Enhanced biomarker capture performance by a novel molecular matrix ("Molecular Net") for liquid biopsy applications can be explained by the Turing effect

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INTRODUCTION

Liquid Biopsy for Cancer Detection

Although tumor tissue is currently the gold standard source for clinical molecular analyses, material shed from cancers into the bloodstream and other body fluids is showing promise to overcome the limitations of physical tissue biopsy. Molecular and cellular traces of the presence of tumors exist in biofluids [1], and may include nucleic acids, proteins, exosomes, lipids, and circulating tumor cells [2]. "Liquid biopsy" is the term used to describe diagnostic procedures performed on cancer-derived material captured in a blood or other body fluid sample [3]. The starting point for liquid biopsy analysis is the capture of tumor-derived material, for downstream analysis.

Over the past few years, the discovery of these molecules, cells and particles have shown the potential for changing the practice of solid tumor biopsy to liquid biopsy. Whilst the technology to increase the sensitivity of measurement of these typically rare components has increased significantly [4], many of the capture techniques (e.g. ultracentrifugation for exosome isolation [5]) have not progressed beyond the research laboratory.

If the limitations of the liquid biopsy approach can be overcome, particularly in the area of biomarker capture, it holds clear advantages over "traditional" tissue biopsies. These include:

- Collecting a biofluid sample is minimally invasive and avoids the complications of invasive tissue biopsies
- Less invasive sampling allows repeatable and consistent assessment throughout the course of therapy
- Liquid biopsy uses fresh tumor-derived material, unaffected by the need for preservatives
- The sample is more likely to provide the complete genomic and proteomic profile of the tumor because it is not dependent upon accurately localizing and biopsying the tumor(s) within the non-tumor tissue [6]
- Liquid biopsy's potential clinical applications are broad and span the patient's entire cancer journey — from screening asymptomatic individuals to assist the early detection of cancer, to monitoring of treatment outcomes, and profiling resistance to targeted therapies in late-stage cancer treatment [7].

Magnetic beads coated with capture molecules such as antibodies or streptavidin are recognised as key tools in biomarker isolation for liquid biopsy [8], however they have primarily been used for single biomarker capture, and not multivariate biomarkers. The surface area of the bead limits the number and type of capture molecules that can be applied to each bead. This is seen as a limitation to the development of magnetic beads as components of multivariate assays [8].

A number of methods have been used in an attempt to enhance the binding capacity of beads. These methods include modifying the chemical functionalization of the bead surface, varying the bead size and physically compressing the bead [9].

Here we present a novel approach for producing a high capacity biomarker capture bead that comprises a multilayered three dimensional matrix that contains an enhanced surface area as well as structural pores that effectively combines ligand capture and size exclusion properties that can be applied to isolate any cancer-derived targets in a scalable, rapid and cost-effective method. This can be used as the sample preparation for a diagnostic liquid biopsy assay in high throughput commercial and hospital pathology laboratories.

Molecular Nets – A novel biomarker capture technology

In order to overcome the limitation of single layers of antibody molecules coated onto magnetic beads that are standardly used for biomarker capture, one of us (ES) designed and developed a covalently-linked multilayered three-dimensional matrix comprising one or more capture molecules (initially antibodies, but we soon realised this could be extended to other biomarker capture molecules), with linkers to bind the capture molecules together and spacers to significantly enhance the surface area available for ligand binding for specific and sensitive analyte capture, enrichment, purification, detection and measurement from a biofluid sample.

Examples of capture molecules that can be incorporated into the matrix include antibodies, nucleic acid sequences, enzymes, proteins and peptides. Linker molecules can be homo-bifunctional, hetero-bifunctional, trifunctional and multifunctional types. Examples of spacer molecules include polyethylene glycol, polymers, nucleic acids, albumin, Fc regions of antibodies and peptides.
Variables in assembling the matrix include the type and number of capture molecules, the chemical

properties, size and number of the spacer and linker molecules, the order in which the spacer and linker molecules are used and the ratio of spacer molecules to linker molecules and capture molecules. The use of specific capture molecules, linkers and spacers in a Molecular Net determines its binding and conformational properties, and its surface structure and form.

We found that concentration, placement and spacing of the capture molecules, linker molecules and spacer molecules confer a characteristic topology on the Molecular Net surface, a characteristic density within each layer, and a characteristic porosity. Types of porosities that can be generated by varying these conditions include nanopores, micropores, sieving pores and pockets. Porosity is influenced both by the choice of, and method of incorporation of, specific capture molecules, linker molecules and spacer molecules in each layer of the Molecular Net.

A diagrammatic representation of the interacting molecular components is shown at Figure 1, with antibody molecules as the capture molecules.

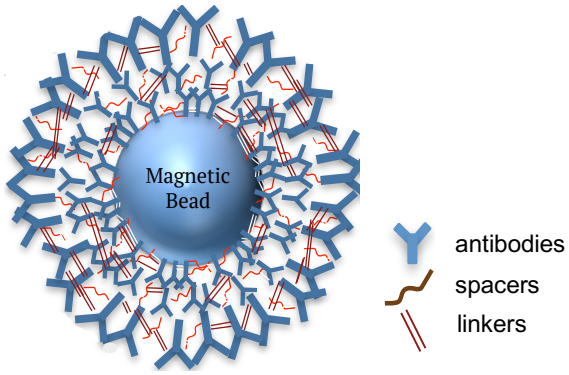

Figure 1. A diagrammatic depiction of the molecular composition of the Molecular Net matrix coated on a magnetic bead. The matrix consists of ligands (represented as antibody molecules **Y***), linkers and spacers.*

Scanning electron micrographs of two different forms of the Nets arising from different component incorporation coated on spherical magnetic beads are shown at Figure 2.

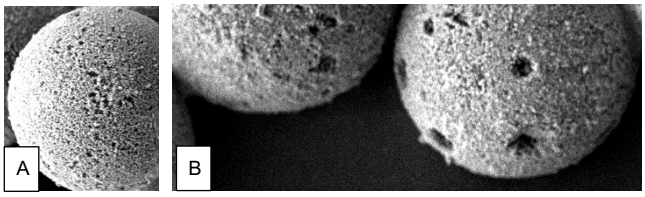

Figure 2. Scanning electron micrographs of magnetic beads coated with two different Molecular Nets, demonstrating that different molecules provide different surface patterns and porosities. A. A Molecular Net composed of streptavidin for the capture of diverse biotinylated biomolecules. B. A Molecular Net (EXO-NET™) composed of antibodies to a range of exosome-associated proteins as ligands.

Molecular Nets demonstrate superior capture ability in a range of biological applications. They have been used to capture viruses, bacteria and DNA from body fluids (data not shown). One application (exosome capture using EXO-NET™) is shown at Figure 3.

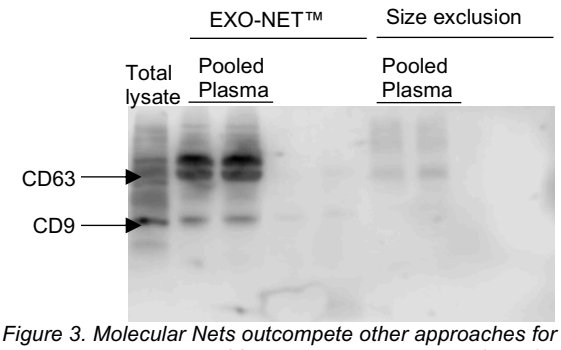

Figure 3. Molecular Nets outcompete other approaches for exosome capture. Most exosomes contain the transmembrane proteins CD9 and CD63. EXO-NETs are composed of several exosome-specific antibodies along with spacers and linkers coated onto a magnetic bead. The Western blot shows that significantly more exosomes were isolated from pooled human plasma samples using a 15 minute incubation with EXO-NET beads compared to a commercial exosome isolation kit based on size exclusion.

In addition to demonstrating superior performance compared to commercial products, the exosome capture Molecular Net demonstrated outperformance against ultra-centrifugation (the recognized research gold-standard method), in a significantly reduced time (15 minutes versus 6-12 hours).

Molecular Nets based on the Turing effect

The porosity and surface topology of a Molecular Net may be random, random or irregularly interspersed, depending upon the molecular components. The Molecular Nets were designed in accordance with the considerations to generate the Turing effect for biological pattern formation in order to enhance their efficacy.

In a seminal paper, the British mathematician Alan Turing outlined a new theory by which pattern formation might arise from the dynamic interplay between reaction and diffusion in a chemical system [10]. Turing hypothesized that the patterns observed during embryonic development arise in response to a spatial pre-patterning arising from biochemical molecular interactions. To generate these pre-patterns, Turing considered the molecules (or 'morphogens') to be interacting in such a way that, in a well-mixed system, there would be a spatially uniform steady state which was stable to perturbations. He then proposed that, in a non-mixed system, this steady state would be driven unstable by diffusion. This is counterintuitive, as diffusion is usually a stabilizing and a homogenizing process. In effect, what Turing showed was that from the interaction of two stabilizing processes (activation and inhibition), an instability could emerge by perturbing a homogeneous stable fixed point, via an activator-inhibitor mechanism. As the perturbation grows, he proposed that non-linear reactions

balance the diffusion terms, yielding an asymptotic, spatially inhomogeneous, steady state. The ability of a biochemical system to generate nascent Turing patterns is therefore derived from reaction-diffusion interactions that can be described by equations. Turing's equation took the form:

$$\frac{\delta u}{\delta t} = D\nabla^2 u + f(u),$$

where u is a vector of chemical concentrations, D is a matrix of constant diffusion coefficients (usually diagonal) and $f(u)$ the reaction kinetics (typically nonlinear) [10]. Appropriate boundary and initial conditions, which are often periodic, and perturbations of the homogeneous steady state, respectively, are applied to close the system [11].

This 'Turing instability' thus constitutes a universal mechanism for the spontaneous generation of spatially organised patterns. It formally applies to a wide category of phenomena, which can be modelled via reaction–diffusion schemes. These mathematical models describe the coupled evolution of spatially distributed species, driven by microscopic reactions and freely diffusing within the immersive medium. Diffusion can potentially seed the instability by perturbing the homogeneous state, resulting in the emergence of a patchy, spatially inhomogeneous, density distribution [12]. The Turing mechanism thus outlines how the amplification of resonant frequencies in reaction-diffusion systems can result in the formation of chemical standing waves, commonly known as Turing patterns.

Gierer and Meinhardt suggested that Turing patterns could be generated by a system composed of just two diffusing chemical species: a short-range activator (A) and a long-range inhibitor (I) [13]. In this system, an activator molecule activates both itself and the inhibitor molecule, whereas the inhibitor downregulates both itself and the activator.

The dynamic relationship between activators (A) and inhibitors (I) is shown schematically in Figure 4. These activator-inhibitor networks can be almost universally used to explain biological patterns [13].

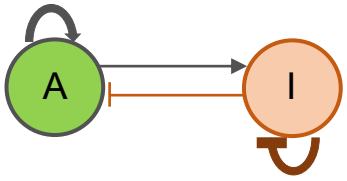

Figure 4. The AI system adapted from Gierer and Meinhardt (1972). Activators (A) upregulate both themselves and inhibitors (I), whereas inhibitors downregulate both themselves and activators.

Turing proposed that cells respond to this pre-patterning by differentiating in a threshold-dependent way. Thus, Turing hypothesized that the patterns seen in nature, such as pigmentation in animals, branching in trees and skeletal structures, are reflections of inhomogeneities in underlying biochemical signalling. Scholes *et al.* described it thus: "Turing patterns generally alter local concentrations of biochemical components, resulting in self-organized spatial patterns such as spots, stripes and labyrinths" [14].

Translating this concept to the Molecular Nets, it can be readily seen that linkers represent the activators and spacers the inhibitors, and the labyrinths of Scholes et al are the pores in the Molecular Net matrix.

The tension between the spacers and linkers in the Net, in which the capture molecules are passive bystanders, sets up the instability required to generate inhomogeneities within the matrix as it forms a network. This then allows the matrix to form a Turing-like instability, with resulting specific patterns and pores.

The influence of solid surfaces on the Turing Effect

Until now, the discussion has focused on the interactions of biochemicals diffusing in an unbounded (liquid) system, but this does not apply to the Molecular Nets when they are coated onto a solid matrix such as a magnetic bead. What happens when the matrix mixture is applied to the solid surface of a spherical magnetic bead, constraining one dimension of the forming matrix? Does the Turing effect still apply? Turing himself proposed the integration of mechanical aspects in pattern formation, but restricted his own studies to purely chemical processes, since "…the interdependence of the chemical and mechanical data adds enormously to the difficulty" [11]. When placed on a regular lattice or on a continuous spatial support the reacting chemical species can only diffuse along certain allowed routes. This has been seen as inhibiting the formation of Turing instability, and therefore likely to inhibit the pattern formation.

However, Asllani *et al.* determined that topology driven instabilities (resembling a Turing instability) can develop also even when the system cannot produce a Turing-like instability, due to it being on a symmetric or continuous spatial support [12]. In fact, they argued that different patterns can be generated depending on the characteristics of the spatial support on which the reaction–diffusion system is defined. In particular, transitions from travelling waves to asymptotically stationary stable patterns, reminiscent of the Turing instability, can occur. Thus the choice of support for the Molecular Nets may alter the binding characteristics of the Nets. This is supported by the work of Brinkmann *et al.* who demonstrated through modelling that even simple interactions between underlying tissue mechanics and chemistry can spontaneously lead to robust and complex mechanochemical patterns [15]. Citing previous studies, they proposed that mechanical patterns are not merely passive results of chemical

pre-patterns, but instead play a central and active role in tissue and other biological pattern formation. The observation that mechanical cues can influence and control chemical patterns, has led to the rapidly evolving study of mechanotransduction in cell biology.

Different mechanical cues such as curvature, stretch, strain, or compression have been theoretically shown to work as long-range inhibitors in spontaneous pattern formation. Importantly, there is also increasing experimental support for mechano-chemical interactions as an important driving force in biological patterning.

Multiplex networks in layers, whose mutual connections are between twin nodes, have been used to model complex networks on a solid surface [12], such as the Molecular Nets on spherical magnetic beads.

In a system of two chemical species, the conditions on the dispersion relation imply the existence of exactly two possible patterning network types: the AI network, and the activator-substrate (AS) network [16]. When systems of three or more species are considered, the variety of possible Turing networks increases dramatically [17]. The analytical complexity of these larger networks meant that little was known about them until all possible types of three and four species Turing networks were enumerated in a computational study by Marcon *et al.* in 2016 [17]. This study revealed that several widely-held assumptions about the kinds of systems which can generate Turing patterns (such as the requirement for diffusing species to have different diffusion coefficients) in fact applied only to two species systems, and implied that the set of potential Turing patterning systems is much more diverse than is commonly supposed. An understanding of Turing patterning beyond the two-species case is essential to understand the patterns formed by the Molecular Nets, as they typically consist of three or more chemical entities in several layers upon a solid substrate.

It could be argued that the Molecular Nets could not form Turing instabilities as not only do they contain multiple species on a solid surface but they do not satisfy all the conditions for reaction-diffusion systems. However, Smith and Dalchau state that there exist systems which violate several of the conditions required for Turing instabilities, but nonetheless still generate stable patterns [16]. The question of which systems will ultimately form stable patterns is a highly non-linear one, and “generalised Turing systems" can arise from a greater variety of conditions than have previously been thought including two-species activator-activator (AA) networks, and networks consisting of a long-range activator and short-range inhibitor [15].

Conclusion

We propose that Molecular Nets comprising species of biochemicals linked together by a variety of linking molecules and kept apart by spacer molecules, work to capture biological molecules from biofluids more efficiently than other currently used bead-based capture methods by virtue of the fact that they form Turing patterns that mimic biological patterns and structures. These patterns and structures are much more efficient at biological interactions than other synthetic, two dimensional systems, such as antibody-coated beads, that do not form Turing patterns. This potentially explains their superior specificity, efficiency and affinity.

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