the velocity of the liquid at $r=R_2$ matches that of the inner wall of the glass tube ($v_{\theta}=R_2\cdot\Omega$), where Ω is the angular velocity of the tube. The resulting velocity profile is a nonlinear function of the form

$$v_{\theta} = Ar + B/r$$

where

$$A = \frac{\Omega}{\frac{R_1^2}{R_2^2} + 1} \quad \text{and} \quad B = \frac{\Omega R_1^2}{\frac{R_1^2}{R_2^2} + 1}$$

From this velocity profile, shear stress can be calculated as

$$\tau_{r\theta} = \mu r \frac{\partial}{\partial r} \left(\frac{v_{\theta}}{r} \right)$$

where μ is the viscosity of water at 20 °C. At a speed of 5 krpm, the calculated shear stress ranges from 0.5246 to 0.5574 Pa (Figure 1B). Although this model provides insight into the shear stress range, the fluid flow will be perturbed by the cross vector of gravity with centrifugal force for a tube oriented at 45°,^[20] which might also facilitate protein folding; this angular setting also proved optimal for the aforementioned applications of the VFD. The calculated values of shear stress are similar to the levels previously reported to cause protein unfolding,^[22] and we hypothesized that short periods of VFD processing could allow equilibration of protein folding.

Experiments with native hen egg white were conducted to determine if shear forces could refold denatured hen egg white lysozyme (HEWL) in complex environments. The separated whites were diluted in PBS and heat-treated at 90 °C for 20 min. The resultant hard-boiled egg white was dissolved in 8 M urea, rapidly diluted, and then VFD-processed at the indicated rotational speeds and times (Table S1 and Figure 2A). The total protein concentration, as determined by bicinchoninic acid assay, was 44 $\mu\text{g mL}^{-1}$. The recovery of HEWL activity was then demonstrated by a lysozyme activity assay (Fig-

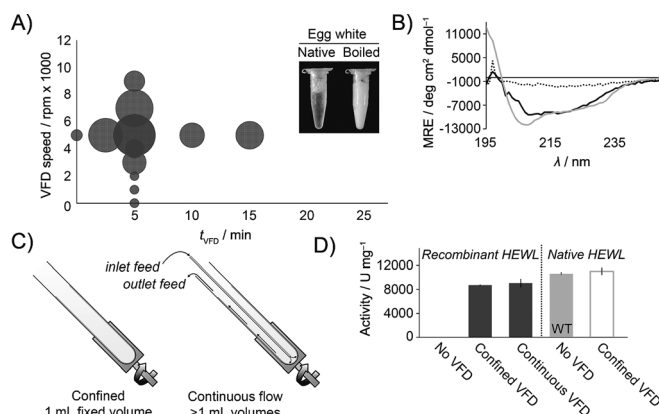


Figure 2. Determination of secondary structure and activity of hen egg white lysozyme (HEWL) processed by VFD. A) Lysozyme activity per mg protein following VFD processing of boiled egg white (90 °C, 20 min) at a fixed VFD speed of 5 krpm or a fixed 5 min refolding time. The relative circle size represents the lysozyme activity, with larger circles representing higher recovered activities, plotted as a function of VFD time and speed. B) CD spectra of recombinantly expressed, reduced, and denatured HEWL before (---) and after (—) VFD refolding; —: native HEWL. C) In continuous-flow mode, the protein solution is introduced through a thin, hollow metal tube to the bottom of the sample tube, and the folded protein can be collected at the top of the VFD. D) Lysozyme activity per mg protein following VFD refolding of recombinantly expressed HEWL and native HEWL. Under these conditions, VFD treatment of recombinant lysozyme recovered over 82% of activity in both confined and continuous-flow modes, compared to wild-type protein (WT). VFD treatment of wild-type, active lysozyme isolated from eggs does not adversely affect its activity (white). Throughout this report, error bars indicate standard deviation ($n=3$).

ure S1). Refolding HEWL within the egg white at 5 krpm recovered glycosidase activity even after a short 2.5 min spin, but continued shear forces unfolded the protein. As mentioned above, shear-stress-mediated protein unfolding has been observed for other proteins previously. Optimization of VFD times, speeds, and concentration of chaotropic additives is required to find conditions allowing re-equilibration of protein folding without causing loss of the protein structure. VFD processing for 5 min at 5 krpm in 1 M urea resulted in optimal HEWL refolding as monitored by lysozyme activity (Figure 2A). This experiment establishes the basic parameters for protein refolding by VFD.

To demonstrate refolding of recombinantly expressed, reduced HEWL, the cell pellet was first reconstituted in lysis buffer containing 2-mercaptoethanol, denatured in 8 M urea, and rapidly diluted into PBS (1:100). Buffers used for the expression and purifications of all recombinant proteins are described in Table S2. Second, the diluted protein (1 mL, 44 $\mu\text{g mL}^{-1}$ protein) was immediately transferred to the VFD sample tube and spun at 22 °C and 5 krpm for 5 min. Circular dichroism (CD) spectra of the VFD-refolded, recombinant HEWL demonstrated restoration of secondary structure from proteins isolated from inclusion bodies. After VFD processing, the CD spectra of identical HEWL samples demonstrated partial recovery of secondary structure compared to the native lysozyme (Figure 2B). Yields of functional protein determined by lysozyme activity assay are shown in Figure 2D.

HEWL could also be refolded by continuous-flow VFD. This approach delivers additional sample through an inlet at the cylinder base. The sample (50 mL), added at a flow-rate of 0.1 mL min^{-1} , demonstrated significant recovery of HEWL activity for scalable, high volume applications (Figure 2C). The recombinant HEWL recovered $>82\%$ of its activity following VFD treatment. As expected, HEWL isolated from inclusion bodies without VFD processing failed to show any lysozyme activity (Figure 2D). The continuous-flow approach could be readily scaled up and parallelized for industrial applications requiring treatment of very large volumes.

After refolding denatured lysozyme in both complex (egg white) and simple (purified recombinant protein) environments, the next experiments focused on refolding the protein caveolin-1, as an example of a protein requiring an inordinate amount of processing time by conventional approaches (e.g., four days of dialysis). A caveolin variant without its transmembrane domain (caveolin- ΔTM) was recombinantly expressed, and the inclusion body was purified under denaturing conditions. Purified caveolin- ΔTM was diluted and then underwent a short (1 h) dialysis to decrease the urea concentration to 1 M. The protein was then VFD-treated for 0, 10, or 30 min at 5 krpm at a concentration of $186 \mu\text{g mL}^{-1}$. The CD spectra of the VFD-processed caveolin- ΔTM showed pronounced minima at 208 and 220 nm, which are indicative of α -helical secondary structure.^[23] Solution turbidity also decreased sharply following VFD treatment, which illustrates VFD solubilization and refolding of partially aggregated proteins (Figure 3B). ELISA experiments examined binding by the refolded caveolin- ΔTM to HIV glycoprotein 41 (gp41), a known caveolin binding partner.^[24–26] VFD processing significantly restored protein function, as shown through binding to gp41 (Figure 3C).

Larger-sized proteins initially failed to refold, despite VFD treatment. For example, the catalytic domain of cAMP-dependent protein kinase A (PKA, 42 kDa) is significantly bulkier than HEWL (14 kDa) and caveolin- ΔTM (17 kDa) and did not refold from inclusion bodies after treatment with similar protocols to the above experiments. To refold full-length PKA *in vitro*, we adapted the VFD to provide a closer mimic of cellular folding. In cells, the nascent polypeptide can fold as the N terminus extrudes from the ribosome, whereas *in vitro* refolding must address the entire protein at once.^[27] Thus, we attempted to focus shear stress on the N terminus of His-tagged PKA by binding the unfolded protein to Ni^{2+} -charged immobilized metal affinity chromatography (IMAC) resin. The His_6 -PKA-IMAC complex (1 mL , $0.2\text{--}1 \text{ mg mL}^{-1}$) was then subjected to refolding by VFD. Following VFD treatment, His-PKA separated from the IMAC resin and recovered 69% of its kinase activity (Figure 4). Interestingly, the remaining His-PKA eluted from the IMAC resin by imidazole yielded far less active protein (Figure S3). We hypothesize that protein folding makes the His_6 epitope less accessible for strong binding to the IMAC resin subjected to shear by the VFD. Under conventional conditions, IMAC resin binds to folded PKA, but we recovered protein remaining on the IMAC resin after VFD treatment to demonstrate its lack of folding and catalytic activity (Figure S3). Thus, as the VFD folds the protein, PKA elutes from the affinity resin. Nega-

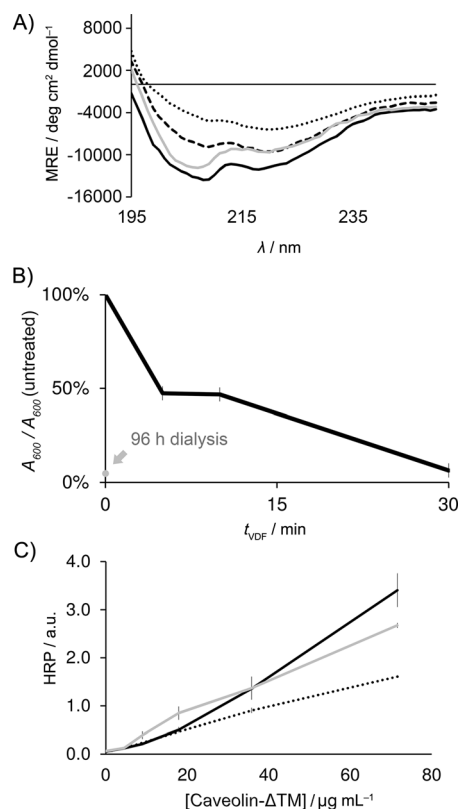


Figure 3. Determination of secondary structure and activity of caveolin- ΔTM processed by VFD. A) Circular dichroism (CD) spectra of caveolin- ΔTM following VFD-induced refolding or conventional dialysis. B) Turbidity of caveolin- ΔTM , measured by the fractional absorbance at 600 nm compared to the untreated sample, also at 600 nm. The arrow indicates absorbance of caveolin- ΔTM following 4-days of dialysis. Both 30 min of VFD-based refolding and 96 h of dialysis significantly decrease the turbidity of the protein solution. C) Binding of caveolin- ΔTM to gp41 determined by ELISA with untreated, VFD-treated, and conventionally refolded by dialysis. All other conditions and buffers remained identical. Although some binding occurs without VFD treatment, caveolin- ΔTM binds with greater affinity after refolding by VFD.: untreated, - - - - -: 10 min VDF, —: 30 min VDF, —: 96 h, dialyzed.

tive control experiments with identical conditions but with uncharged IMAC resin, decreased quantities of charged resin, or 500 mM imidazole to block the Ni^{2+} - His_6 tag interaction showed only low levels of kinase activity (Figure 4).

As reported here, protein refolding by VFD requires optimization for each protein. Buffers, chaotrope additive, protein concentration, and processing time were optimized for HEWL, caveolin- ΔTM , and PKA. The refolding of HEWL from the complex mixture of boiled egg whites appeared less efficient than recovery of the folded protein isolated from inclusion bodies. In egg whites, the mechanical energy of the VFD could be misdirected to the other $>96\%$ of proteins present.

This report offers significant advantages over conventional approaches to protein refolding. First, VFD-mediated refolding requires much smaller solution volumes (ca. 1% of the volumes required for conventional dialysis). Second, this key step in protein production occurs more than 100 times faster than overnight dialysis, with >1000 -fold improvements for proteins such as a caveolin. Notably, introducing high shear in thin fluid

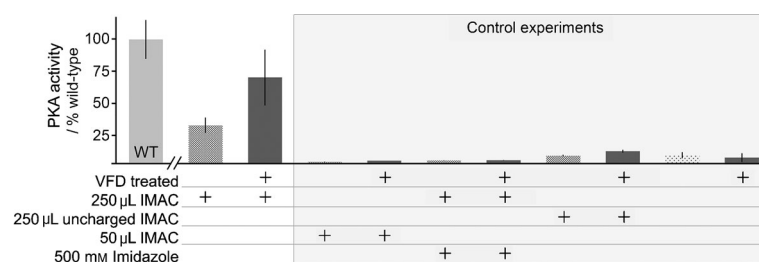


Figure 4. Facilitating VFD refolding of PKA by pre-binding the His₆-tagged protein to IMAC resin. PKA (1.7 mg) was pre-incubated with 250 or 50 µL IMAC resin in 6 M guanidine-HCl prior to dilution to 1 M guanidine-HCl and VFD treatment. Excess PKA was removed by an imidazole (1 mM) wash buffer. The PKA activity per µg of protein was measured by a nicotinamide adenine dinucleotide (NADH) enzyme-linked assay and is shown here as a percentage of untreated, wild-type PKA activity (WT). Dark gray bars denote samples that are VFD-treated. In this assay, the consumption of ATP by PKA catalysis results in a lactate dehydrogenase-coupled decrease in the levels of NADH, monitored through measuring absorbance at 340 nm. The control experiments included low quantities of resin (50 µL IMAC) and two controls for non-specific binding to the resin in the absence of the Ni²⁺-His₆ tag interaction (500 mM imidazole added, and uncharged IMAC resin lacking Ni²⁺). Imidazole was diluted to 50 mM before the kinase assay.

films is a low energy, inexpensive process and can be readily implemented for relatively low cost in conventional biochemistry laboratories.

The advantages of VFD refolding open new possibilities for increasing protein yields in simple cell lines. By using the constant soft energy of the thin microfluidic films, the VFD can untangle complex mixtures, aggregates, and insoluble inclusion bodies. In industrial and academic applications, high concentrations of a chemical inducer like IPTG could drive over-expressed proteins into insoluble inclusion bodies before VFD-based refolding. Conversely, most conventional processes avoid inclusion bodies by optimizing growth conditions and special cell lines at the expense of higher yields and purer protein isolated directly from bacterial cells. As reported here, the continuous-flow mode of the VFD or parallel processing with multiple VFD units could allow scale-up to accommodate much larger solution volumes. Thus, the approach could drastically lower the time and financial costs required to refold inactive proteins on an industrial scale. The VFD sample tube itself can also be modified to amplify or otherwise direct the intensity of shear forces applied. For example, modified surfaces with high contact angle and/or with textured features could enhance the turbulent flow, altering the applied shear stress. Harnessing shear forces to achieve rapid equilibration of protein folding could be expanded to a wide range of applications for research and manufacturing.

Acknowledgements

We thank Professor Abraham Lee for discussion and advice on fluid modeling. This work was supported by the National Institute of General Medical Sciences of the NIH (R01 GM100700-01) and

the Australian Research Council (ARC DP1092810 and DP130100066).

Keywords: green chemistry · protein expression · protein folding · shear stress

- [1] H.-P. Meyer, D. Schmidhalter in *Innovations in Biotechnology* (Ed.: E. Agbo), InTech, Rijeka, **2012**, pp. 211–250.
- [2] E. Freire, A. Schön, A. Velazquez-Campoy, *Methods Enzymol.* **2009**, *455*, 127–155.
- [3] S. Futaki, *Adv. Drug Delivery Rev.* **2005**, *57*, 547–558.
- [4] S. E. Radford, *Trends Biochem. Sci.* **2000**, *25*, 611–618.
- [5] P. Jemth, S. Gianni, R. Day, B. Li, C. M. Johnson, V. Daggett, A. R. Fersht, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 6450–6455.
- [6] L. Wang, S. K. Maji, M. R. Sawaya, D. Eisenberg, R. Riek, *PLoS Biol.* **2008**, *6*, e195.
- [7] L. Wang, D. Schubert, M. R. Sawaya, D. Eisenberg, R. Riek, *Angew. Chem. Int. Ed.* **2010**, *49*, 3904–3908; *Angew. Chem.* **2010**, *122*, 3996–4000.
- [8] L. F. Vallejo, U. Rinas, *Microb. Cell Fact.* **2004**, *3*, 11.
- [9] K. Tsumoto, D. Ejima, I. Kumagai, T. Arakawa, *Protein Expression Purif.* **2003**, *28*, 1–8.
- [10] L. Feng, N. Vijayasankaran, A. Shen, R. Kiss, A. Amanullah, *MAbs* **2010**, *2*, 466–479.
- [11] G. Hannig, S. C. Makrides, *Trends Biotechnol.* **1998**, *16*, 54–60.
- [12] D. C. Andersen, L. Krummy, *Curr. Opin. Biotechnol.* **2002**, *13*, 117–123.
- [13] R. J. St. John, J. F. Carpenter, T. W. Randolph, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 13029–13033.
- [14] M. W. Qoronfleh, L. K. Hesterberg, M. B. Seefeldt, *Protein Expression Purif.* **2007**, *55*, 209–224.
- [15] B. G. Lefebvre, M. J. Gage, A. S. Robinson, *Biotechnol. Prog.* **2008**, *20*, 623–629.
- [16] B. G. Lefebvre, A. S. Robinson, *Biotechnol. Bioeng.* **2003**, *82*, 595–604.
- [17] X. Chen, J. F. Dobson, C. L. Raston, *Chem. Commun.* **2012**, *48*, 3703–3705.
- [18] R. A. Boulos, F. Zhang, E. S. Tjandra, A. D. Martin, D. Spagnoli, C. L. Raston, *Sci. Rep.* **2014**, *4*, 3616.
- [19] L. Yasmin, X. Chen, K. A. Stubbs, C. L. Raston, *Sci. Rep.* **2013**, *3*, 2282.
- [20] A. Mallock, *Proc. R. Soc. A* **1888**, *45*, 126–132.
- [21] L. Rayleigh, *Proc. R. Soc. London Ser. A* **1917**, *93*, 148–154.
- [22] I. B. Bekard, P. Asimakis, J. Bertolini, D. E. Dunstan, *Biopolymers* **2011**, *95*, 733–745.
- [23] V. P. Saxena, D. B. Wetlaufer, *Proc. Natl. Acad. Sci. USA* **1971**, *68*, 969–972.
- [24] A. G. Hovanessian, J.-P. Briand, E. a. Said, J. Svab, S. Ferris, H. Dali, S. Muller, C. Desgranges, B. Krust, *Immunity* **2004**, *21*, 617–627.
- [25] X. M. Wang, P. E. Nadeau, Y.-T. Lo, A. Mergia, *J. Virol.* **2010**, *84*, 6515–6526.
- [26] S. Majumdar, A. Hajducski, R. Vithayathil, T. J. Olsen, R. M. Spitler, A. S. Mendez, T. D. Thompson, G. A. Weiss, *J. Am. Chem. Soc.* **2011**, *133*, 9855–9862.
- [27] M. S. Evans, I. M. Sander, P. L. Clark, *J. Mol. Biol.* **2008**, *383*, 683–692.

Received: July 30, 2014

Revised: December 1, 2014

Published online on January 23, 2015