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Activity-Based Probes as Molecular Tools for Biomarker Discovery

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Biomarker discovery and validation techniques have been extensively developed together with the "omics" Era. Nevertheless, there is still a reduced number of biomarker candidates that surpass clinical trials every year. One of the drawbacks of biomarker discovery deals with the quantification of disease-related entities and its correlation with disease predisposition, disease stages or treatment response. Thus, activity-based profiling emerges as an exceptional tool for biomarker discovery and validation and this review highlights advances in this field.

Introduction

The definition of biomarker was stated by the National Institutes of Health Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[1] Advances in biomarker discovery are closely related to the technological development of powerful analytical tools. Nevertheless, there is a substantial translational gap from biomarker discovery to their clinical applications and only a few receive regulatory approval every year.[2-7] Early stage biomarker candidate selection is usually achieved by comparing transcript or protein abundances between diseased and normal tissues. However, these factors might not correlate well with activity and in some cases are irrelevant for the pathological process, especially when analyzing enzymes, due to the complex regulation of enzymatic activity.[3, 8-14] Technical limitations are also a factor when analyzing low-abundance and membrane proteins.[15-17]

Activity-based protein profiling (ABPP) is a proteomic technique pioneered by Cravatt and collaborators in which small active-site directed probes called activity-based probes (ABPs) are used to target only the active form of enzymes. The field of ABPP and its applications has been extensively reviewed in the past.[18-25] ABPP can be applied to the comparison of enzyme activities between normal and diseased cells, with the goal of identifying enzyme activities that differ between the two phenotypes and propose them as potential new biomarkers (Fig. 1).[26]

In this review we assemble the research developed in the field of ABPs applied to the discovery of new biomarker candidates and to the study of previously identified biomarkers, with special focus on hydrolases, a group of enzymes for which most of ABPP research was aimed at. Variations of ABPP and ABPs are also discussed, including gel-free approaches, the use of microarray platforms and capillary electrophoresis for downstream analysis and two-photon probes. Overall, we hope to highlight the outstanding potential of this molecular tool in the field of biomarker discovery.

Fig. 1 – Application of ABPP to biomarker discovery. ABPs are incubated with proteomes from normal and diseased tissues. Separation of the targeted enzymes by SDS-PAGE identifies bands corresponding to enzymes for which activity differs between the two phenotypes. Mass spectrometry analysis is then used to identify the enzymes of interest.

Serine Hydrolases

A typical genome contains 2–4% of genes encoding for proteolytic enzymes and over one third are serine proteases that are involved in protein turnover, digestion, blood coagulation and wound healing, fertilization, cell differentiation and growth, cell signaling, the immune response, and apoptosis.[27, 28] Wiedl *et al.*[29] used an ABPP approach to study serine hydrolase (SH) activities as potential biomarkers for lung cancer since the serine hydrolase superfamily has previously been related to cancer.[30] The fluorophosphate derivative 6-N-biotinylaminohexyl isopropyl phosphorofluoridate (FP-1) was used as the ABP (Fig. 2)[31] and SH activity was evaluated in 40 pairs of human lung adenocarcinoma and matching non-neoplastic lung tissues.

Approximately 40 SHs were identified, the majority of them being esterases and proteases. Two potential biomarker candidates that have not been previously associated with non small cell lung cancer were identified, namely

S-formylglutathione hydrolase, also known as esterase D (ESD), an enzyme involved in the detoxification of formaldehyde, and abhydrolase domain-containing protein 11 (ABHD11), a previously uncharacterized enzyme. A decrease in the activity of ESD significantly predicts the presence of high-grade lung adenocarcinomas.

No difference in ESD transcript levels or protein abundance was found when comparing normal and tumor tissue, which highlights the importance of using ABPP for activity quantification. The increased activity of ABHD11 predicts the presence of lymph node metastases as well as the development of distant metastases in these patients. A higher activity of this protein in the majority of malignant tissues was also observed, indicating that ABHD11 may play a role in the pathogenesis of lung adenocarcinoma. Overall the results show ABPP's capability to discover previously uncharacterized enzymes with no known function as potential new biomarkers.

The fluorophosphonate and fluorophosphate groups have been widely used in ABPP experiments since they inhibit the majority of serine hydrolases in a potent and irreversible way, while remaining mostly inert to cysteine, aspartyl, and metallohydrolases.[32, 33] The first derivatization of the FP group into an ABP was accomplished by Liu *et al.*[34] by attachment of a biotin tag and the resulting ABP was used as an agent for the profiling of SH expression and function. The results showed that the inhibition requires the enzymes to be in a catalytically active state.

Cravatt and co-workers[33] used FP probes in the profiling of serine hydrolase activity in human cancer cell lines. The differences in enzyme activity of the 57 groups of identified enzymes made it possible to distinguish cancer cells with different tissues of origin and in distinct states of invasiveness. Most of the identified enzymes belonged to the cancer secretome, the totality of proteins released by cells and tissues,[35, 36] suggesting that this subcellular fraction is an important source of new biomarkers. The SH activity patterns were also studied in breast and melanoma cancers by Jessani *et al.*[30, 37] using rhodamine tagged FP probes and in-gel visualization of probe-enzyme complexes. Among other results, the authors identified active urokinase, a serine protease which is an established marker of human cancer progression.

Knowing that urokinase is subject to complex post-translational regulation, an analysis of mRNA levels was performed. mRNA levels failed to correlate with urokinase activity, which shows that they do not represent an accurate measure of enzyme activity as provided by ABPP. Cheng *et al.*[38] created fluorogenic probes that measure BlaC activity for rapid detection of *Mycobacterium tuberculosis* (Mtb, Fig. 3). BlaC is a β-lactamase specifically expressed by Mtb which possesses a central role in the biochemical mechanism responsible for pervasive b-lactam-antibiotic resistance. Fluorogenic probes that target β-lactamases have been built in the past, but these are not specific for BlaC. The crystal structure of BlaC shows that this β-lactamase has a bigger and more flexible active site than most other β-lactamases and could potentially accommodate more bulky lactam substrates. Addition of a 2-cyclopropyl and 7-OMe substitutions to an umbelliferone-based, non-specific β-lactamase fluorogenic substrate yielded a probe which displayed remarkable specificity for BlaC and was able to detect 10 CFU BCG from unprocessed human sputum in the presence of high levels of other b-lactamase expressing clinically prevalent bacteria. The use of this probe for Mtb detection showed high sensitivity and specificity, comparable to those of nucleic acid based diagnostic methods, and could provide a low-cost alternative for Mtb detection in resource-limited areas.

Fig. 2 – FP-1, the flourophosphonate biotinylated ABP used by Wiedl *et al.* in the profiling of serine hydrolases.

Fig. 3 – Fluorescence generation after beta-lactamase activation of fluorogenic probes that target BlaC (adapted from Cheng *et al.*[62]).

Another example of the FP warhead in biomarker discovery using ABPP is given by Cavalli *et al.*[39] with the report of the design, synthesis and characterization of the first ABP to target autotaxin (ATX), a secreted glycoprotein involved in the hydrolysis of lysophosphatidylcholine into lysophosphatidic acid, which has been shown to possess a role in tumor progression. The probe consisted of four components: a lysophospholipid-based recognition element to target ATX, a trapping device to covalently bind ATX upon activation, a hydrophilic (ethylenedioxy)diethylamine linker and a fluorescent reporter group.

This probe managed to specifically label recombinant ATX and its isoforms in plasma in an activity-dependent manner. These results showed that the use of an ABP could be an effective tool to monitor ATX levels in plasma. Several studies showed that ATX could serve as a biomarker of b-cell tumors, in particular follicular lymphoma, in which ATX levels are associated with tumor burden and show variation patterns with the course of the disease, with little influence from inflammatory states, unlike other biomarkers for lymphoma.[40] Thus, ATX-ABPs could potentially function as diagnostic tools for this type of tumors as a monitor reagent for ATX levels in body fluids.

Cysteine Proteases

Cysteine proteases have been implicated in a variety of diseases including cardiovascular, inflammatory, viral and immunological disorders and cancer.[41, 42] Lyo *et al.*[43] used an acyloxymethylketone-based ABPP to identify and localize active forms of cathepsins in the pancreas and spinal cord during pancreatitis (Fig 4). These enzymes are involved in pancreatitis initiation by controlling trypsinogen processing in the pancreas and are potential biomarkers of this condition. Active cathepsins were selectively detected in the pancreas and spinal cord of mice with cerulean induced pancreatitis, showing active enzyme localization to be in the acinar cells and macrophages of the pancreas and also spinal microglial cells and neurons.

Fig. 4 – ABP used by Lyo *et al.* in the measurement of active cathepsins in pancreatitis.

A separate experiment with intrathecal administration of the ABP showed an increased activity of cathepsins within neurons and microglial cells of the spinal cord where inputs from primary spinal afferent neurons innervating the pancreas are received, a novel finding which might suggest a role of centrally acting cathepsins in the generation of pancreatic pain. This experiment used a probe with an acyloxymethylketone (AOMK) reactive group, which is a well established warhead with exceptional reactivity to target the active site of cysteine proteases.[33, 44] Cathepsin B is a promising diagnostic and prognostic biomarker of various cancers and may be a useful marker predictive of response to chemotherapy.

Recently, Chowdhury et al.[45] developed prodrug-inspired fluorogenic peptides as efficient ABPs for cathepsin B that proved to be capable of selectively assessing cathepsin B activity in cell lysates under conditions favoring cysteine cathepsin activity (Fig. 5).

Metalloproteases

Metalloproteases (MP) present a special challenge in ABPP approaches since there is no active-site nucleophile, but rather a metal-activated water molecule to perform hydrolysis. For these enzymes, a reversible inhibitor is used to form a reactive intermediate with the enzyme's active site, followed by formation of a covalent bond between a photocrosslinker and a proximal nucleophilic residue outside of the active site.[8, 24, 46]

Saghatelian *et al.*[47] used this approach by building ABPs containing an hydroxamate group to target MPs, benzophenone as a photocrosslinker and rhodamine as a tag. These probes labeled the active forms of the target proteases with great sensitivity and identified a membrane-associated glycoprotein, neprilysin, which was highly up-regulated in invasive melanoma cells, but not in healthy cells. While these results showed a potential role of neprilysin in melanoma and other instances of it being upregulated in cancers have been described, this enzyme has also been considered a negative regulator of tumorigenesis, which means that further investigation about its role is necessary.

The hydroxamate is a strong zinc chelating agent that tightly binds to the MP active site.[10] Sieber *et al.*[48] created a library of structurally diverse photoreactive hydroxamate probes with complementary MP reactivity aiming to improve the general strategy of MP proteomic profiling. For this purpose, an optimal binding scaffold, succinyl hydroxamate, was chosen, since it is capable of accommodating a broad range of active site structures found in the MP family and provides a better interaction than hydroxamate alone. Alkyne groups were used as surrogate groups for later addition of reporter tags by click chemistry to reduce adverse interactions between bulky tags and the target enzymes. These probes were used to compare MP activity profiles of human cancer cell lines including membrane proteomes from invasive and noninvasive breast carcinoma and melanoma lines. Several enzymes showed different activity patterns, like alanyl aminopeptidase (AlaAP) and neprilysin, which were highly active in invasive, but not noninvasive, melanoma cells. This warrants further investigation on these enzymes as potential candidate biomarkers. AlaAP is a potential marker for transdifferentiation of melanocytes into metastatic melanoma. The probes were also able to identify ADAM proteins, with critical roles in cell surface signaling events, a class for which no ABPs had been previously reported.

Fig. 5 – Mechanism for the generation of fluorescence by produg-inspired fluorogenic peptides after hydrolysis performed by cathepsin B.

Other Enzymes

Carmony *et al.*[49] reported the synthesis and characterization of fluorescent ABPs that selectively target LMP2, an immunoproteasome subunit, by derivatization of a previously synthesized LMP2 inhibitor, UK-101.[50] The immunoproteasome is a variant of the constitutive proteasome which is normally expressed in cells of hematopoietic origin but can also be induced in other cell types. This variant of the proteasome functions primarily in the adaptive immune response, but it has also been implicated in disorders like cancer and is a potential cancer biomarker. A fluorescein tag was added to UK-101 to create UK101-Fluor, an ABP which was able to penetrate cultured cells and label the immunoproteasome, yielding a fluorescent signal. In order to create a non-invasive imaging probe, a second ABP, UK101-B660, was created with a near-infrared fluorescent group BODIPY 650/665. This probe also showed selective labeling of active LMP2. Overall, the authors showed that an immunoproteasome inhibitor can be derivatized into ABPs and used as imaging probes to visualize catalytically active LMP2 in living cells. With LMP2 serving as a potential tumor biomarker, the NIRF LMP2 targeting probe could find use for in *vivo* screening in animal models.

Wang *et al.*[51] described a click chemistry ABPP strategy for labeling of dimethylarginine dimethylaminohydrolase (DDAH). This enzyme regulates the blood levels of asymmetric N,N-dimethyl-L-arginine (ADMA), an endogenous inhibitor of nitric oxide synthases and an emerging biomarker for endothelial dysfunction in cardiovascular disease.[52] Dysregulation of DDAH leads to endothelial dysfunction in hyperhomocysteinemia, renal failure and diabetes. The authors synthesized an N-but-3-ynyl-2-chloro-acetamidine probe to measure DDAH activity. Combination with biotin-PEO3-azide probe introduced the biotin tag and allowed detection of the probe-enzyme complexes (Fig. 6). It was demonstrated that the probe selectively labels active DDAH in cultured mammalian cells and that it can be blocked by the presence of competitive reversible and irreversible inhibitors. Given its small size and simplicity it could present itself as a powerful tool in other endothelial dysfunction studies.

Fig. 6 – Reactive clickable chloro-acetamidine probe containing an alkyne handle and complementary biotin probe containing an azide handle.

Non-directed ABPP Biomarker Discovery

Non-directed ABPP uses generally reactive electrophiles as reactive groups to probe complex proteomes with the potential of targeting previously unidentified enzymes.[53]

Barglow and Cravatt[17] synthesized a group of 18 ABPs containing an alpha-chloroacetamide reactive group and a variable dipeptide binding group. These were initially tested in three soluble mouse tissue proteomes (brain, heart and liver) where labeling of several enzyme families occurred, including aminolevulinate Δ-dehydratase (ADD) and creatine kinase (CK), two enzymes for which no previous ABPs had been built. The probes were then used to compare the enzyme activities in the mouse liver of wild-type and mice lacking the leptin gene, which present extreme obesity, insulin resistance and elevated gluconeogenesis. Several differences in enzyme activity were observed. Some were known obesity markers like monoacylglycerol (MAG) lipases and carboxylesterases. One of them, hydroxypyruvate reductase (HPR) presented a 6-fold greater labeling in the obese mice and could be a novel marker of obesity since it has been suggested to play a role in converting serine to glucose, contributing to the elevated gluconeogenic state of the obese mice. It is important to notice that in an analogous experiment using 2D-electrophoresis this enzyme was not detected, possibly due to co-migration with other abundant proteins, which once again shows the advantage in using ABPP in these experiments for the measurement of low abundance proteins and also the potential of non-directed approaches to reveal previously uncharacterized probe-enzyme interactions.

Adam *et al.*[16] used a group of sulfonate probes capable of targeting several distinct enzyme families to analyze the differences in enzymatic activity between estrogen receptor- positive (ER+) and –negative (ER-) human breast cancer cell lines (Fig. 7). Several proteins reacted with the ABPs in both types of cancer cells. One protein, an omega-class glutathione S-transferase, which had no previous link to breast cancer, was more than tenfold upregulated in ER- when compared with ER+. Further experiments showed that the probe-enzyme reaction occurred in the active site of the enzyme. For breast carcinomas the ER- phenotype is usually associated with metastatic phenotypes and aggressive forms of cancer.[54]

Fig. 7 – Example of a biotinylated sulfonate ester ABP used by Adam *et al.* in a non-directed approach to target enzymes from different families.

Alternative and Gel-free Activity-based protein profiling

Gel-based ABPP provides a robust and efficient method to quickly assess enzyme activity. However, problems with sensitivity and low-abundance and/or co-migrating proteins due to low resolution are important limitations of this method.[55]

Several gel-free options for downstream analysis in ABPP have been used in enzyme activity study, namely liquid chromatography, microarrays and capillary electrophoresis. Here we describe some of the work performed with these approaches and instances of their application in biomarker discovery and evaluation.

ABPP-Liquid Chromatography-MS and ABPP-MudPIT

Liquid chromatography-MS-ABPP has been used to identify ABP-binding sites in target enzymes in order to profile enzyme active sites. In a noteworthy example performed by Evans and Cravatt,[56] proteomes were labeled with a biotinylated ABP with a tobacco etch virus (TEV) protease cleavage site. Labeled proteins were enriched using avidin beads and digested with trypsin. The tryptic peptides were obtained by filtration. Adding the TEV releases the probe bound peptides corresponding to the enzyme's active site. Both groups of peptides were then analyzed by parallel LC-MS/MS runs.

Previous methods described for this purpose only analyzed ABP labeled tryptic peptides, discarding the rest of the protein, which complicated MS analysis. In order to achieve an in-depth analysis of enzyme activities in breast tumor biopsies Jessani *et al.*[57] tested an integrated ABPP-multidimensional protein identification technology (MudPIT) platform (Fig. 8). Thirty three primary human breast tumor and normal breast tissue biopsies were analyzed using rhodamine tagged FP ABPs. A first step uses ABPP labeling and reading by 1DE to obtain the target enzyme's activity signatures and allow rational selection of representative members of breast tumor classes for in-depth proteome analysis. The second step involves the use of a biotinylated probe, enrichment of probe labeled proteins using avidin, on-bead trypsin digestion and multidimensional LC-MS/MS analysis of the tryptic peptides. Over 50 serine hydrolase activities were identified by this method, including proteases, lipases, esterases and uncharacterized hydrolases. Several proteome subunits were also labeled by the probes. Among the identified proteins, fibroblast activating protein, KIAA1363 and platelet-activating factor acetylhydrolase 2 were elevated in ER(-)/PR(-) tumor, when compared with ER(+)/PR(+) and normal tissues. ER(-)/PR(-) is generally considered the most aggressive form of breast cancer and the most difficult to treat. For certain enzymes like KIAA1363 for example, activity and mRNA levels were highly uncorrelated. ABPP-MudPIT has good reproducibility, is suitable for comparative measurement of enzyme activities and can discover disease-associated enzyme activities that might not be detected by other methods. This method also solves some of the limitations of using human samples due to their limited quantity, high complexity and heterogeneity.

Microarrays

Despite addressing several limitations of the gel-bassed ABPP techniques, LC-MS/MS still suffers from problems like poor throughput and requiring high sample quantities. Microarray techniques are able to answer these problems by using small sample volumes and allowing multiple analyzes to take place in a single

Fig. 8 - ABPP-MudPIT; Phase I (A,B,C) – Standard ABPP approach to map probe-enzyme interactions; Phase II: D. Incubation of the biotin tagged probe(s) with the sample proteome; E. Purification using avidin beads; F. Tryptic digestion of the targeted enzymes; G. Mass spectrometry analysis of tryptic peptides for target enzyme identification.

array plaque simultaneously. Most microarray techniques developed to study enzyme activity rely on the display on an enzyme substrate that generates a readable signal upon cleavage by the enzyme or an immobilized enzyme with which appropriate substrates are incubated.[58-60] One of the first works combining microarray technologies and ABPP was reported by Grace *et al.*[61] who used a microarray with immobilized enzymes which was incubated with fluorescently tagged mechanism-based inhibitors of the target enzymes, thus providing a readout of the target enzyme's activity.In an effort to discover new probes for enzyme activity, Uttamchandani and Moochhala[62] combined concepts in microarray technologies and ABPP and reported the first small molecule microarray-facilitated approach for high-throughput identification of photocrosslinking based probes to target enzymes. The target enzyme was γ-secretase, which performs proteolysis of amyloid precursor protein, yielding amyloid β-protein, a central pathogenic feature of Alzheimer's disease.[63] A library of 198 biotinylated compounds based around the hydroxylethylene scaffold was immobilized onto avidin-functionalized slides to generate the corresponding small molecule microarrays. These showed to be able to sensitively report activity-based binding events of aspartic proteases after a screening with fluorescently labeled proteomes. The best hits were then converted into probes by click chemistry and successfully labeled a proteolytic active subunit of γ-secretase in a gel-based assay.

Sieber *et al.*[33, 64] described an antibody-based microarray platform for ABPP that performs the isolation, detection and identification of probe-labeled proteins in a single step with greater sensitivity and resolving power than gel based methods, with less sample consumption. The process involves treatment of proteomes with fluorescent ABPs and visualization of the probe-enzyme complexes on glass slides displaying anti-enzyme antibodies as capture reagents (Fig. 9).

This allows the simultaneous profiling of several enzyme activities and could be used in the discovery of new biomarkers and further studies on previously established ones. This method was tested against proteases that are upregulated in human cancer like prostate specific antigen (PSA), a clinical biomarker of prostate cancer. The analysis of PSA in microarrays yielded a strong signal with a sensitivity limit of 2-8ng of enzyme/mL. The detection limit of this protease was 50 times lower than for gel-based assays, in which detection is hindered by two co-migrating enzymes.

PSA is the most well established secretome biomarker, belonging to the family of kallikrein and kallikrein-related peptidases (KLKs) which have been implicated in several aspects of cancer pathophysiology.[36, 65] The secretome comprises all the proteins secreted by cancer through the several existing secretory pathways, which might include growth factors, enzymes, and cytokines, among other proteins. The investigation of cell secretomes constitutes a critical strategy in the identification of new candidate biomarkers.[35] Further developments in this field could be achieved by use of high surface area organisilicate nanoporous films, which provide large and accessible surface areas and amplification of signal, while being easy to fabricate, inexpensive and portable, being ideal for high density binding of biological probes, at the expense of sensitivity. Harris *et al.*[66] used this strategy to study trypsin as an analogue of thrombin, a trypsin-like serine protease, which is an important biomarker in cardiovascular diseases. Immobilized trypsin target peptides were utilized as sensor probes, which induce a detectable change in fluorescence upon cleavage by trypsin. The films enabled analysis of trypsin with high areal binding density of the peptide probes while ensuring adequate inter-peptide spacing to avoid quenching among the dye molecules and steric hindrance to the target enzyme.

Fig. 9 – Microarray ABPP: A. Incubation of ABPs with the target proteome; B. Capture of the probe-enzyme complexes on glass slides displaying anti-enzyme antibodies; C. Downstream analysis (adapted from Sieber *et al.*[79]).

Capillary Electrophoresis

Okerberg *et al.*[67] described a gel-free platform for ABPP combining capillary electrophoresis with laser-induced fluorescence detection (CE-LIF), a technique with high sensitivity, high resolution and potential for high-throughput separations, using LC-MS/MS for identification.

A validation experiment using 8 purified pre-labeled serine hydrolases added to eight different proteome samples showed similar migration times and peak heights for all eight proteins, including the ones in problematic samples like undiluted plasma. Further testing revealed an absolute detection limit of 1 x 10^{-19}mol. For protein identification, ABP-labeled peptides were enriched and concentrated by using monoclonal antibodies and analyzed by LC-MS/MS. Three different mouse proteomes were studied with 49 serine hydrolases being identified. Remarkably, an SDS/PAGE analysis showed the three proteomic samples as being substantially similar, while the CE-LIF screening platform showed very distinct serine hydrolase profiles. CE-LIF was able to separate the majority of 11 proteins from the kallikrein family, which showed up as a single band in SDS-PAGE. The kallikrein family members have been implicated in tumorigenesis, angiogenesis and inflammation, showing great therapeutic potential and also for discovery of new biomarkers.[68]

Xu *et al.*[69] developed a novel single-cell chemical proteomics (SCCP) approach using an ABP to identify membrane proteins on neurons. The target protein, GB1, a subunit of the GABAB receptor, was labeled with a trifunctional click chemistry based ABP containing a warhead that shared the pharmacophore with GB1 receptor antagonists, diazirine as a photocrosslinking group and a fluorescent BODIPY tag for detection. The labeled single cells were encapsulated in buffer droplets stored in a PDMS chip holder. The droplets were deposited in a PDMS microwell array, from where they were injected into a capillary electrophoresis-laser induced fluorescence system after cell lysis and denaturation.

This technique allowed detection of GB1 in single cells and showed that the levels were different between different cell strains and also within different cells of the same strain, highlighting the

heterogeneous nature of the tissues. The ability to discriminate differences in membrane proteins of different cells is important in fields like neuroscience and cancer biology, where tissues display high heterogeneity. Since biomarker discovery usually relies on the analysis and comparison of protein expression and enzyme activity, the ability to discriminate between different individual cells of the same tissue could be a major advantage in some studies.

ABPP using Nanoparticles

Welser *et al.*[70] described nanoparticle based platforms for protease activity detection in the context of studying proteases as potential biomarkers. A typical nanoparticle enzyme sensor consists of a biological substrate specific to the target enzyme immobilized on the nanoparticle surface. The active target enzyme induces a change in the substrate which causes a change in the environment of the nanoparticle resulting in a detectable signal change. Nanoparticles could offer several advantages when compared with fluorophores for example, like superior optical properties, and increased stability. However, since some of these techniques function in an analogous way to substrate-based assays, they could provide less specificity than an ABPP approach.

Warren et al.[71] used a similar approach by developing synthetic biomarkers for noncommunicable diseases. These release ligand-encoded reporters upon reaction with its target and are detected in a biological fluid by paper test strips, which are rapid to use and have a low cost. The authors studied thrombosis and colorectal cancer, using thrombin and mettaloproteinases as targets, respectively, by conjugating substrate-reporter tandem peptides sensitive to these enzymes on the surface of nanoparticles. After administration, the nanoparticles probe diseased tissues where the local-upregulated target enzymes cleave the its surface peptides, releasing reporters which are concentrated in the urine and detected by a custom lateral flow assay, a variant of paper tests. While not directly related to ABPP, these experiments might provide the basis to develop similar nanoparticle-ABPP assays into fast and inexpensive diagnostic tests.

Two photon ABPs

Hu *et al.*[72, 73] developed a new class of fluorescently quenched ABPs based on quinine methide chemistry, including a variant with incorporated two-photon dyes enabling the two-photon imaging of enzyme activities for the first time. These probes release a quinone methide intermediate upon cleavage that reacts in a non-specific way with other medium nucleophiles, therefore keeping fluorescence in the place of reaction, unlike fluorogenic probes, which diffuse away from the catalytic active site of the target enzyme, resulting in amplification of the signal and greater resolution. These probes are highly modular, facilitating attachment of different warheads, tags, cell penetrating peptides and other components, which makes them applicable to other enzymes and imaging techniques. Taking advantage of this property, two-photon ABPs were also created. The fluorescently quenched probes were used for in-cell bioimaging of apoptosis. After incubation of cell lysates with the probes and induction of apoptosis, an activity-dependent exponential increase in fluorescence was observed. The two-photon probes were used to target tyrosine phosphatases. Two photon microscopy has increased penetration depth, lower background signal and reduced photodamage and photobleaching, providing numerous advantages for in-vitro enzyme imaging experiments. Following this work, a novel highly fluorescent two-photon dye was created, which upon attachment of electron withdrawing phosphate groups becomes only weakly fluorescent, resulting in the first fluorogenic two-photon pair capable of real-time detection of phosphatase activities. The phosphate groups were caged with 2-nitrobenzyloxy groups to increase permeability and allow better control of probe functioning. An alkyne moiety was added to allow attachment of cell-penetrating peptides through click-chemistry. These probes are internalized by cells and after UV irradiation release the phosphatase-responsive probe (uncaged phosphate groups). Dephosphorylation by endogenous phosphatases leads to generation of fluorescence (Fig. 10). The probes were capable of detecting endogenous phosphatase activities in live mammalian cells and drosophila brains. The use of cell-penetrating peptides allowed for organelle and tumor specific delivery of the probes. Several phosphatase activities work as markers of disease and to evaluate the efficacy of treatments, which makes new approaches to measure and study the activity of these enzymes invaluable in research and in the field of diagnostics and therapeutics.[74-76]

Fig. 10 – Mechanism of activation of the two-photon ABP created by Li et al.

Conclusions

The field of biomarker research has reached huge proportions and together with the advances on technological platforms led to the examination of a wide range of proteins and genes and gathering of an unprecedented amount of information on disease mechanisms at the molecular level. Nevertheless, several pitfalls emerged so that translation to its clinical utility has been slower than expected. While from the technical point of view, the methodologies need to be improved toward more sensitive and reproducible assays, the diversity on the population concerning for eg. age, gender or pathology, leads to a very low rate of success on the validation of new biomarkers. Activity-based protein profiling has emerged as a powerful technique for proteome analysis and, unlike classical proteomics, allows the quantification of enzymes in their active catalytic state. With the adequate design of specific targeted probes, ABPP paves the way to better isolation techniques that ultimately will lead to selective, efficient and easy to handle assays for biomarker discovery, validation and diagnostic/prognostic tool development.

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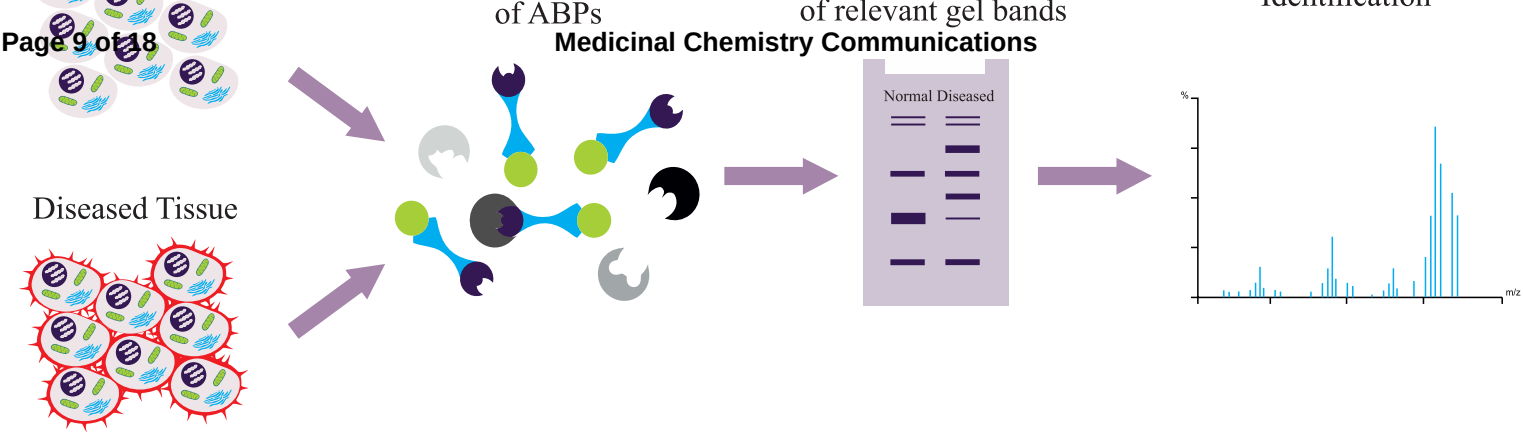
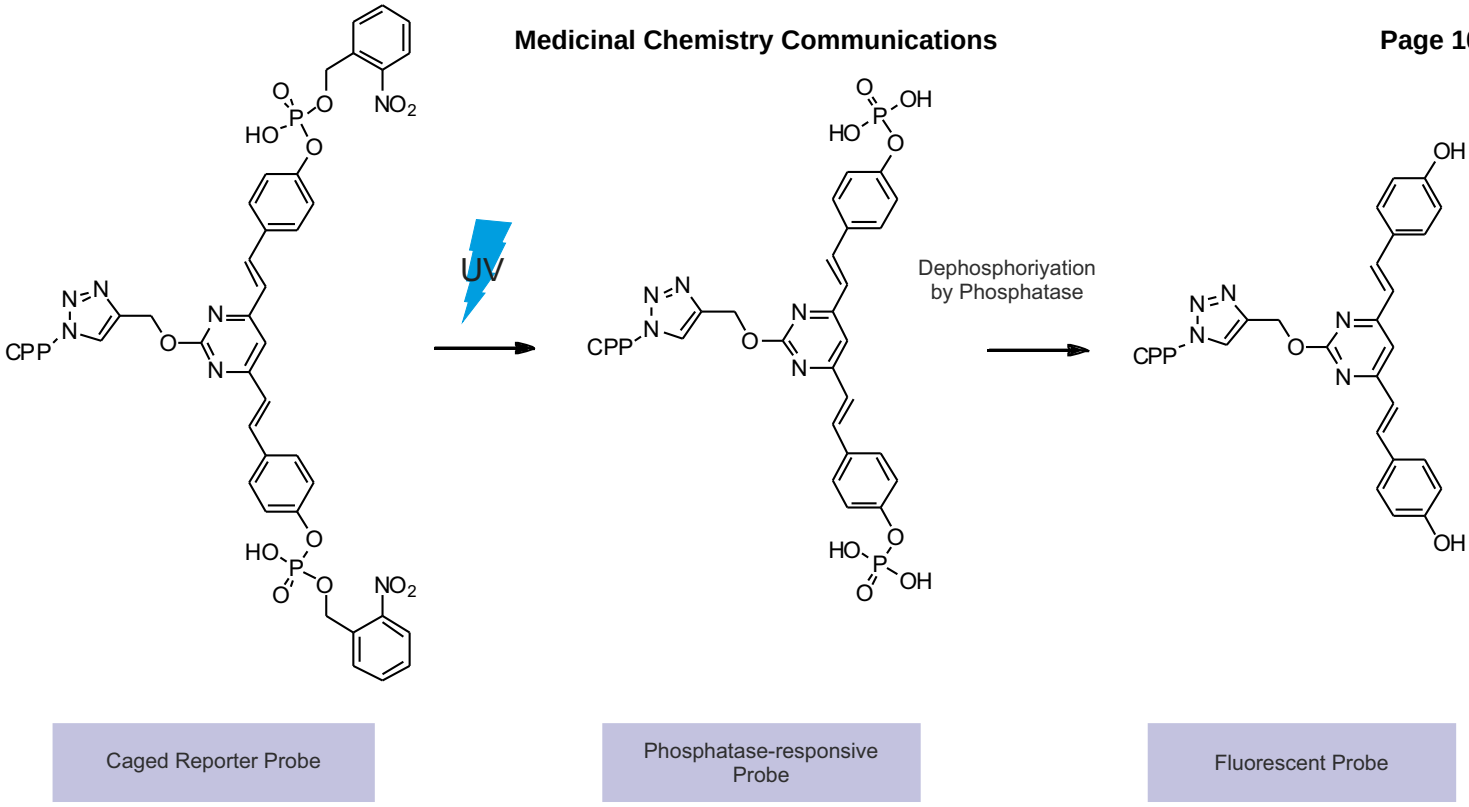
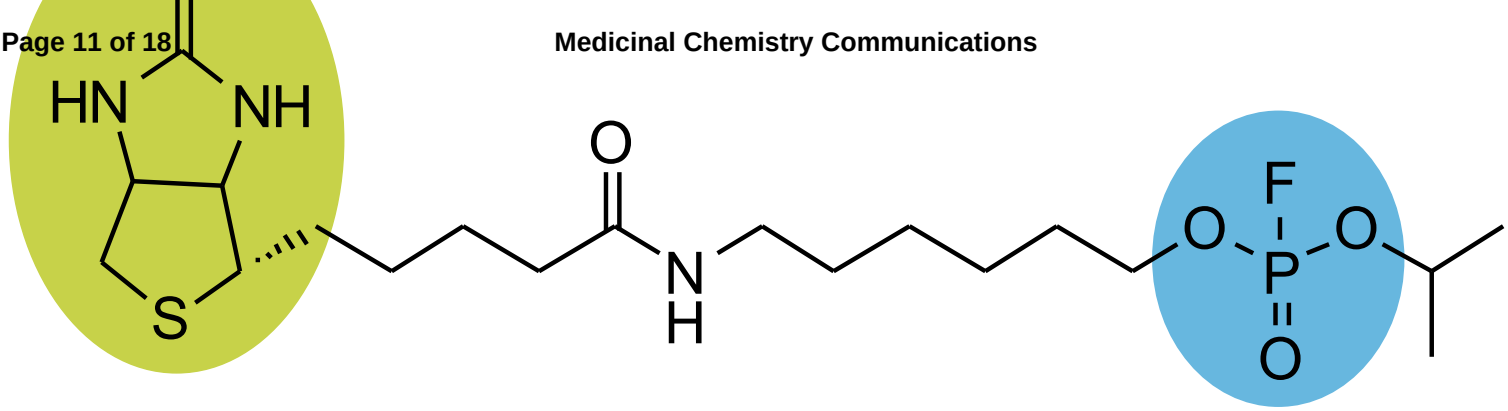


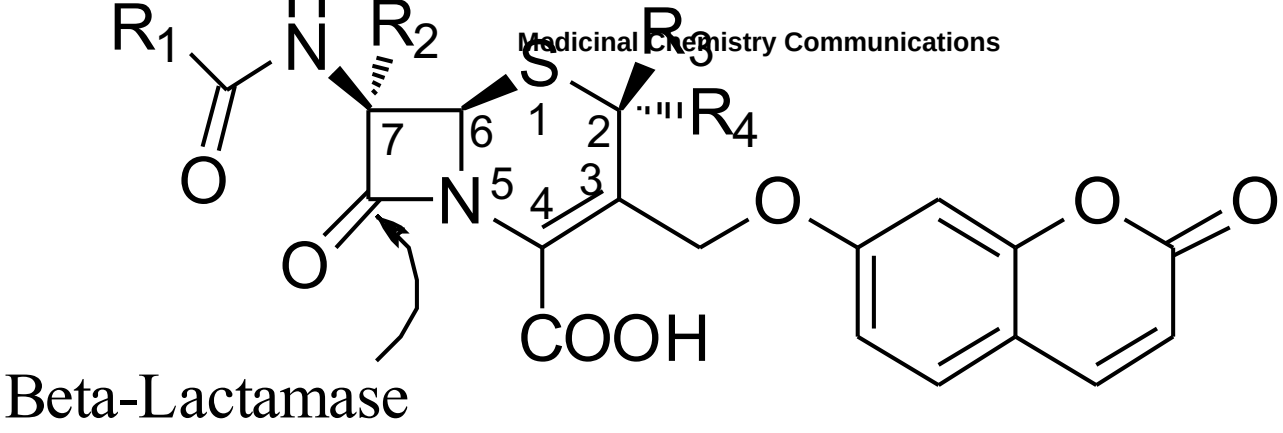


Caged Reporter Probe

Phosphatase-responsive Probe

Fluorescent Probe

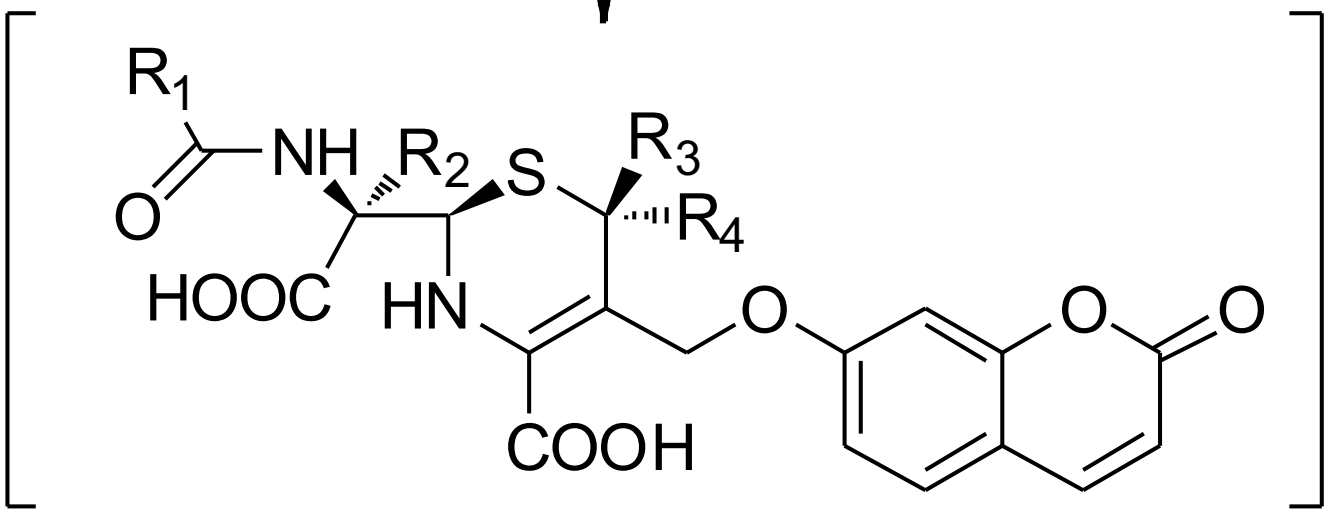


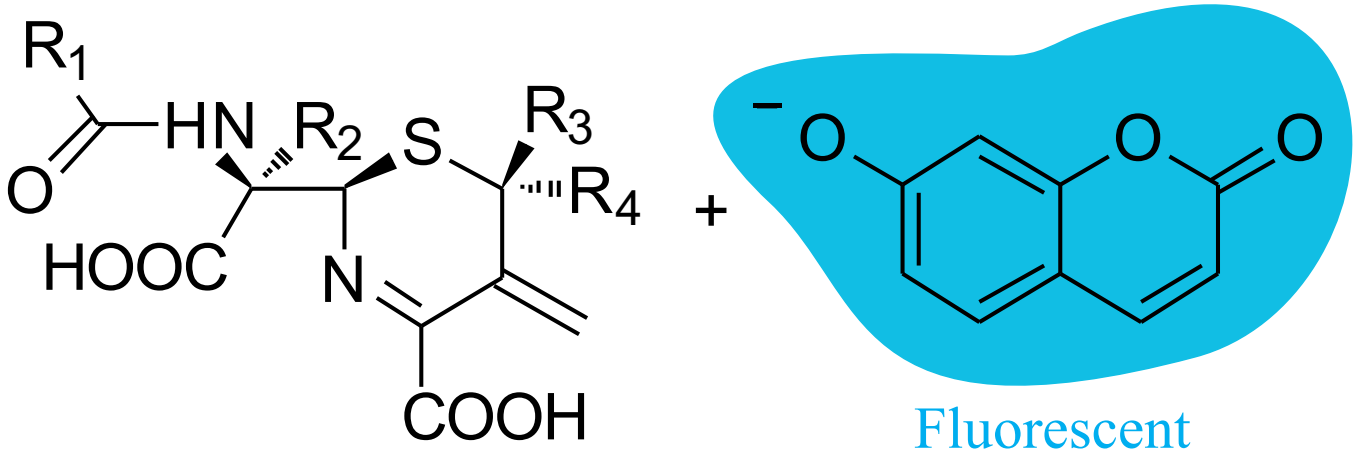


Beta-Lactamase

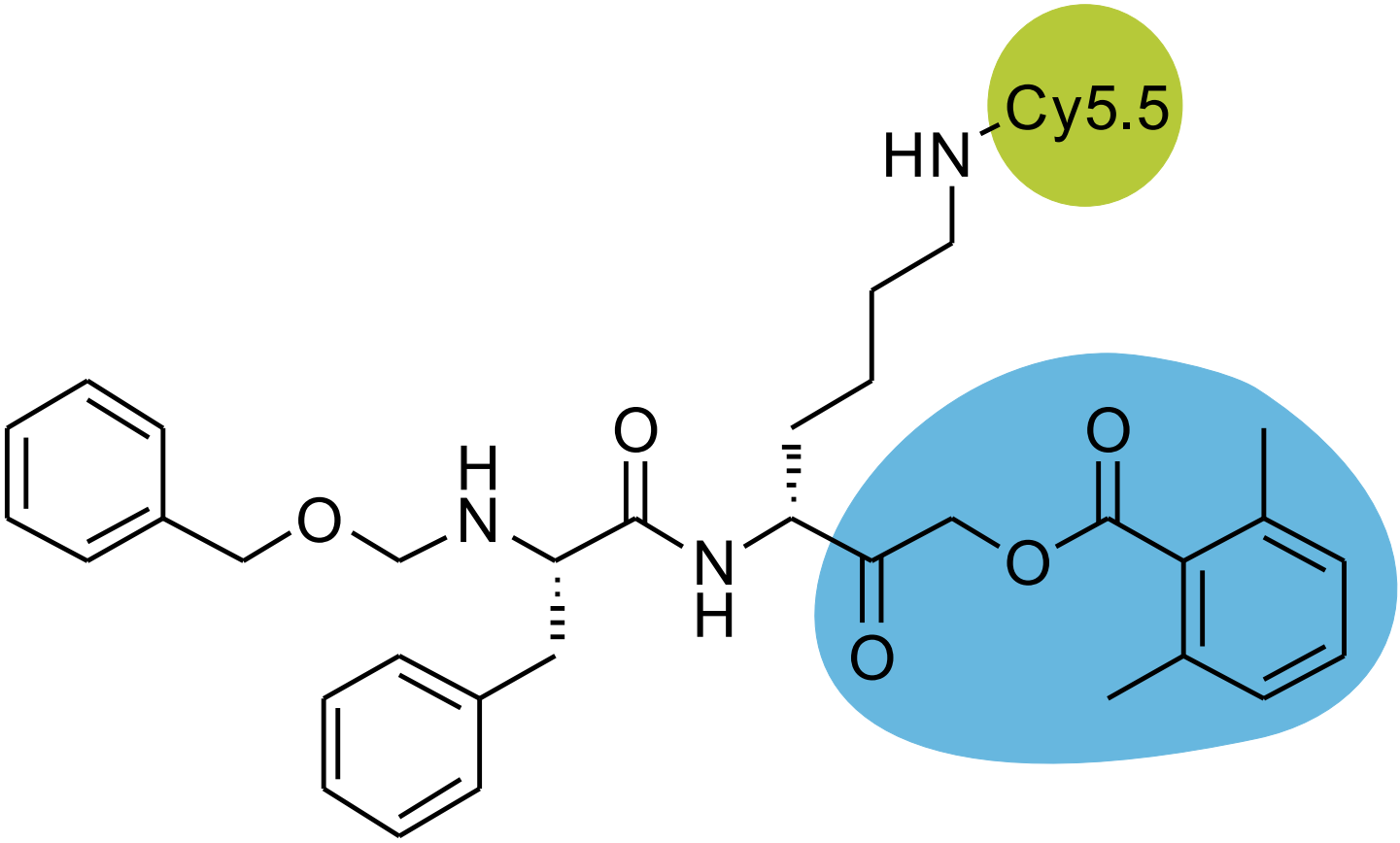
(Non-fluorescent)

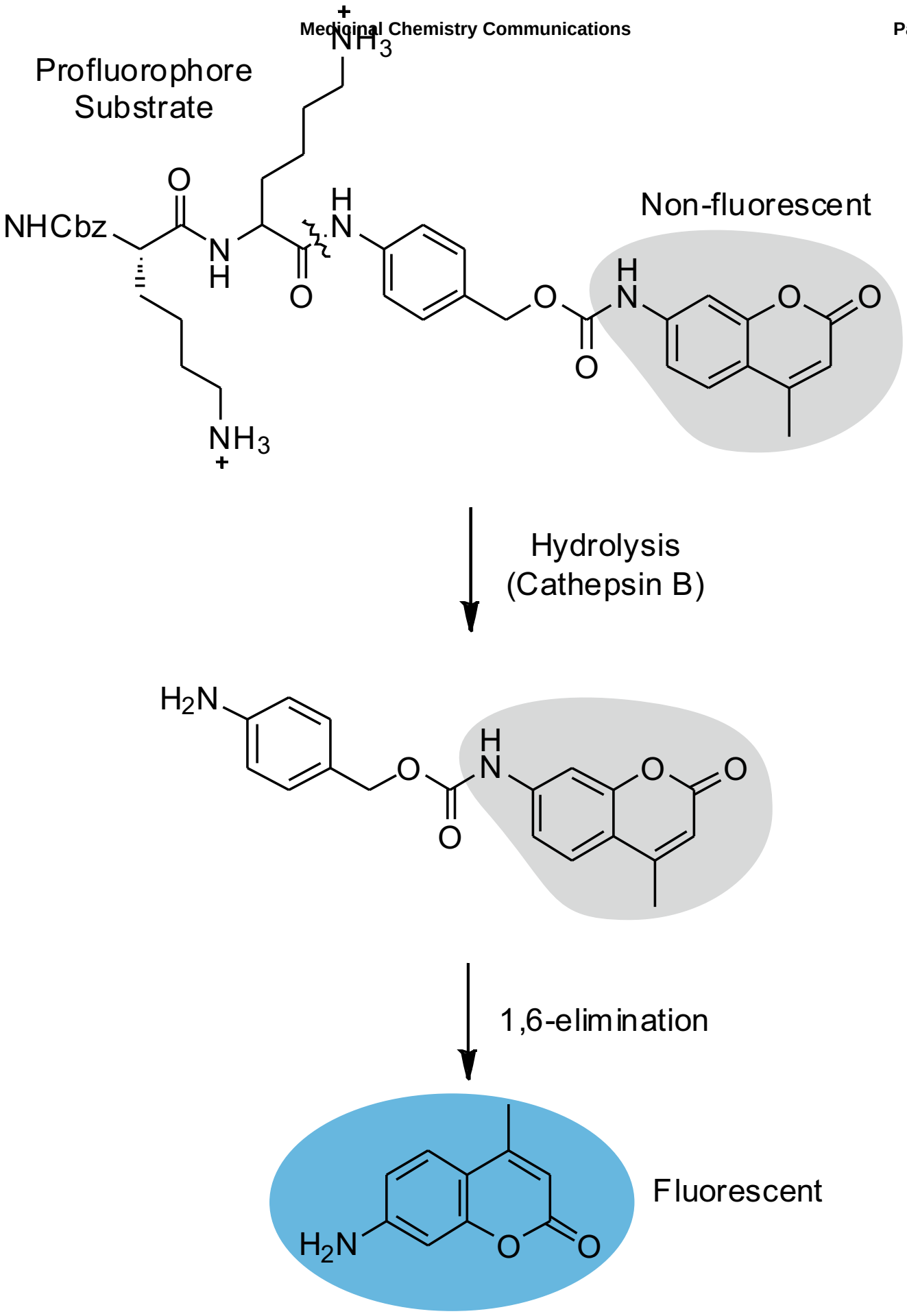
R1 = Bn; R2 = OMe; R3/R4 =

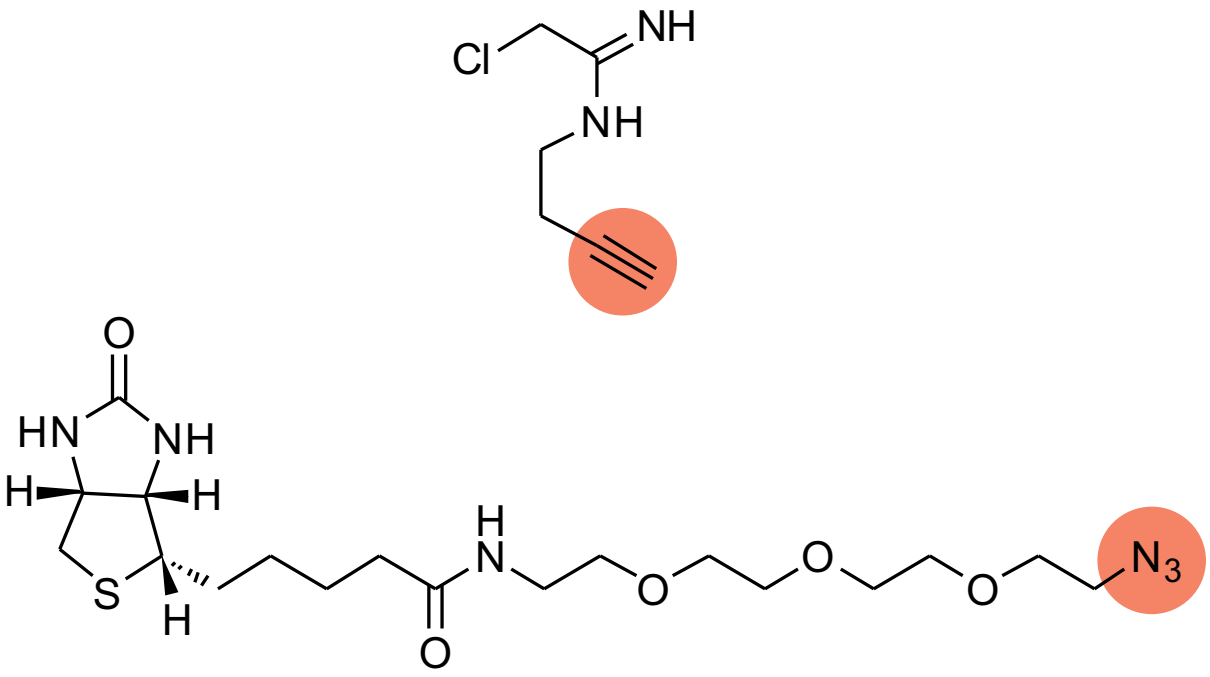


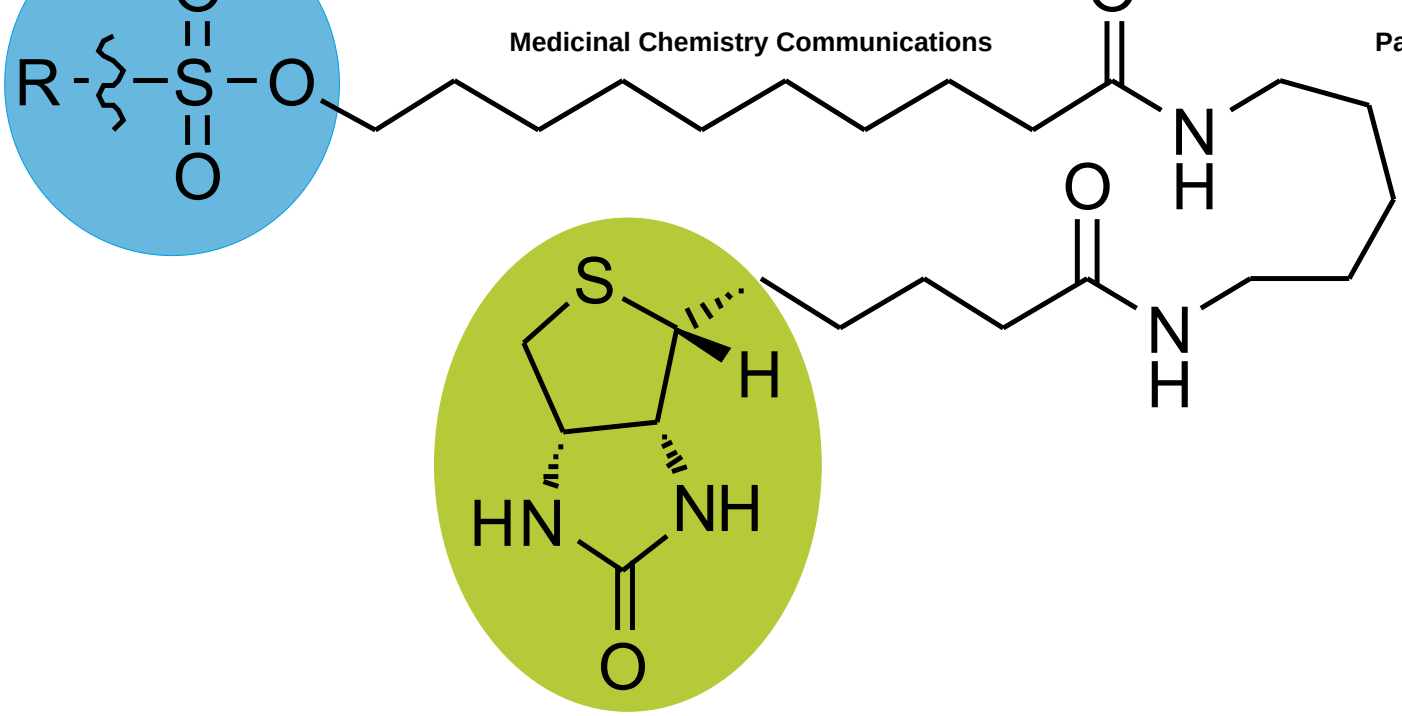


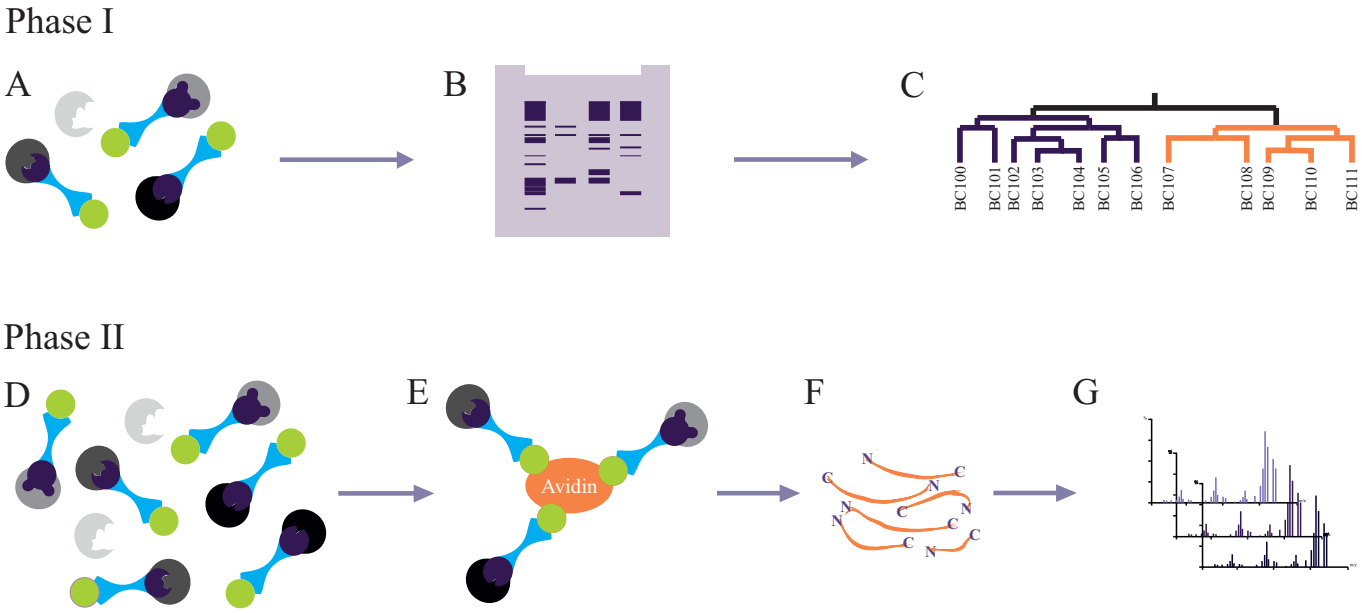
Fluorescent



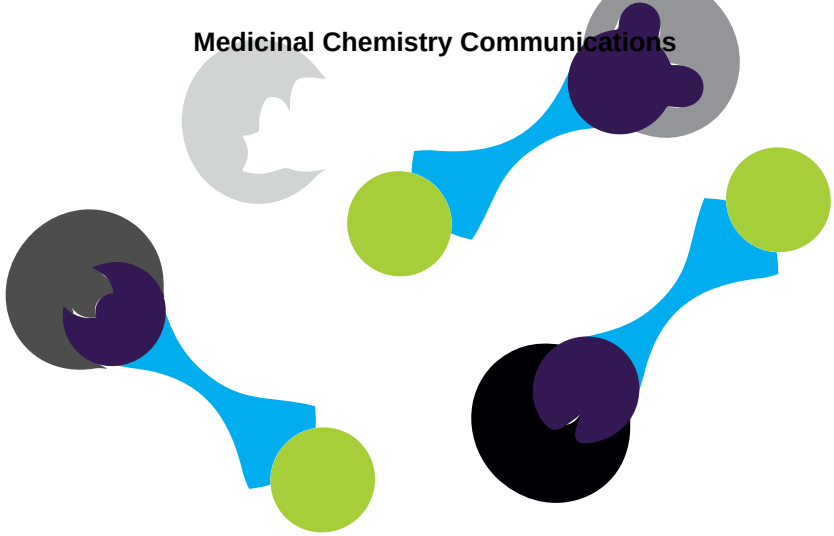




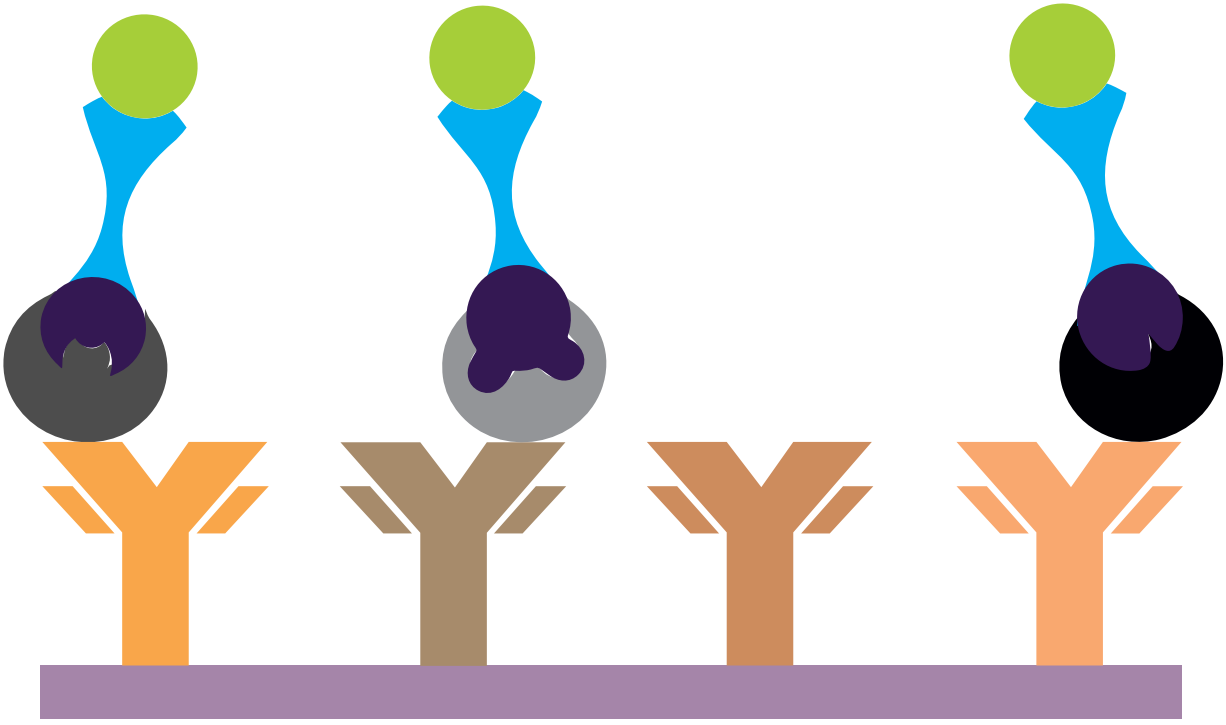




A



B



C