FUNCTIONAL PROTEOMIC SIGNATURES OF THE UBIQUITIN / PROTEASOME PATHWAY

Prospecting for new drug targets and diagnostic biomarkers in cancer

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REPORT SUMMARY

This report presents preliminary findings on the application of ProFACT's surface library – SeraFILE™, to the functional proteomic characterization of an important cellular pathway, the Ubiquitin/ Proteasome pathway (UPP). The results show:

- Markedly contrasted signatures of matched cancer to normal adjacent tissue,
- Differential pools of Proteasome regions and regulatory factors,
- Altered catalytic rates, both higher and lower, on several different surfaces,
- Evidence of conformational isoforms resistant to small molecule inhibitors,
- Evidence of soluble regulatory factors that potentially could be isolated.

As the feasibility of sufficiently compartmentalizing complex protein mixtures has been established, future objectives shall focus on exploiting the exciting finding that SeraFILE™ also induces altered catalytic activities.

Defining the full complement of protein expression, function, and interaction is recognized as the field of proteomics. As abnormal cells and especially cancer produce a unique set of proteins, these differences can be identified and applied advantageously towards new diagnostics and therapies. However, the proteomic state of the art discovery methods – largely driven by Mass Spec, have focused on identifying early disease-state biomarkers, and have had limited utility in the acceleration of drug development.

Drug candidate selection requires a mechanistic understanding of the protein target of action, information not readily obtainable from peptide constituent annotation. Ideally, proteomic profiles (or signatures) can facilitate biomarker discovery and directly couple to screening methods in drug development. For this, functional proteomic signatures – the subject of this report, are required.

ProFACT's SeraFILE™ surface library affords high-resolution partitioning of complex protein extracts into discrete fractions that can be characterized further. The benefit of applying SeraFILE™ is that complex protein specimens can be examined simultaneously to generate approximately 80 sub-proteomes; the resulting protein profiles offering a much more comprehensive signature of disease-specific differences.

The ubiquitin/proteasome pathway (UPP) is a highly conserved proteolytic system that degrades damaged proteins, critical cell cycle regulators and signal transduction molecules. Transitions in growth, differentiation and cell fate are dependent on this pathway, which also underlies its significance in aberrant cell growth, and its importance for drug discovery. The activity of the UPP is regulated during the cell-cycle, stress response and apoptosis. Increased UPP activity has been described in several cancers, and a systemic inhibitor is clinically in use (Velcade®) for the treatment of multiple myeloma.

In Dr. Madura's laboratory at UMDNJ, methods were developed for isolating proteins that are conjugated to ubiquitin (UBA). Likewise, methods for purifying catalytically active proteasomes (UbL), which is the protease that degrades ubiquitinated proteins, were also developed. Because proteasomes are compositionally diverse, proteomic characterizations may have a bearing on the regulation of important cellular proteins, and ultimately in the identification of drug targets.

In addition to generating descriptive functional signatures, proteasome catalytic rates are altered upon exposure to the SeraFILE™ surface library. This discovery reveals the presence of alternate proteasome sub-states, which could be the result of dissociation of positive and/or negative regulating factors from the proteasome. Potentially, these functional isoforms may have value as pharmacological targets.

The resulting SeraFILE™ derived sub-proteomes can be resolved further on UbL and UBA affinity-type matrices, to more specifically interrogate UPP function. The synergy offered by these new surface-binding technologies provides powerful new techniques for examining the expression and functional activity of critical cellular proteins.

Because quantification of activity rather than protein presence is the most informative aspect of UPP in cancer, novel functional biomarkers of diagnostic value may be identified, and more importantly, new pharmaceutically relevant targets for cancer drug development are foreseen.

SIGNIFICANCE

Most key regulatory proteins are degraded by the ubiquitin/proteasome pathway (UPP) [1]. Significantly, the activity of this pathway is increased in disease [1, 2]. In Dr. Madura's laboratory, highly efficient and rapid methods for purifying ubiquitinated proteins, and catalytically active proteasomes that degrade ubiquitinated proteins were developed. Consequently, the tools jointly developed now permit the characterization of the substrates of the proteasome, and regulators of the proteasome. No other methodology can capture such a significant number of regulatory proteins that function in diverse cellular roles including transcription, cell-cycle control, tumor suppression, stress-response, DNA repair, and signal transduction.

Prior studies showed a striking difference in the activity and expression profile of the UPP proteolytic system in breast cancer (using UbL and UBA matrices) [1, 2]. We describe here that a novel surface-library called SeraFILE™, developed by ProFACT Proteomics, resolved proteasomes into distinct functional fractions, and could also alter proteasome kinetics. The combination of these technologies offers a formidable new approach for altering the catalytic function of an **information-rich** set of proteins, and provides a gateway to drug discovery.

The ability to both identify key factors, and alter their protein function, is especially innovative, as it provides a seamless path for developing activators and inhibitors of critical biochemical functions in disease.

The Proteomics - Drug Development Gap

Systems biology attempts to reveal disease relationships by uncovering differences in the *identity*, *function* and *quantity* of proteins [3]. No single technology adequately characterizes all three of these aspects, and precedence given to *identity* has been based on available methods, most notably 2D analysis [4] and mass spectrometry [5, 6]. With identification technology well advanced, the need remains for simple and rapid methods to uncover differences in protein function and quantification.

Because of improvements made in pre-fractionation, separations, and sensitivity of mass spectrometry, proteomic investigations today are largely focused on detecting low abundance protein(s) unique to a clinically defined disease state [7, 8]. For this, mass spectrometry drives the discovery engine for early disease-state biomarkers [9, 10].

However, biomarkers discovered in this manner will not likely accelerate drug development or improve therapeutic intervention beyond early diagnosis. For instance, the identification of single proteins as putative biomarkers [11, 12], has generally been unproductive. This is because drug development requires a mechanistic understanding of the protein target of action. However, such information cannot be interpreted solely from mass spectrometry data, where peptides are the prospective markers and functional annotation remains elusive. This gap can be closed by generating proteomic approaches that not only facilitate biomarker discovery, but also provide a link to **functional** screening methods [13].

Therapeutic strategies frequently rely on altering protein catalysis [14]. It is well established that variations in rate mechanisms can give rise to qualitative differences in biological outcomes. The measurement and regulation of enzymes have become key elements in clinical diagnosis and therapeutics [15]. Nevertheless, functional annotation remains challenging as proteins do not have rigid molecular structures. Rather, alternative conformations are in constant transition – among themselves and through interactions with other cellular constituents, thereby imparting functional heterogeneity. As a result, enzymes exist in a continuum of different sub-states, ranging from low activity (attenuated) to high activity (excited) states [16-20]. The prospect of defining motion and plasticity of active sites would have implications on the design of enzyme targeted drugs [21].

Enzyme reporter-type assays, while useful, infer only the weighted average of all of the functionally heterogeneous sub-states. For instance, measurements derived from substrate catalysis reflect a collective averaged representation of conformational and regulatory states. Thus, **Functional Proteomic** endeavors could fulfill an urgent pharmacological need, **if** the regulatory and conformational variability of enzyme activity could be isolated and characterized. Therein lays the strength of SeraFILE™.

SeraFILE™. A new approach for drug discovery and molecular profiling.

The SeraFILE™ inventions (USPTO Application Numbers 60/403,747 & 11/561,251) encompass the surface characteristics and protocols suitable for differential proteomic subfractionation. The library consists of porous silica which is simultaneously passivated and functionalized for substitution; derivative immobilized moieties contain combinations of electrolytic, poly-electrolytic, aromatic, and aliphatic constituents.

The distinction of SeraFILE™, compared to conventional ion-exchange and affinity chromatography, is that the surfaces in combination with the protocols exploits a unique amalgamation of homogeneous and weak (or low) binding energy and are not subject to the predominant influence of the high abundance proteins. This enables selectivity to be modulated by the presentation and architecture of the charges present on the surface; t**he net result is a differential sub-proteome for each subfraction pool without the need for radical depletion steps.**

While HPLC has been reported as a suitable first dimension in lieu of IEF for hybrid LC-1DE profiling, it is limited to a small quantity of productive subfractions [22]. Furthermore, unlike serial HPLC, with SeraFILE™ the same elution conditions are mild and consistent for each surface in the library, facilitating a simple and direct handoff to structural and functional interrogation.

The application of the SeraFILE™ library combines elements of biochemical and functional analysis quantitatively providing:

- A modality that is open-ended and industrially productive,
- Reduced protein complexity with maintenance of native, functional conformations,
- New profiling techniques which generate signatures across a multiplicity of sub-proteomes and interrogations,
- A means to characterize enzyme regulation and related functional sub-states from disease,
- Discovery strategies that enrich catalytic activity and directly couple to drug development.

The discovery that SeraFILE™ offers both physical partitioning of complex protein mixtures, as well as activity-based discrimination of these constituents is the basis for a USPTO provisional patent filing. A key advantage of this approach is that it provides a powerful venue for enabling **activity-based** drug design that can be coupled directly to the process of identifying novel disease-specific targets.

As a platform technology, SeraFILE™ delivers:

i. Differential Sub-proteome Pools. The SeraFILE™ surface library generates approximately 80 sub-proteome pools.

ii. Disease Signatures. Sub-proteomes reveal both activity signatures, and abundance profiles for comparison. In our model case, measurement of proteasome function revealed markedly contrasting activity and abundance profiles in human breast cancer and normal tissues.

iii. Bioassay Profiles. Bioassay profiles (as opposed to protein abundance profiles) provide the basis for defining the functional and conformational diversity of enzymes. Herein we report proteasome activity upon surface treatment revealed up to 10-fold reduced activity, and 4-fold higher activity on specific matrices.

iv. Surface Induced Sub-states. The SeraFILE™ surface library imparts functional alterations (activation or inhibition) which can be measured in multiwell bioassays. These alterations reveal the existence of functional sub-states that can be purified, and would represent important pharmacological targets.

v. Pathway Characterization. Biological pathways can be characterized by generating sub-proteomes whose functional properties can be examined. We determined that the activity of the Ubiquitin/Proteasome Pathway is altered following application to different SeraFILE™ matrices. Consequently, the availability of functional data derived from SeraFILE™, opens novel paths toward drug development.

vi. Drug Assays Using Bioactive Pools

SeraFILE™ can alter well-defined biochemical activities. This key discovery offers a powerful link to therapeutic development, and constitutes a fundamental advance over conventional system biology approaches. We note that other approaches that seek to uncover patterns and changes in protein expression profile [23], are limited by the absence of functional information.

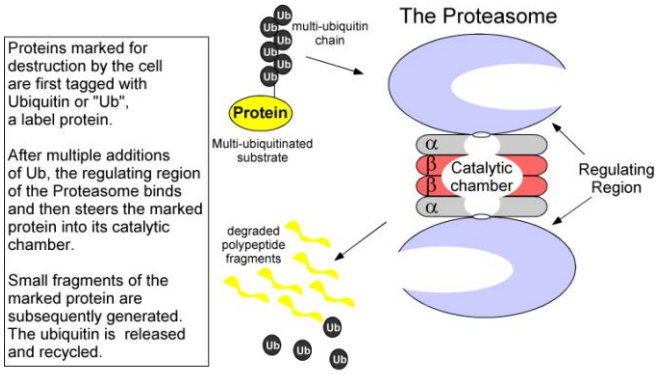
The Ubiquitin/Proteasome Pathway (UPP)

The UPP is among the most conserved mechanisms in eukaryotic evolution, and is required for conditional degradation of important cellular factors, and the elimination of damaged proteins [24]. In this pathway ubiquitin is attached to proteins [25, 26], and the proteasome is the protease that degrades proteins that are linked to ubiquitin [27]. The enzymology has been well-characterized, and is known that three key enzymes catalyze the covalent attachment of Ub to a substrate [26, 28].

Almost 800 ubiquitin-transfer (E2) and ubiquitin-ligating (E3) enzymes have been identified in human, and it is believed that the compositional diversity offered by forming distinct E2 + E3 assemblies facilitates highly specific targeting of a very large number of cellular proteins. However, the identification of each E2/E3 'combination' has been elusive. What is also unclear is how ubiquitinated substrates are delivered to the proteasome. However, there is compelling evidence that the proteasome itself interacts with regulatory factors that influence its ability to recognize and degrade ubiquitinated proteins.

One such factor is called Rad23 (an area of expertise in the Madura laboratory) that contains a motif (UbL) that binds proteasomes, and another domain (UBA) that binds ubiquitinated proteins. These features allow Rad23 to function as a shuttle-factor that delivers ubiquitinated substrates to the proteasome. The UbL and UBA domains function independently, and were developed into high-affinity reagents for purifying proteasomes and ubiquitinated substrates.

UbL: A highly efficient proteasome affinity reagent. UbL domains from Rad23 proteins bind the proteasome [29, 30]. The UbL domains in two human Rad23 proteins form differential binding to proteasomes [31]. UbL domains were expressed in *E. coli* as fusions to glutathione S-transferase (GST). GST-UbL efficiently purified catalytically active proteasomes from human tissue extracts. Furthermore, regulatory factors that formed sub-stoichiometric interactions with the proteasome were also detected [32-36].



Simplified representation of the Ubiquitin-Proteasome Pathway:
A cell's protein disposal and recycling system

Evidence that the proteasome is compositionally dynamic. Proteasomes purified by conventional chromatography contain ~ 35 subunits [37].

However, when proteasomes were affinity-purified with GST-UbL (or FLAG-Rad23) over a hundred proteins could be detected [10]. While many of the subunits represented proteasome subunits, mass spectrometry results showed that a significant number of co-purified proteins were proteasome-associated regulatory factors [30, 35, 38-40].

To directly test this hypothesis we purified proteasomes and confirmed its interaction with previously identified regulatory factors by immunoblotting. Although these accessory factors are not *bona fide* proteasome subunits, they are required for efficient protein degradation. Because the activity of the UPP is perturbed in most cancers, identifying compositional differences in proteasomes could provide an opportunity to identify disease-related alterations, and develop more specific proteasome inhibitors [3, 9, 10, 41, 42, 43].

To further test the idea of compositional variability, we examined proteasome composition in yeast strains in which DNA damage sensitive mutants were available. Mutations in Rad23 and Rad6 showed significant changes in the levels of specific proteins co-purified with proteasomes In other studies, a unique collection of associated proteins were co-purified with each UbL, suggesting an interaction with compositionally distinct proteasomes (Chen and Madura, Cancer Res. 2005). Thus, the data generated in Dr. Madura's laboratory supports the accepted view that the proteasome function is variable and is compositionally diverse.

RESULTS AND DISCUSSION

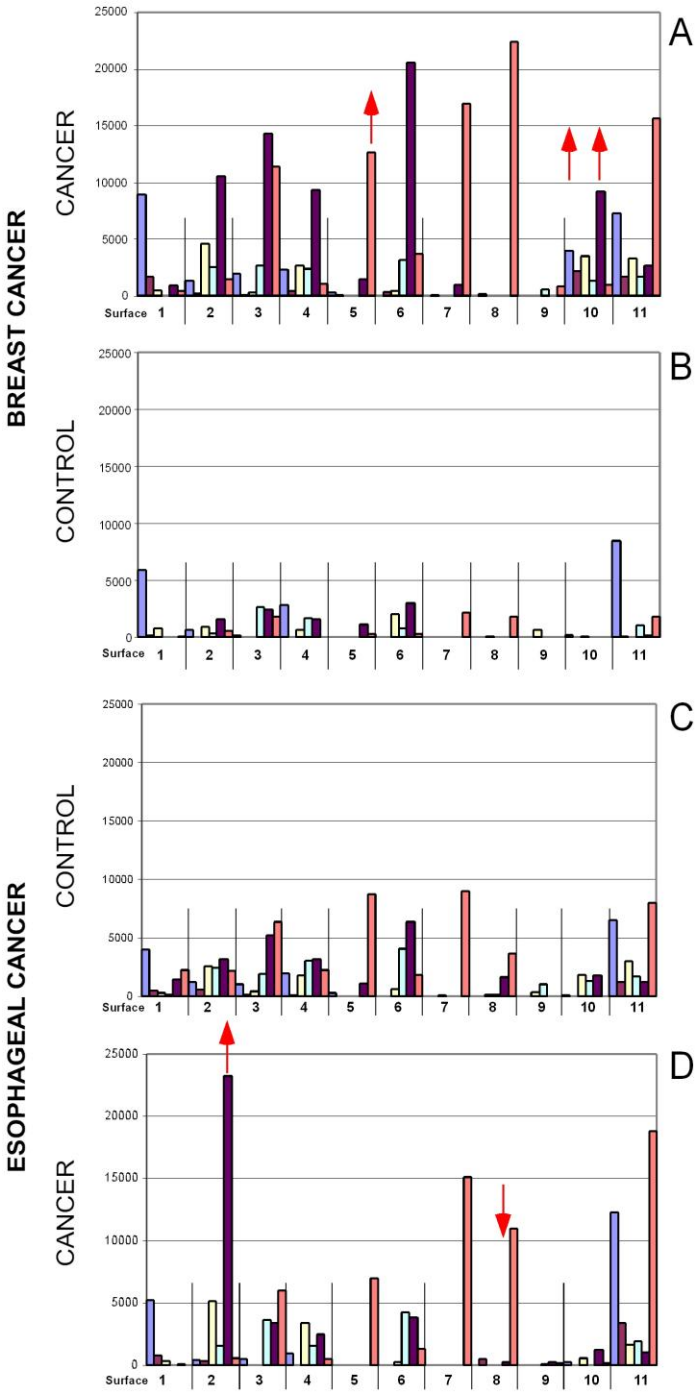
Proteome sub-fractionation on SeraFILE.

A surface-library comprising 11 different SeraFILE™ surface architectures was prepared (ProFACT Proteomics). The characteristics of these matrices were examined under different adsorption and elution conditions, using yeast proteins, human blood cell extracts, and cultured mammalian cell extracts. Concentration-dependent binding, binding capacity and stability of bound proteins was determined. The fraction of irreversibly bound proteins, activity of proteasomes, and the presence of proteasome-associated factors were determined.

To examine the ability of this library to fractionate a complex protein extract, we characterized proteins isolated from breast and esophageal cancers and patient-matched control tissues. Samples were normalized to total soluble protein concentration prior to surface application. For each of 11 SeraFILE™ matrices we examined flow-through (blue), four different wash/elution conditions (dark blue, yellow, light blue and black), and a final sample representing UPP activity that remained bound to the matrix (red). Control tissue extracts from breast (B) and esophageal patients (C) were examined, and a similar pattern (but not amplitude) of UPP activity was detected on the various SeraFILE™ matrices (B and C). Direct measurement in unfractionated protein extracts showed that proteasome activity was ~ 4-fold higher in esophageal tissue, compared to breast tissue. Consequently, higher levels were detected following fractionation of esophageal extracts on SeraFILE™.

UPP activity (chymotryptic) increased significantly in cancer samples in most sub-fractions, for both breast and esophageal cancer (A and D), consistent with our previous studies. Altered UPP activity in a few notable sub-pools where differences compared to control as well as between the two cancers is indicated with a red arrow. Other differences are apparent.

Approximately 5,000 arbitrary units of proteasome activity were present in unfractionated control extracts that was applied to each SeraFILE™ surface. Yet, analysis of the fractionated samples indicated much higher activity in several sub-fractions (for instance flow-through fractions from matrix 1 and 11; B). On other matrices activity was reproducibly reduced for both breast (matrix 10; B) and esophageal



cancers (matrix 9; D). Proteasome inhibition by the matrix was reversible, because high activity was recovered on matrix 10 in breast cancer extracts (A), and because of the non-overlapping effect seen with other cancer extracts.

In another example (data not shown) a second paired breast cancer/control reproduced these observations that overall UPP activity is increased in cancer and SeraFILE™ differentiation of proteasome activity is apparent.

Furthermore, surfaces 3, 6, and 11 in both instances promote an elevation in overall proteasome activity relative to the control.

Though preliminary, this data supports our view that these profiles may have diagnostic and/or therapeutic value in that there are some similarities as well as some differences; similarities observed over multiple samples may lead to drug targets, differences may be indicative of cellular origin – maybe distinguishing between lobular and ductal.

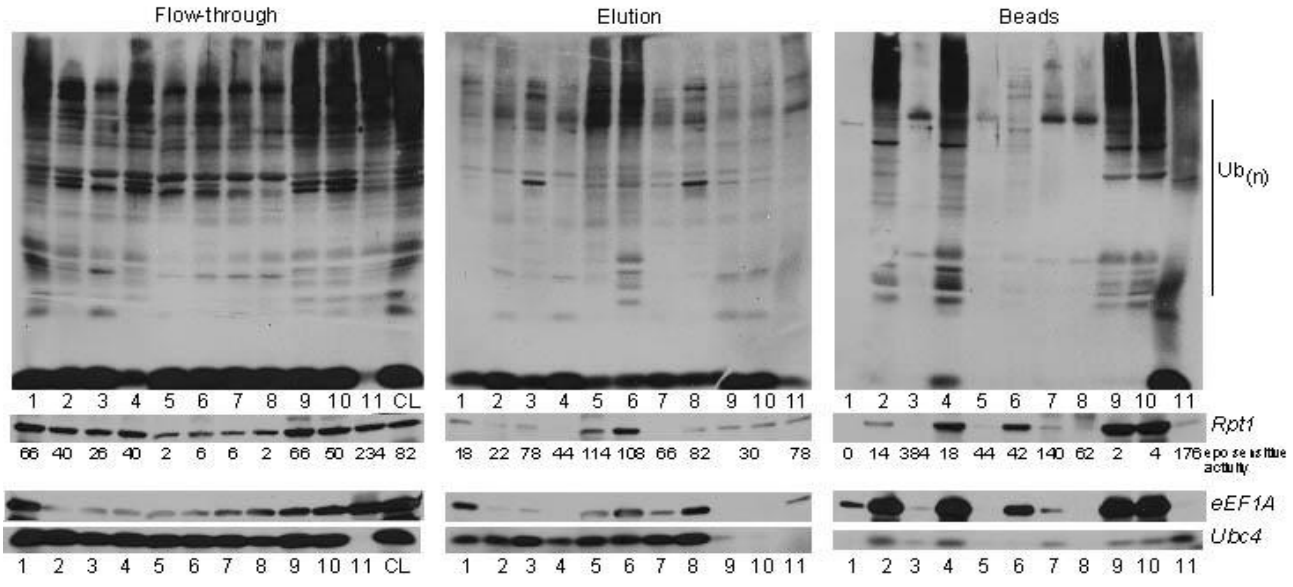
The figure below shows the characterization of yeast protein sub-pools generated from the SeraFILE™ surface-library. Notice that different amounts of high molecular weight ubiquitinated proteins were detected in the Flow-through fractions (left panel). No mono-ubiquitin (strong band at the bottom of the upper panel) was detected in the flow-through from matrix-11. The distribution of eEF1A was also highly variable. In the eluted fractions (middle panel) high level of multi-ubiquitinated proteins were released primarily from matrix 5 and 6.

In the bound fractions (right panel), significant levels of multi-Ub proteins remained on matrix 2, 4, 9, 10 and 11. Mono-Ub was also retained on matrix-11. In the lower panels, the elution and retention of Rtp1, eEF1A and Ubc4 is shown.

Proteasome activity was measured in all fractions and the values are displayed below the second (Rpt1) panel. These studies revealed unequal distribution of proteasome activity in the various fractions. The amount of proteasome activity in an equal amount of unfractionated extract (lane CL) was 82 arbitrary fluorescence units. We especially note the 19S regulatory region (inferred by presence of subunit Rtp1 and multi-Ub proteins) is surface bound on matrices 9 & 10, while the flow-through activity from 9 & 10 is about 80% of control. This is suggestive that the 20S catalytic region dissociates from 19S upon exposure to one or more SeraFILE™ matrices. If validated, new drug strategies that target assembly/stability may be forthcoming.

For the purposes of this investigation, the SeraFILE™ derived sub-proteomes are shown to be sufficiently differentiated as determined by two important performance metrics: proteolytic activity rates, and relative abundance of UPP components. These preliminary findings support our claim that this is a superior approach for cancer detection, assessment of prognosis, and identification of targets for drug development, as methods are well developed and all basic technologies are in place.

Figure Description: Yeast protein extracts were applied to 11 SeraFILE™ matrices and the interactions examined in a SDS-polyacrylamide gel. Only activity that is sensitive to epoxomycin, a specific proteasome inhibitor is shown. **Left**; Identical protein samples were applied and the unbound fraction is shown. CL in the last lane represents the input protein for each matrix. Four panels were probed with antibodies against ubiquitin, Rpt1 (19S proteasome subunit; Top), eEF1A (translation elongation factor that promotes co-translational degradation), and Ubc4 (a stress-responsive Ub-conjugating E2 enzyme; bottom). **Middle**; A single elution (elevated pH) step was used and the released proteins were examined. **Right**; proteins that remained bound to the matrices were released in boiling SDS and examined.



Strikingly, we detected higher, and lower, proteasome activities on many surfaces. For instance, the Flow-through from matrix 11 contained 3-fold higher activity than the input, while proteasomes bound to matrix 3 (right panel) showed almost 5-fold higher activity than the input. These findings have been extensively replicated in yeast extracts, human blood and primary tissues. Furthermore, a similar activation of bacterial alkaline phosphates on SeraFILE™ surfaces (in total extract) was reproducibly established [manuscript in preparation].

Such observations suggest a powerful link to drug development, constituting a fundamental advance over conventional system biology approaches; the process of discovery is directly coupled to screening for function. In one example from yeast lysate, the proteasome surface activity on matrix # 3 was approximately 60X higher than the control. In this conformationally excited state, the inhibitor epoxomycin was marginally effective, reducing activity only by 15%. Thus, this isoform which is resistant to the inhibitor, suggests that functional variants could be identified and subjected to drug candidate libraries. In the other fractions, activity was reduced as expected by 95-100% validating that specific matrices will compartmentalize functional isoforms. This establishes a mechanism to pre-select targets, based on data-mining functional profiles, which would tailor drug screening more closely to disease phenotype.

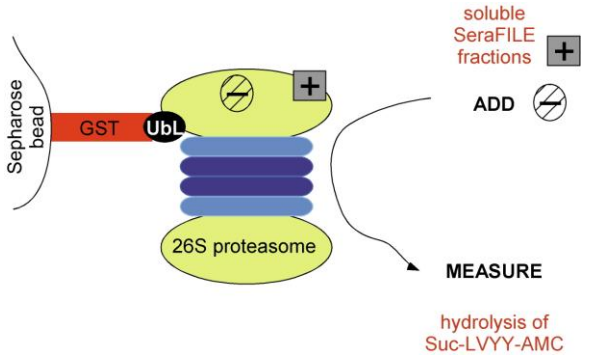
There are two straightforward interpretations for these altered activity results. First, the ligand architecture on the SeraFILE™ surfaces may bind and alter enzyme function. This can be determined by examining the effect of the surfaces on purified proteasomes.

Second, proteasomes are bound to both positive and negative regulators, and application to SeraFILE™ surfaces induces selective dissociation of either positive or negative regulators, yielding proteasomes that are either more, or less active than the input value. This finding would be extremely valuable, as it provides a basis for identifying regulatory molecules. If this were to occur, we would suspect the presence of a soluble negative regulator. Similarly, mixing experiments can be conducted with various fractions to identify soluble regulators of proteasome function. For instance, would the highly active proteasomes bound to matrix 3 be inhibited if the Flow-through fraction (from matrix-3) were added back?

Our preliminary findings demonstrated that **SeraFILE can alter proteasome function**. This remarkable finding provides a powerful foundation for identifying candidate proteins that alter proteasome function.

*Promising support for this approach is suggested by the following study. SeraFILE™ surface #3 was generated and incubated with yeast protein extract. The untreated matrix had **zero** non-specific hydrolytic activity with the fluorogenic-reporter substrate. Addition of protein lysate generated **903** arbitrary fluorescence units of activity. Removal of the unbound fraction yielded **3732** units of activity on the matrices (considerably higher than the activity present in the original lysate). Adding back fresh protein extract reduced this activity to **1900** units within 60 min. One simple interpretation of this result is that a soluble factor can inhibit proteasome activity. If verified, this suggests by re-combining sub-proteome pools, important regulating factors can be discovered (see Model below).*

The identification of candidate molecules could be achieved either by purification using conventional chromatography, or by affinity capture using purified proteasomes. As noted earlier, Dr. Madura's laboratory developed GST-UbL matrix which forms a high affinity interaction with human proteasomes [30, 44]. Fractions from the SeraFILE™ matrices can be applied to purified proteasomes to assess if soluble factors can activate or inactivate it. If these regulatory molecules exist (shown as + and – objects in Model below) they should bind the purified proteasomes, and be amenable to identification by mass spectrometry.



CONCLUSIONS AND FUTURE DIRECTIONS

ProFACT Proteomics has developed SeraFILE™, a unique proprietary surface-technology that permits high-resolution compartmentalization of complex protein mixtures. SeraFILE™ also allows rapid binding/elution equilibrium properties, stability, and reversible binding to proteins. This methodology is readily adaptable to any biochemical system for which a useful measurement of function is available.

An **important future objective** is to exploit the finding that SeraFILE™ matrices alter proteasome activity, either by dissociating regulatory factors, or through proteasome interaction with the SeraFILE™ surface architecture. The ability to directly alter proteasome activity provides an exceptional path to drug discovery.

Future developments for this novel approach are foreseen:

The examination of numerous **matched pairs of cancer/control** specimens to generate activity and expression profiles, and identify putative biomarkers.

The determination of whether all **three proteolytic activities** of the proteasome (peptidyl-glutamyl, tryptic, chymotryptic) are altered on SeraFILE™ surfaces. The effect of known proteasome inhibitors can also be tested.

The identification and isolation of **positive and negative regulators** of the proteasome from SeraFILE™ derived sub-fractions - challenged with purified proteasomes.

Validation that the 20S catalytic and 19S regulatory regions of proteasomes separate on certain SeraFILE™ matrices, using affinity purified intact proteasomes. If SeraFILE™ affects proteasome assembly/stability, it would provide a formidable way to generate **drugs that target assembly/stability**, rather than activity - which represents the current class of drugs.

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