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ARTICLE *in* JOURNAL OF PHYSICAL CHEMISTRY LETTERS · NOVEMBER 2012

Impact Factor: 7.46 · DOI: 10.1021/jz3018682

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In Vitro Enzyme Comparative Kinetics: Unwinding of Surface-Bound DNA Nanostructures by RecQ and RecQ1

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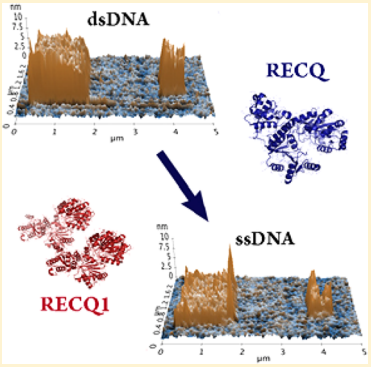
Supporting Information

ABSTRACT: Many cellular processes entail the separation of nucleic acid strands. Helicases are involved in the separation of the double-stranded DNA, a process fueled by ATP hydrolysis. We investigated the reaction mechanism of two homologous helicases, the bacterial RecQ and the human RECQ1, in vitro, that is, within confined DNA monolayers. We generate arrays of engineered DNA sequences by atomic force microscopy (AFM) nanografting and monitor the enzyme activity on the surface by means of differential, highly precise AFM measurements of the DNA height variation. The latter is associated with the unwinding action of the enzyme onto the surface-bound DNAs because it arises from the different mechanical properties of single- versus double-stranded DNA that are sensibly detected by AFM. Our results highlight different kinetic behaviors for these enzymes under the same experimental conditions.



SECTION: Biophysical Chemistry and Biomolecules

Helicases are molecular motors, which typically use the energy from ATP hydrolysis to catalyze the breaking of the H-bonds between nucleotides of complementary DNA/RNA strands. The multiplicity of helicases existing in nature (31 DNA helicases in humans[1]) reflects the multiplicity of cellular processes in which helicases are involved: from DNA replication to DNA transcription, translation, repair, chromosome partitioning, and so on.

Among helicases, the RecQ family is well known for its relevant role in the biological processes that enforce genomic stability.[2,3] This class of helicases is highly conserved in evolution and in human cells: five human RecQ homologues, named RECQ1, BLM, WRN, RECQ4 and RECQ5, have been identified. Whereas it is known that inherited mutations in the genes encoding for BLM, WRN, and RECQ4 can cause cancer-prone disorders (Bloom syndrome, Werner, and Rothmund–Thomson syndrome, respectively[4−6]), no mutation in RECQ1 and RECQ5 genes has been associated to heritable cancer predisposition disorder to date. However, it has been recently highlighted that RECQ1 may have a role in cancer survival and might be a potential target for cancer therapy.[7−9]

Despite its high abundance in human cells and its putative role as a cancer biomarker, RECQ1 is one of the less studied human RecQ helicases to date, its function is still unclear and its unwinding behavior still debated. Recent biochemical and structural studies evidenced the relevance of topological effects in the interaction between RECQ1 helicase domains and DNA substrates (substrate specificity and formation of oligomers), and only few kinetic data relative to the enhancement of RECQ1 unwinding activity in the presence of human replicative protein A have been reported so far.[10−16]

To gain new insights on the mechanism of action of such enzymes and to identify and quantify their interactions with DNA substrates it is required to follow and understand the biochemical reaction of unwinding with high sensitivity and high spatial resolution.

Here we propose a novel strategy based on atomic force microscopy (AFM) to study at the molecular level the unwinding action of the RECQ1 helicase in laterally confined monolayers of dsDNA substrates. We exploited the different mechanical properties of ssDNA and dsDNA to detect, by AFM height measurements, the duplex opening due to helicase unwinding activity as a function of the incubation time. To validate our assay, we then compared the RECQ1 results with the kinetic behavior of its homologous and more extensively studied *E. coli* RecQ helicase.[17−26]

A schematic picture of the assay is given in Figure 1. By means of AFM nanografting, that is, by local molecular displacement and substitution, we immobilized a dsDNA forked substrate inside a monolayer of self-assembled thiolated

Received: November 15, 2012
Accepted: November 16, 2012

   dx.doi.org/10.1021/jz3018682 | *J. Phys. Chem. Lett.* 2012, 3, 3532−3537

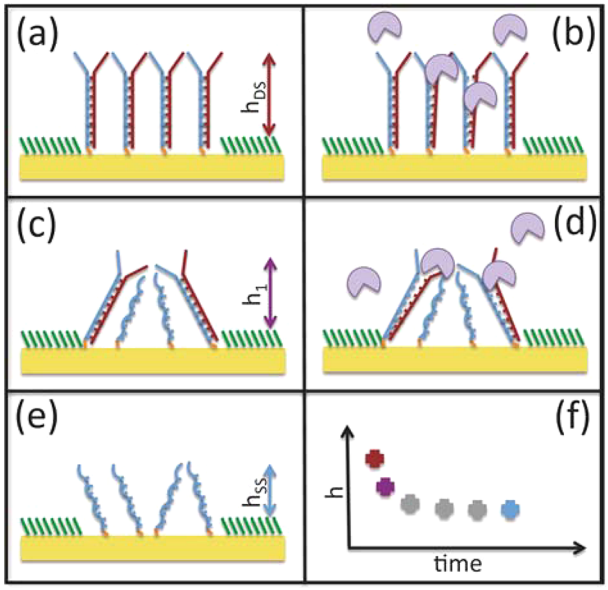


Figure 1. Schematics of the Helicase assay on DNA nanografted monolayers.

oligoethylene glycol (OEG) molecules on a flat, gold film surface. (The procedure is described in the Supporting Information (SI).) By AFM imaging, we then measured the relative height of the dsDNA structure (h_{ds}) with respect to the top of the ordered OEG carpet (Figure 1 a) that has the function of discouraging unspecific adsorption.[27] In a second step, we let the enzyme work for a time t_1 (Figure 1 b), we stop the reaction by an accurate washing, and we repeat the height measurement (Figure 1 c). If the enzyme worked properly, then the initial dsDNA structure would be converted into a mixed monolayer of ssDNA and not unwound dsDNA. Because the mechanical response of single-stranded and double-stranded DNA is very different due to the different strand rigidity (the persistent length varies from 2 to 50 nm, respectively, at the used salt concentration), the measured height will be lower than that before $h_1 \leq h_{ds}$. By repeating the procedure for increasing incubation time (Figure 1d), the dsDNA in the patch will be finally converted to a fully unwound ssDNA monolayer (Figure 1 e), the measured height of which matches the height of the initial ssDNA patch. The plot of the variation of the height as a function of the incubation time (Figure 1 f) gives the kinetic signature of the unwinding process. One of the separated strands remains in place while the other (the red one in the Figure) goes into solution, where it becomes too diluted to affect any further process.

The proof of the working principle of our helicase assay is given in Figure 2. The immobilization of ssDNA gives a resulting patch emerging from the thiolated-OEG molecular carpet by ~3.5 nm, as shown by the AFM image and the corresponding height profile (Figure 2a,b). This value will serve as the reference value for the ssDNA. After the hybridization with the complementary strand, the height increases to ~8 nm due to the higher rigidity of the dsDNA forked substrate (Figure 2c,d). An incubation of 3 h in a 50nM RECQ1 solution

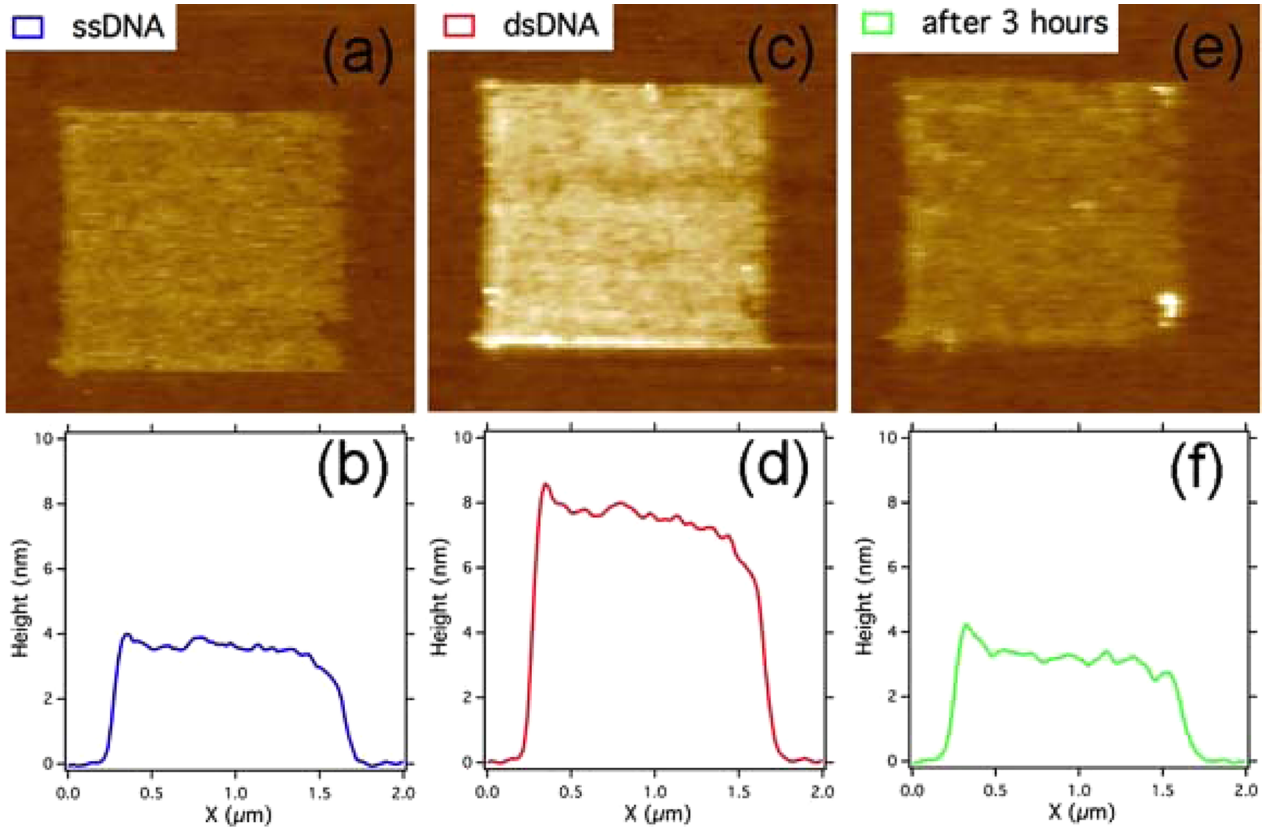


Figure 2. Atomic force microscopy topographic images and corresponding *average* line profiles of a patch (size of the images is 2 × 2 μm). As grafted (ssDNA), after the hybridization (dsDNA), and after 3 h of 50nM RecQ1 helicase incubation in the unwinding buffer (20 mM Tris, 2 mM DTT, 5 mM $MgCl_2$, 10 mM KCl, 5 mM ATP, pH 7.5).

dx.doi.org/10.1021/jz3018682 | *J. Phys. Chem. Lett.* 2012, 3, 3532–3537

results in the complete unwinding of the DNA substrate with a consequent recovery of the previous ssDNA height, as shown in the AFM image and corresponding height profile in (Figure 2e,f). The experiment reported in Figure 2 shows that enzymes acting on a dsDNA substrate vertically immobilized on a surface and laterally confined are capable of unwinding the forked DNA and that the height variation from dsDNA to ssDNA can be measured with high precision, confirming that our technique can follow the action of the enzyme. Similar results have been obtained on patches of different sizes, from 300 nm to 2 μm, as reported in SI, Figure S1.

To extract kinetic information on the unwinding time course, we choose to monitor as a function of the incubation time the ratio h_{REACT}/h_{ds} (hereafter $R(t)$) between the patch height after the reaction (representative of the unwound substrate) and the dsDNA height. In Figure 3 (lower panel), we report the

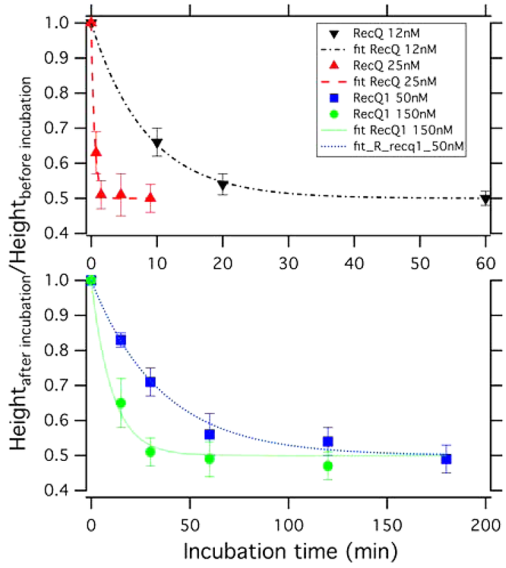


Figure 3. Variation of the ratio between the height before and after the reaction as a function of the incubation time of *E. coli* RecQ (upper panel) and human RECQ1 (bottom panel).

variation of $R(t)$ as a function of the incubation time for two different concentrations of bacterial RECQ1 helicase (50 and 150 nM). We can clearly observe a decay of this ratio until it reaches the expected value of the fully ssDNA patches (h_{ss}/h_{ds} = 0.50 ± 0.02, see Figure S2 in the SI). To check that the effective unwinding is not damaging the DNA surface, we rehybridized the patches after the incubation and we recover the starting dsDNA value within standard deviation for the rehybridized patches (h_{hyb}/h_{ds} = 0.95 ± 0.05; see Figure S2 in SI). Fitting the data with an exponential decay ($(R(t) = A + (1 - A)\exp(-k_{OBS}*C*t)$ dotted lines, where A = 0.5 is the mean experimental value of the ratio h_{ss}/h_{ds} and C is the concentration of the enzyme in solution), we can extract an observed apparent rate k_{OBS} of the unwinding process on the surface-bound DNA structures. In the specific case, the k_{OBS} values result in being 5.6 ± 0.6 × 10^{-4} $(min*nM)^{-1}$ (50 nM incubation) and 6 ± 2 × 10^{-4} $(min*nM)^{-1}$ (150 nM incubation). We repeated the experiments with the bacterial RECQ1 homologue, the *E. coli* RecQ helicase. In the upper panel of Figure 3, we report the variation of $R(t)$ for two different concentrations (12 and 25 nM). Because RECQ1 helicase has been recently demonstrated to act as a dimer,[9] whereas the *E. coli* RecQ is known to act as a monomer,[12] we used a lower concentration of bacterial RecQ with respect to human RECQ1 to favor a direct comparison of the results.

The k_{OBS} values result in being 10 ± 6 × 10^{-3} and 24 ± 8 × 10^{-3} $(min*nM)^{-1}$ for 12 and 25 nM RecQ concentrations, respectively. The human RECQ1 enzymatic reaction is much slower than the one of the bacterial RecQ, and the height ratio decreases slowly, even though we increased the concentration of enzymes in solutions. The apparent observed rate k_{OBS} that can be extracted from our assay contains the information related not only to the unwinding rate but also to the binding rate and the diffusion rate of enzymes onto the surface and onto the DNA patches. To prevent, or at least reduce, the effect of the diffusion onto the surface, we started the reaction by ATP addition after a preincubation time of 15 min. After the preincubation of RecQ without ATP and washing only with high salt buffer (Tris-Hcl 10 mM, 1 mM EDTA, 1 M NaCl, pH 7.1), we observed the presence of a quite homogeneous layer on top of the patches, which resulted to be ∼4 nm higher, as shown in the SI, Figure S3. This is consistent with the binding of a single layer of enzymes on the DNA, and it is compatible with a 1:1 (or nearly 1:1) ratio between DNA and enzyme. We can then assume that the difference in k_{OBS} can be ascribed to the processivity of the two enzymes.

To get a straight comparison between the two unwinding activities, we can assume that the height variation scales linearly with the dehybridization efficiency of the patch and hence calculate the estimated fraction of unwound substrate as a function of time for the 25 nM bacterial RecQ assay and the 50nM human RECQ1 assay (Figure 4).

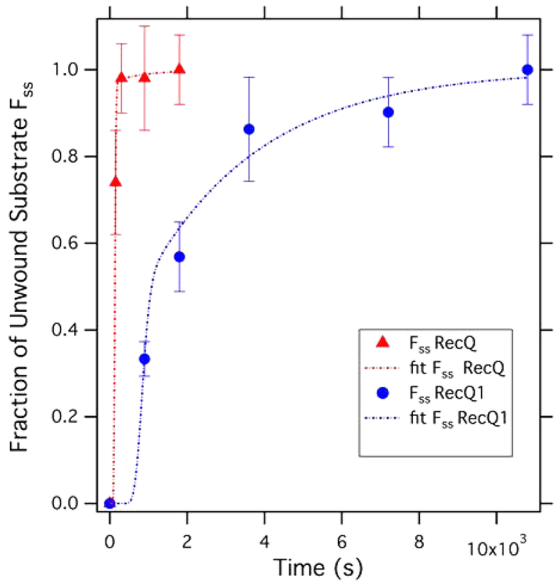


Figure 4. Comparison between the processivity of *E. coli* RecQ and human RECQ1 enzymes. Estimated fraction of the unwound substrate as a function of incubation time.

Our experiments show no dependence on the presence of a protein trap, as often used in standard helicase assays.[17,28] Indeed we performed assays adding 1 μM excess ssDNA in the incubation solution to prevent initiation of DNA unwinding by any free enzyme or rebinding of any enzyme that dissociates during the course of unwinding, and the experiments with and without the trap give similar results within standard deviation.

dx.doi.org/10.1021/jz3018682 | J. Phys. Chem. Lett. 2012, 3, 3532−3537

(See Figure S4 of the SI.) This indicates that we work under single turnover conditions; therefore, we can apply the same approach used in single turnover DNA unwinding experiments[28] to fit the estimated fraction of unwound substrate as a function of the incubation time. The approach used by Lucius et al. takes into account two distinct processes occurring during the assay: the unwinding process, characterized by a rate constant k_U, and the formation of nonproductive bound DNA-enzyme complexes, characterized by a rate constant k_{NP}. Within this approximation it is possible to derive an analytic expression describing the time-dependent formation of the fraction of unwound ssDNA molecules. The analytic expression can be resumed into eq 1 and used for fitting the experimental data to extract the unwinding rate for the bacterial and the human helicase.

In eq 1, k_U and k_{NP} represent the unwinding rate and the nonproductive binding rate, respectively. x is the fraction of productively bound complexes, n is the number of steps required to unwind fully the L bp of the duplex (the step size m will be L/n) and $\Gamma(n, z)$ and $\Gamma(n)$ are the incomplete gamma function of n and z and the gamma function of n, respectively. (See ref 28 for more details.)

$$f_{ss}(t) = \left[\left(1 - \frac{\Gamma(n, (k_U)t)}{\Gamma(n)}\right) - e^{-k_{NP}t}(1 - x)\left(\frac{k_U}{k_U - k_{NP}}\right)^n\left(1 - \frac{\Gamma(n, (k_U - k_{NP})t)}{\Gamma(n)}\right)\right] \quad (1)$$

The kinetic parameters obtained by the fitting are reported in Table 1. We fixed value m = 4 bp step^{-1} for both RecQ and RECQ1, as reported in refs 17 and 28 for RecQ and RecBCD, assuming, therefore, the same step size for the two helicases (no appreciable changes in the final unwinding rate have been derived, assuming a different step size from 1 to 4 bp step^{-1}). With this assumption, we derive a final unwinding rate of the bacterial RecQ of 0.24 ± 0.04 bp s−1 in our assay (0.036 ± 0.008 bp s−1 for RECQ1).

Table 1. Kinetic Parameters Determined by Fitting the Experimental Data in Figure 4 with Equation 1[a]

	k_U (1/s)	k_{NP} (1/s)	m (bp/step)	x
RecQ	$6 \pm 1 \times 10^{-2}$	$10 \pm 5 \times 10^{-4}$	4	1.0 ± 0.1
RECQ1	$9 \pm 2 \times 10^{-3}$	$3 \pm 2 \times 10^{-4}$	4	0.5 ± 0.2

[a]Fitting values are reported with their standard deviation error. Chi-square analysis gives a confidence level of 80% (70%) for RecQ (RECQ1).

Whereas for human RECQ1 the unwinding rate information is quite poor,[14,16] for bacterial RecQ, we can compare our measurements with the apparent unwinding rate obtained by fluorescence cross-correlation spectroscopy (FCCS) and other fluorescence-based assays by which values ranging between 0.7 and 3 bp s^{-1} have been measured.[21−23] Other experiments (FRET/stopped flow[17] and optical tweezers[24]) report values of unwinding rate around 80 bp s^{-1}, but the discrepancy can be reasonably explained by considering that our technique, as for FCCS assays, is poorly sensitive to early catalytic events because what we monitor are the fully unwound substrates. In addition, spontaneous rehybridization events can slow down the unwinding process, contributing to the reduced unwinding rate.[21,25]

Finally, whereas all reported assays are working in solution, in our study the DNA substrate is tethered at one end within a densely packed surface structure: steric hindrance effects could therefore reduce the enzyme diffusion and processivity. The inhibition of the diffusion mechanism of enzymes inside highly packed nanografted DNA monolayers was demonstrated in our group for restriction enzymes, as reported in refs 29 and 30 In the case of helicases, however, we did not evidence any appreciable change on the action of the enzyme as a function of DNA surface density. (See Figure S5 in the SI.) This is rationalized by considering that in the case of restriction enzymes the consensus sequence was in the middle of the DNA chain, whereas for the helicase assays the DNA substrate exposes at the top a 12 bp fork to which the enzyme binds. Once it is bound, the enzyme acts as a molecular motor fueled by ATP hydrolysis that translocates along the DNA chain and unwinds, more or less at the same time.[24] Hence, this "fueled" translocation through the DNA sequence can help the enzyme to overcome the hindrance effect of the densely packed chains, regardless of the density of the brush itself.

Despite the discrepancy found on the absolute value of RecQ unwinding rate, the data collected in our comparative assay evidence a ∼10 time slower unwinding activity of the human RecQ1 with respect to its bacterial homologue RecQ. This result is in agreement with the findings in ref 16, where a higher RECQ1 concentration was found to be needed to unwind the same fraction of substrate unwound by the RecQ.

It is also interesting to note that the fraction of productively bound complexes in the human RECQ1 case is ∼50% lower than that for *E. coli* RecQ (see Table 1, column 5) consistently with the dimeric activity of RECQ1 and with a reduced binding affinity of the human RECQ1 due to the absence of the C-terminal part.[2,26] Moreover, if the RECQ1 acts as a dimer, its effective diameter will be almost twice the one of bacterial RecQ, and this can result in a less efficient slippage inside the DNA brush. However, the different kinetic behavior can be also ascribed to the fact that helicases may process much faster in vivo than in vitro due to the presence of accessory proteins, which aids in the destabilization of the fork junction (e.g., human replication protein A (hRPA)[14]), and this is of increasing importance as the complexity of the cell increases.

In summary, we have reported here a novel method for the study of enzymatic unwinding reactions onto confined DNA nanografted monolayers. The effective unwinding of DNA by means of *E. coli* RecQ and human RECQ1 helicase has been measured highlighting their different processivity behavior. The lower processivity of human RECQ1 can be attributed to a reduced binding affinity, to its dimeric activity, or to the intrinsic properties of our in vitro assay. It is clear that additional factors might be needed to stimulate RECQ1 activity in vivo. Our platform highlighted the possibility of studying biochemical reactions on surfaces, and it is open to multi-substrate reactions. Engineering and immobilizing different sequences on the substrate can help the specificity study in a comparative assay. Further studies including changes in sequence length, ATP concentration, and hRPA concentration are needed to allow a more precise estimation of the kinetic parameters and a better understanding of the unzipping mechanism.

dx.doi.org/10.1021/jz3018682 | J. Phys. Chem. Lett. 2012, 3, 3532−3537

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods. Effect of the DNA patch's size, enzyme preincubation, presence of a DNA trap, and dsDNA density on the helicase assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Dr. Bojana Lucic (ICGEB, Trieste, Italy) for providing the proteins, to Dr. Denis Scaini (University of Trieste, Trieste, Italy) for useful discussion, and to Dr. Matteo Castronovo (CRO, Aviano, Italy), Dr. Mauro Melli (Lawrence Berkeley National Laboratory, Berkeley, USA), Dr. Alessandro Bosco, and Dr. B. Sanavio (Sincrotrone Trieste S.C.p.A., Trieste, Italy) for critical manuscript discussion and revision.

This work was supported by FIRB 2011 grant "Nanotechnological approaches toward tumor theragnostic" (P.P. and L.C.), ERC grant "Monalisa Quidproquo" to G.S., and a grant from the Associazione Italiana per la Ricerca sul Cancro (AIRC) to A.V. (AIRC5088).

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