

Biomarkers beyond proteomics and genomics: microvesicles as indicators of cancer progression

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Protein- and gene-based biomarkers are critical indicators for evaluating certain biological processes that give insight into the patient's pathologic condition; however, only a few of these candidate biomarkers have progressed beyond the initial stage and many are plagued with problems including failed translation to bedside care and poor specificity that led to many false-positive results. The discovery that microvesicles shed by cancer cells are released in bodily fluids has paved the way to a paradigm shift in biomarker detection. The ability to target and study these nanovesicles using designed synthetic peptides is valuable in helping clinicians and patients with treatment decisions and improving the quality of personalized medicine.

Current status of cancer biomarkers

The use of biomarkers as a method to detect and quantify variations in biological processes has shown great promise, providing the ability to evaluate a biological condition at an early stage. Many biomarkers have provided clinicians with the tool to aid in the detection, diagnosis, prognosis, and treatment, as well as the response to treatment. Currently, efforts are focused on investigating a range of cancer biomarkers to improve the understanding of cancer development, increase therapeutic options, and enhance the efficacy of treatment for cancer patients. There are four main categories of biomarkers: (a) detection, (b) diagnostic, (c) prognostic and (d) predictive. Detection of biomarkers give insight on identifying the cancer, e.g. prostate-specific antigen, used to detect prostate cancer at an early stage. [1] Diagnostic biomarkers more readily describe the risk of a certain cancer, e.g. the presence of mutated breast cancer type 1 and type 2 susceptibility proteins

(BRCA1 and BRCA2). [1] Prognostic biomarkers provide critical information for long-term options. [2] While predictive biomarkers help outline the specifics of a cancer type to determine whether a patient will be susceptible to a certain form of treatment. [3]

Cancer cells show a variety of distinct genotypes, including point mutations, changes in the DNA content, translocations, and gene deletions. These alterations can be monitored with technology such as high throughput DNA sequencing and microarrays, and even subtle tumour changes can be observed. For example, the *adenomatous polyposis coli* (APC) gene, which usually suppresses cancer by preventing polyp formation, is shortened and nonfunctional in the majority of several subtypes of carcinoma patients, such as colorectal and esophageal adenocarcinoma. A high level of the hypermethylated APC gene in serum has been linked to mortality, and is useful as a biomarker to monitor progression and response to treatment. [4]

In advanced tumour stages, particular cells and macromolecules can be found in the blood stream, such as regulatory T-cells (T-regs) and the prostate specific antigen (PSA). T-regs regulate self-tolerance and control the innate and acquired immunity response. Increased T-reg activity is linked to low immune responses to tumour antigens, leading to tumour growth, and has been found in several cancer types, including lung, breast, and pancreatic. PSA is a protease found in healthy prostate epithelial cells and at elevated amounts in prostate disease states. [4]

As much promise as there has been in the current development of biomarkers using proteomics and genomics, problems still remain with these approaches. For example, there are several shortcomings with using PSA as a biomarker, including the decrease in sensitivity with aging and obesity. Monitoring DNA methylation and histone acetylation also provides a valuable but un-optimized tool to

follow cancer progression. Although recent advances in technology and the understanding of cancer have paved the way for a myriad of biomarkers to be developed, there is still an unmet need for reliable biomarkers that could potentially allow for the identification and diagnosis of cancer before the onset of the earliest symptoms.

Microvesicles as biomarkers of cancer metastasis

Extracellular nano-sized particles called microvesicles ($d = 100 - 1000$ nm) and exosomes ($d = 30 - 100$ nm) are highly curved lipid vesicles that bud from the plasma membrane (microvesicles) or are released by multivesicular bodies from the cytoplasm into the extracellular matrix (exosomes). These extracellular vesicles, collectively referred to as microvesicles, are shed into bodily fluids (e.g. urine, blood, ascitic tissue), where studies report this stimulation as an effect of proteins altering membrane curvature or lipid asymmetry. In fact, this lipid de-regulation is suggested to cause the externalization of phosphatidylserine on the outer leaflet of the plasma membrane. Although microvesicles are known to shed from normal cells under physiological conditions, an overexpression of this shedding has been observed in those with cancer. Recently, the shedding of microvesicles has been directly correlated to cancer metastasis in B16 mouse melanoma cells. Their primary functions describe their impact on cancer progression, i.e. evading immune responses, targeting distal parts of the body, promoting angiogenesis necessary for tumour survival and growth, and transferring oncogenic receptors and proteins through the body. [5]

The particle size and the exposure of phosphatidylserine on the vesicle membrane surface are the two properties that distinguish microvesicles from

normal cells, potentially providing a strategy for specific targeting. No longer considered an artifact, these microvesicles have the potential to function as a biomarker, paving the way to identify peptide probes for the detection of metastatic behaviour-pivotal due to the high incidences of cancer metastasis-related deaths. Since these nano-sized lipid vesicles are ultramicroscopic, there is a need for new technology to detect these potential metastatic cancer biomarkers. Flow cytometry, electron microscopy and dynamic light scattering all have instrument limitations that hinder their capability for accurate vesicle counting on the nanoscale. [6] Our approach is to design peptides with a conjugated fluorophore to specifically target these vesicles using different techniques, which are performed to identify peptide-bound lipid vesicles. We focus on using small peptides to select for nano-sized lipid vesicles due to their feasibility and minimally invasive properties.

The transmembrane protein Synaptotagmin-I (Syt1; PDB: IUOW) was

selected as inspiration for our peptide design. Loops 1 and 3 of Syt1 are reported to insert into the lipid membrane bilayers, suggesting that membrane insertion is critical in membrane-sensing behaviour. This function is facilitated by electrostatic interactions between the acidic lipid head groups and the conserved basic residues of loop 3, which are driven by the formation of a Ca^{2+} complex. We took advantage of nature's lead and selected a 10-residue sequence derived from Syt1 loop 3 because it is a major contributor to membrane curvature sensing. To enhance rigidity, which may influence membrane binding, the Syt1 peptide was cyclized [Figure 1].

The Syt1 peptide binding to highly curved vesicles has been observed through *in vitro* fluorescence assays using synthetic lipid vesicles composed of 8:2 palmitoyl oleoyl phosphatidylcholine (POPC) and palmitoyl oleoyl phosphatidylserine (POPS). A fluorescence enhancement assay is described as the following: an increase in fluorescence intensity upon peptide-lipid binding correlating to a change in the

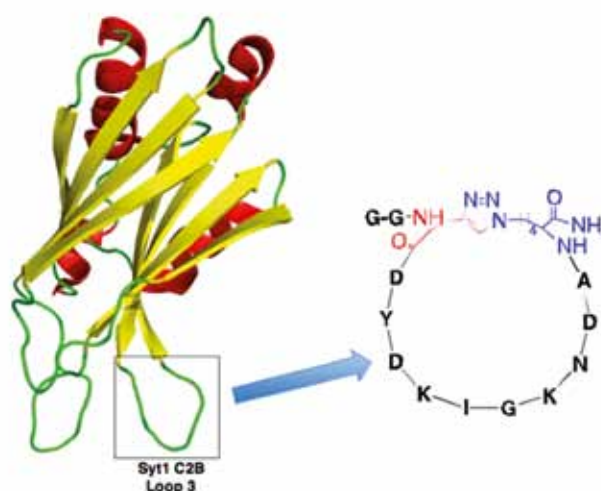


Figure 1. Pictorial view of the Synaptotagmin I protein (Syt1) with Loop 3 (boxed) and the peptide C2BL3C, depicting the inspiration behind the design of the curvature sensing cyclic peptide. Peptide cyclization on solid support was performed using 'Click' chemistry.

environment of the fluorophore; from a polar, aqueous solvent to a hydrophobic lipid vesicle; thus, fluorescence enhancement relative to the untreated peptide indicated peptide-lipid interaction. The relative fluorescence enhancement was observed to be comparable to both known curvature-sensing proteins: Syt1 and the ALPS motif-bearing Golgi-microtubule-associated protein-210. By performing a fluorescence anisotropy titration, the Syt1 peptide bound stronger to smaller vesicles independent of calcium. This observation was expected since the cyclic peptide was derived from only one loop of the C2B domain, where both loops are necessary to form the calcium complex. [7]

An *ex vivo* microscopic assay also showed the Syt1 peptide preferentially binding to highly curved microvesicles using nanoparticle-tracking analysis (NTA); a technique used as a proof-of-concept for curvature selection. NTA

is a microscope that analyses nanoparticles in real-time and measures their sizes using Brownian motion, independent of the nanoparticle refractive index and density. The Syt1 peptide was observed to preferentially bind to vesicles with diameter range of 30 – 75 nm (e.g. exosomes derived from a stressed rat model) to larger particles [Figure 2]. These results showed the Syt1 peptide preferably targeting highly curved vesicles perhaps due to the insertion in lipid packing defects exposed on the membrane surface, facilitating membrane stabilization by electrostatic interactions. These findings help contribute to the optimization and design of identifying peptides that could translate their curvature-sensing behaviour to select for highly curved, shedding microvesicles.

Future prospects

We have demonstrated the ability to detect highly curved lipid vesicles using designed synthetic peptides.

Motifs from other curvature-sensing proteins will be investigated to improve the selection for nanovesicles. Combining these motifs in identifying, designing, and optimizing peptides will enhance our discovery for generating probes to select for highly curved vesicles. These motifs include insertion moieties, scaffolding, and electrostatic stabilization. The proteins that are of high priority for us are those that contain the Bin-Amphiphysin-Rvs (BAR) domain because it has both the insertion and scaffolding motifs; combined features that hold big promise for designing curvature-sensing peptides. Further development of such technology holds great promise for new biomarker detection methods beyond the traditional proteomic and genomic approach.

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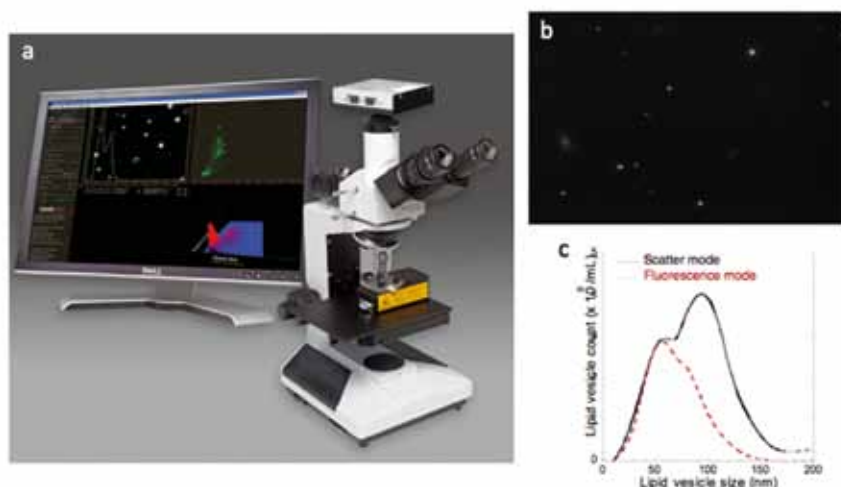


Figure 2. Detection of peptide-tagged lipid nanovesicles by nanoparticle tracking analysis (NTA). a) Nanosight NTA LM10-HS courtesy of Nanosight Ltd., Amesbury, UK, b) screen shot of heterogeneous lipid vesicles viewed under fluorescence mode after treatment with fluorescent C2BL3C, and c) plot showing the size distribution of lipid vesicles viewed under scatter mode (solid black line) and the sizes that are preferred and labeled by C2BL3C as viewed under fluorescence mode (broken red line).