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Technical Note

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Hsp70 Trap Assay for Detection of Misfolded Subproteome Related to Myelodysplastic Syndromes

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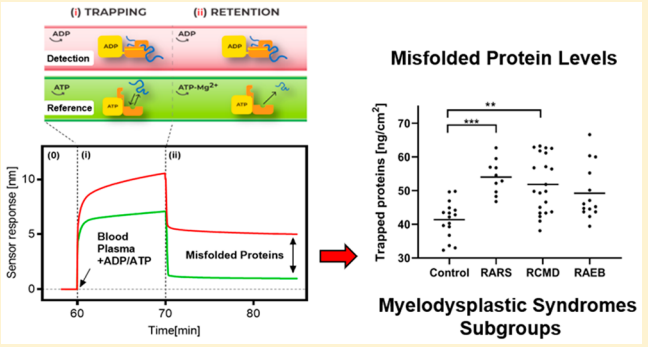
Supporting Information

ABSTRACT: The onset and progression of numerous serious diseases (e.g., various types of malignancies, neurodegenerative diseases, and cardiac diseases) are, on a molecular level, associated with protein modifications and misfolding. Current methods for the detection of misfolded proteins are not able to detect the whole misfolded subproteome and, moreover, are rather laborious and time consuming. Herein, we report on a novel simple method for the detection of misfolded proteins employing a surface plasmon resonance (SPR) biosensor and heat shock protein 70 (Hsp70) that recognizes and traps misfolded proteins in a nucleotide-dependent manner. We use this method for the detection of misfolded proteins in blood plasma of patients with various subtypes of myelodysplastic syndromes (MDS) and healthy donors. Our results reveal significantly elevated levels of misfolded proteins in the two stages of MDS that are most affected by oxidative stress: low-risk (RARS) and intermediate-risk (RCMD) patients. This approach can be extended to a variety of diseases and provides unique insights into the thus far unexplored area of blood proteome.



Myelodysplastic syndromes (MDS) are a diverse group of malignant stem-cell diseases that are usually diagnosed in elderly patients.[1] MDS are characterized by mitochondrial dysfunction generating an excess of reactive oxygen species (ROS) that play an important role in the onset of MDS and their progression to acute myeloid leukemia (AML).[2] ROS cause oxidative post-translation modifications (PTMs) of proteins. Progression in PTMs alters protein structure and functions.[3] In addition, the ineffective DNA repair increases levels of mutated oncoproteins with modified binding characteristics.[4,5] Therefore, during MDS, levels of misfolded proteins increase both within and outside a cell.[6] Loss of native structure (misfolding) results in exposure of otherwise inaccessible hydrophobic peptide segments. These segments serve as bait for the multifunctional Hsp70 protein family that bind misfolded proteins, their intermediates, and aggregated proteins and facilitate their refolding. In this work, we refer to this group of proteins as misfolded proteins. As most of the proteins contain such segments, Hsp70 family members can bind to the majority of misfolded proteins in the proteome.[7]

All currently available methods for the detection of misfolded proteins are laborious and time consuming and include complex multiple-step procedures that may lead to a loss of proteins and degradation of the sensitivity of the detection. In these methods, the misfolded proteins in complex with endogenous Hsp70 are first isolated and then released from Hsp70 for subsequent identification. The most frequently used approach for the isolation of Hsp70−misfolded protein complexes is ADP-affinity chromatography; however, native proteins that bind to ADP and are subsequently released for protein identification may be falsely regarded as misfolded proteins.[8] Specificity of the isolation method may be improved by using immuno-purification and chemical probe-based approaches.[9,10] In addition, the levels of endogenous Hsp70 in clinically relevant samples (e.g., cell lysate or blood plasma) are much lower than the levels of misfolded proteins, and therefore, the above-mentioned methods detect only a part of the misfolded subproteome.

In this work, we report on a novel method for the detection of misfolded proteins that combines, for the first time, a surface plasmon resonance (SPR) biosensor and Hsp70 as a recognition element. We use this method for the detection of misfolded proteins in the blood plasma of patients with myelodysplastic syndromes (MDS) and demonstrate a

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correlation between the levels of misfolded proteins and levels of oxidative stress that are different for different stages of MDS.

MATERIAL AND METHODS

SPR Biosensor. A six-channel SPR sensor platform based on the wavelength spectroscopy of surface plasmons combined with dispersionless microfluidics was used.[11] The sensor response is expressed as a shift in the wavelength for which the resonance occurs and is directly proportional to the mass of biomolecules attached to the surface of the sensor. A shift of 1 nm in the SPR wavelength represents a change in the protein surface coverage of 17 ng/cm^2.[12] Immobilization of cytosolic Hsp70 (HspA1A; Sigma-Aldrich) on the sensor surface was performed by its covalent attachment to a ω-carboxyalkylthiolate self-assembled monolayer (SAM; for details, see Supporting Information). The details of the preparation of the SAMs as well as protein immobilization are described in Pimková et al.[13] In order to generate a sensor chip with reproducible sensing characteristics, the same amount of Hsp70 (153 ng/cm^2, corresponding to an SPR sensor response of 9 nm) was immobilized on the sensor surface prior to each experiment. The attachment of the Hsp70 (36 μM) was performed at a temperature of 25 °C under a flow rate of 15 μL/min; all other steps were performed at 20 μL/min.

Concept of the Hsp70 Trap Assay. The proposed assay takes advantage of Hsp70's ability to trap a misfolded protein in the presence of ADP and to release the trapped protein when ADP is replaced by ATP.[7] The assay consists of two steps: (i) Trapping: Misfolded proteins present in diluted blood plasma are trapped by ADP-mediated closing of immobilized Hsp70. (ii) Retention: Buffer with ADP is introduced to maintain Hsp70 in a closed state to hold the trapped misfolded proteins. The amount of trapped proteins is monitored in real time using the SPR biosensor (Figure 1). In order to provide a more robust detection of misfolded proteins, a sensor channel with ATP (which prevents trapping of misfolded proteins) was used as a reference. The amount of trapped misfolded proteins was determined as the difference between the sensor response in the detection and reference channels 15 min after the initiation of the retention step. The amount of released misfolded proteins was determined as the difference between the sensor response in a detection channel before and 10 min after the injection of the elution buffer.

Optimization of the Hsp70 Trap Assay. Given the complexity of preparation and work with isolated misfolded proteins, we performed the optimization of the assay directly in blood plasma.[14] The Hsp70 assay was optimized with respect to the presence of ATP and ADP nucleotides and K^+ and Mg^{2+} cations in respective steps of the assay to maximize the amount of trapped and released misfolded proteins, respectively. In particular, ADP monopotassium salt dihydrate and ATP magnesium salt (Sigma-Aldrich) nucleotides were used in both the trapping and retention steps of the assay. Three concentrations of nucleotides were tested: 1.5, 2.5, and 3.5 mM. The effect of ATP preloading (2.5 mM concentration) for 10 min in a PBS running buffer (1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, and 139.7 mM NaCl, pH 7.4)) prior to the trapping step was also investigated. In order to evaluate the effect of the K^+ cations, we used a PBK running buffer (1.4 mM KH_2PO_4, 8 mM K_2HPO_4, and 139.7 mM KCl, pH 7.4). In order to evaluate the effect of the Mg^{2+} cations on the performance of the assay, a PBK buffer with added Mg^{2+} (Mg^{2+} concentration: 2.5 mM) was used. The correct function of the reference channel (Hsp70 remaining open and not able to trap misfolded proteins) was confirmed by using ATPγS (Sigma-Aldrich), a nonhydrolyzable analogue of ATP.

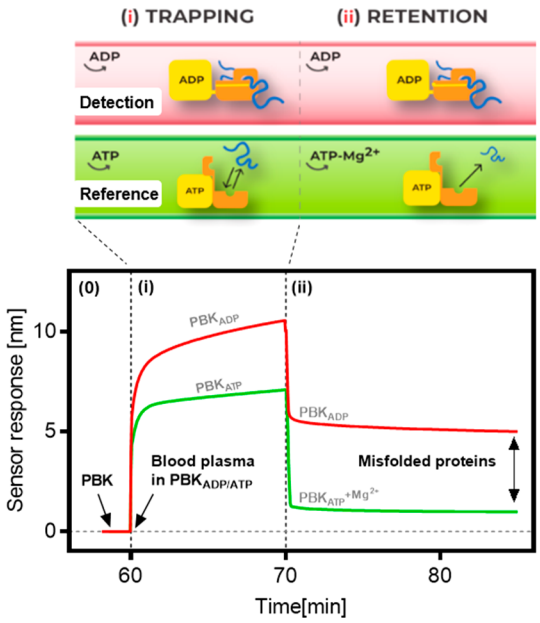

Figure 1. Concept of the SPR-based Hsp70 trap assay. The lower part of the figure shows a typical sensorgram corresponding to the detection (red line) and reference (green line) channels. The upper part depicts the conformation of Hsp70 during two stages of the assay. In the detection channel, the misfolded proteins are trapped and retained by ADP-mediated closing of Hsp70, while in the reference channel no binding of misfolded proteins to Hsp70 takes place.

Plasma Samples. Plasma samples were collected from 45 patients with MDS and 16 age-matched healthy controls. All patients were reclassified according to the WHO classification criteria. The studied group was composed of 10 patients with refractory anemia with ringed sideroblasts (RARS), 21 with refractory cytopenia with multilineage dysplasia (RCMD), and 14 with refractory anemia with excess of blasts subtype 2 (RAEB-2). Pooled plasma (mixed gender) was used for assay optimization. In all SPR experiments reported herein, blood plasma diluted to 10% in a running buffer (PBS or PBK) was used. For the patients' characteristics and details of plasma collection procedure, see the Supporting Information.

Statistical Analysis. Statistical tests were used to examine the differences in data across all groups (MDS subgroups, controls). Kruskal–Wallis and post hoc analysis (a Tukey and Kramer test) were performed using R-software. All the tests for statistical significance were standardized at an alpha level of $P < 0.05$.

Mass Spectrometry Analysis. The proteins trapped by Hsp70 were released from the SPR chip by an elution buffer and digested with trypsin. The composition of the elution buffer was optimized to maximize the efficiency of the elution. The tryptic digest was injected into a Nano-LC system equipped with a C18 column and electrosprayed into a TripleTOF 6600 mass spectrometer (MS). The MS analysis was carried out in a data-dependent mode, and the resulting files were searched using Protein Pilot 5.0. The list of identified proteins was classified in terms of the Gene Ontology (GO) biological functions. For detailed information on LC-MS/MS, see the Supporting Information.

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RESULTS AND DISCUSSION

Influence of Nucleotides on Trapping and Retention of Misfolded Proteins. For the induction of an allosteric change of Hsp70 conformation to trap and retain the Hsp70 misfolded proteins, both ADP and ATP nucleotides were applied in the trapping step (nucleotides added to blood plasma) and in the retention step (nucleotides added to buffer). For brevity, we used a special notation system for the experimental conditions: $A_{(0)}$-$B_{(i)}$-$C_{(ii)}$, where A, B, and C denote the composition of the buffer (presence of nucleotides), and subscripts 0, i, and ii denote different steps of the assay (prior to the assay, the trapping step, and the retention step, respectively). The binding of misfolded proteins under different conditions can be seen in Figure 2A. A substantial

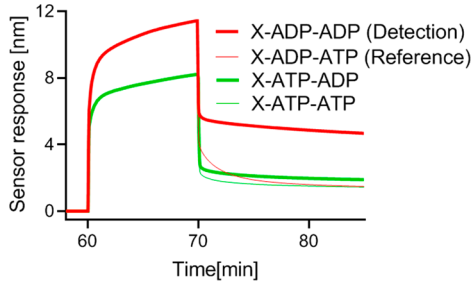

Figure 2. Typical SPR sensorgram corresponding to the trapping and retention steps with various added nucleotides.

binding of the misfolded proteins was observed when ADP was added to plasma, whereas a lower binding was observed when ATP was added to plasma, confirming that ATP keeps Hsp70 in an open state. Therefore, the presence of ADP in the retention step is necessary for the retention of Hsp70 complexes created in ADP-enriched plasma. In addition, Figure 2A reveals that the presence of ATP in the trapping and retention steps ($X_{(0)}$-$ATP_{(i)}$-$ATP_{(ii)}$) or only in the retention step ($X_{(0)}$-$ADP_{(i)}$-$ATP_{(ii)}$) has a rather similar effect on the release of the Hsp70-associated misfolded proteins. Therefore, in further experiments, we used the channel with $X_{(0)}$-$ADP_{(i)}$-$ADP_{(ii)}$ as a detection channel and the channel with $X_{(0)}$-$ATP_{(i)}$-$ATP_{(ii)}$ as a reference channel. To confirm the correct function of the reference channel, we used ATPγS (a nonhydrolyzable analogue of ATP) instead of ATP. The SPR sensor responses for the reference channel using ATP and ATPγS were found to differ only by about 5%.

The amounts of trapped misfolded proteins (expressed in ng/cm^2) obtained for different added nucleotides are shown in Figure 3A. As follows from Figure 3A, efficient trapping and retention of Hsp70 misfolded proteins was achieved in a channel with $X_{(0)}$-$ADP_{(i)}$-$ADP_{(ii)}$ with a high reproducibility (>95%; four chips). The amount of trapped proteins that remained in the complex with Hsp70 in an $X_{(0)}$-$ADP_{(i)}$-$ADP_{(ii)}$ channel at the end of the retention step was 53 ng/cm^2 ,and the corresponding SPR response was 2 times higher than that in an $X_{(0)}$-$ADP_{(i)}$-$ATP_{(ii)}$ channel. The amount of trapped proteins increased with an increase in the concentration of nucleotides (data not shown). However, the increasing concentration of nucleotides also increased the nonspecific interaction of plasmatic proteins in the reference channel. Therefore, a concentration of nucleotides of 2.5 mM was used in further experiments. We observed that ATP preloading ($ATP_{(0)}$-$ADP_{(i)}$-ADP/$ATP_{(ii)}$) had only a minor effect on the

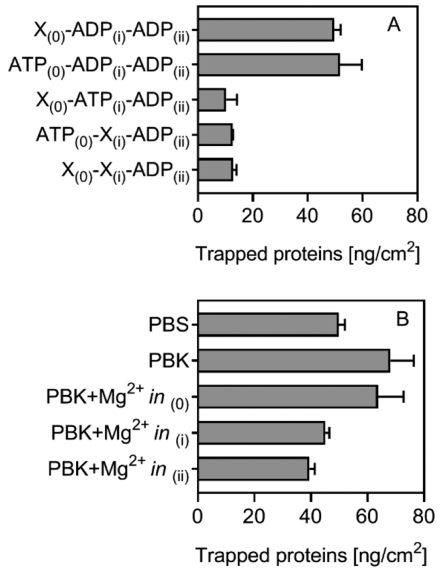

Figure 3. (A) Amount of Hsp70 misfolded proteins retained upon the addition of different nucleotides. Each column represents the amount of trapped proteins after the retention step (average of four channels from four chips). (B) Influence of K^+ and Mg^{2+} cations on Hsp70 trapping and retention of misfolded proteins (average of five chips).

amount of trapped proteins and resulted in a rather low measurement reproducibility (85%; Figure 3A). This may be caused by a slow intrinsic nucleotide dissociation rate, in which ATP hampers the binding of ADP and the entrapment of misfolded proteins.

Influence of Cations on Trapping and Retention of Misfolded Proteins. The assay was performed in two different phosphate buffers that contained sodium salts (PBS) and potassium salts (PBK). The levels of misfolded proteins on an SPR chip were 53 and 70 ng/cm^2 for PBS and PBK buffers, respectively (Figure 3B), indicating that the PBK buffer enables more efficient trapping of misfolded proteins by Hsp70. As follows from Figure 3B, the presence of Mg^{2+} in the PBK buffer decreases the amount of trapped proteins in all the steps of the assay. In order to facilitate dissociation of misfolded proteins in the retention step in the reference channel, we added Mg^{2+} (concentration: 2.5 mM) in the retention buffer used in the reference channel.

Characterization of Trapped Proteins from Pooled Plasma by MS. To demonstrate that the trapped Hsp70 misfolded proteins can be subsequently identified by mass spectroscopy (MS) and to determine the nature and origin of the trapped proteins, we optimized the elution process and characterized the proteins released from the detection channel by LC-MS/MS. In order to enable efficient elution of the trapped Hsp70 misfolded proteins, we tested five buffers with different ATP and Mg^{2+} ratios in the detection channel after the retention step. Using the initial and final baseline, the amount of misfolded proteins released from Hsp70 during a 10 min period was determined. The efficiency of the elution (EE) was calculated as a ratio of the amount of released and trapped proteins (Table S2, Supporting Information). The EE of the elution buffer containing ATP (2.5 mM) and Mg^{2+} (2.5 mM) was 83%. A more effective elution (92%) was achieved by using an elution buffer containing ATP (2.5 mM) and Mg^{2+} (3.75 mM). In the reference channel treated by this elution buffer, we observed only a minor elution effect (8%; 1.36 ±

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0.56 ng/cm^2; nine chips). The trapped proteins from the pooled plasma, eluted from the detection channel, were analyzed by LC-MS/MS. A total of 54 proteins with varying sizes from 9 to 158 kDa were identified (Table S3, Supporting Information; we omitted high-abundant proteins such as albumin, immunoglobulins, and the proteins that were shown to bind nonspecifically to SAMs in our previous study[15]). The identified proteins included 44 proteins secreted to plasma, seven proteins from extracellular space, one cytoplasmic protein, and two membrane proteins. According to the GO database, these proteins are mostly involved in protein folding, inflammatory response, signal transduction, cell adhesion, and blood coagulation (Table S3, Supporting Information).

Screening Plasma of MDS Patients for Hsp70 Misfolded Proteins. We further used the Hsp70 trap assay to determine the levels of misfolded proteins from the plasma of 16 healthy controls and 49 MDS patients divided into three subgroups according to the WHO classification (10 RARS patients, 21 RCMD patients, 14 RAEB-2 patients). The subgroups were compared with each other and with the group of healthy controls. Significant differences in the amount of misfolded proteins (Kruskal–Wallis, P = 0.0002) among the analyzed sample groups were observed (Figure 4). Using

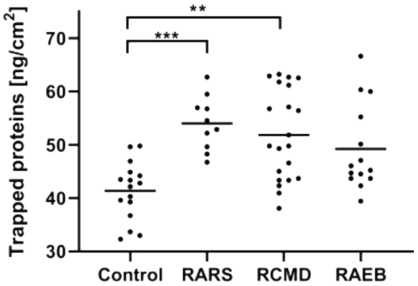

Figure 4. Amount of misfolded proteins (ng/cm^2) for plasma samples of MDS subgroups and healthy controls.

simultaneous tests for general linear hypotheses (a Tukey and Kramer test), we found that sensor responses were significantly higher for the RARS and RCMD group when compared to the healthy donors (***P = 0.0005 and **P = 0.0015).

Our data are in agreement with previous studies reporting different levels of oxidative stress in different MDS subgroups.[2,16] High oxidative stress together with mitochondrial dysfunction play a crucial role in the pathogenesis of RARS and RCMD subgroups. In contrast, the cells in the advanced stage of MDS (RAEB) exhibit low oxidative stress, probably due to the ROS defense mechanisms inherent to cancer cells. It should be noted that the trend in the levels of misfolded proteins agreed well with the trend observed in the previous study in which the levels of carbonylated proteins were measured by means of spectrophotometry after derivatization.[6]

The presented approach detects misfolded proteins that are trapped by Hsp70 (HspA1A). A deeper insight into the misfolded subproteome of blood plasma could be obtained by employing additional subcellular members of the Hsp70 family.

CONCLUSIONS

We developed a novel method for the detection of misfolded proteins based on SPR biosensor technology and the intracellular Hsp70 protein that recognizes and traps misfolded proteins in a nucleotide-dependent manner. We demonstrate that the assay enables the detection of misfolded proteins in blood plasma of MDS patients and show that levels of misfolded proteins correlate with the levels of oxidative stress that differ for different stages of MDS. We also demonstrate that the misfolded proteins can be released from the SPR chip for subsequent identification via MS analysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b04175.

Instrumental condition of MS analysis, result of protein characterization, and a table with MDS patients' characteristics (PDF)

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Author Contributions

O.P. with support from L.C. and M.B. designed and executed the experiments. O.P. analyzed the assay data. J.S. performed statistical analysis of the data. J.C. characterized the used patients' samples. J.H. and J.E.D. contributed to the interpretation of the results and were responsible for the project. All the authors discussed the results and contributed to the final manuscript.

Notes

The authors declare no competing financial interest.

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