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Section I: Emerging Strategies in Cancer Nanotechnology

Early-to-Late Stage Diagnosis: Nanotechnology-Based Interventions

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Introduction

The best chance of winning the war against cancer is to detect the disease at its earliest possible stages prior to there being increased cellular heterogeneity and physical spread of cancer cells from the primary site of origin. Finding cancer early is particularly challenging, as there are fewer numbers of cancer cells, and therefore lower concentrations of biomarkers at the cancer site and in bodily fluids, at an early stage along the natural progression path of the cancer. Furthermore, since most cancers are detected relatively late we often lack the ability to ideally characterize the true properties of early cancers, which are likely quite different than late cancers. Simply put, as there are more cancer cells present in advanced stage disease, in a similar fashion there are likely to be more changes in the genome, epigenome, proteome, and transcriptome when characterized *ex vivo*, as well as more protein targets for molecular imaging probes *in vivo*. All of these challenges can ideally be addressed by nanotechnology-based medical diagnostics as part of the *Nanomedicine* field. For its part, *Nanomedicine* promises unprecedented innovations for early diagnosis, staging, and therapy. It offers capabilities to perform simultaneous cancer detection and treatment in ways unachievable with other strategies. For example, nanotechnology has the potential to greatly impact *in vivo* diagnostics through molecular imaging for early cancer detection, even if, this approach must first be validated through the more tractable problem of impacting the management of later stage cancers. With its capacity to provide enormous sensitivity, multiplexing, throughput, and flexibility, nanotechnology has the potential to profoundly impact cancer patient management in the upcoming years.

Surgery is still the mainstay in medical management for both early and late stage cancers. Preoperative molecular diagnostic screening using both *in vitro* nano-enabled diagnostics tools and nanoimaging can detect and localize the tumor, exclude the patients who have metastasized beyond eligibility for a resection, identify the molecular signatures which can

be used to guide surgical procedure, screen the suitable cases whose biology is surgically most relevant, and orientate the surgeons to enable surgery planning.

Nanotechnology offers many other benefits for cancer early to late stage detection such as detailed single molecule and single cell analysis possibilities instead of 'bulk' measurements (**Figure 1**). Nanotechnology offers: (1) analytical sensitivity, (2) massive biomarker/analyte multiplexing ability, (3) low clinical sample volume operability, (4) capability to continuously monitor health and detect any deviation from it via implantable sensors, (5) capability for simultaneous cancer detection and therapy (theranostics), (6) solutions to visualize oncologic pathogenesis and its response to medical intervention in animal models via intravital fluorescence imaging, bioluminescence, and magnetic resonance imaging (MRI) and finally (7) cost benefits to the patients and the healthcare system at large.

Current Trends in Nanotechnology-Based Intervention for Early to Late Stage Diagnosis

A myriad of preclinical research grade nanobiosensors have already been developed, however, the ultimate goal of multiplexed, low-cost, high-throughput, reliable diagnostic devices for the clinic has yet to be fully realized. Having this capability in the clinic would undoubtedly allow for the improved detection of cancer with potential significant benefits to patients and the health-care system at large.

Often the vast majority of long-term cancer survivors have resectable tumors seemingly confined to the primary site at the onset of diagnosis and hence, they can benefit significantly from curative surgery, supporting that early cancer detection and intervention will increase the overall survival of patients. From a technological perspective, we have great nano-centric tools within our arsenal; disappointingly there are currently no reliable serum biomarkers with the sensitivity and specificity to accurately detect early pre-cancerous lesions. In many ways our technologies are ahead of our understanding of the underlying cancer biology. Furthermore, the heterogeneous nature of cancer and the inherently

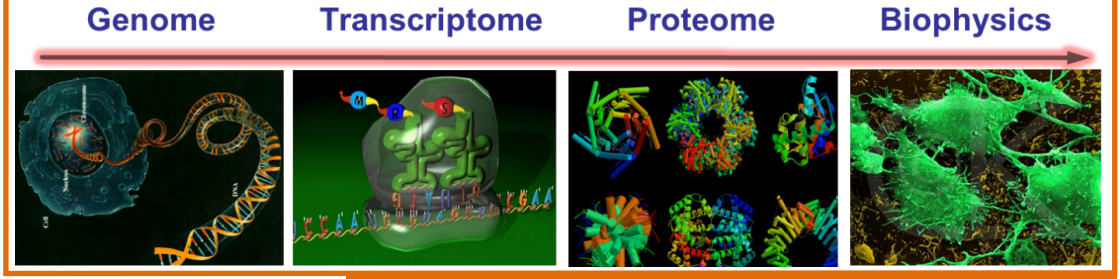

Figure 1. Nanotechnologies for comprehensive cancer cell analysis, ideally at single cell and single molecule sensitivity levels.

complex stromal microenvironment also present a challenge for identification of potential biomarkers. Hence, early diagnosis of tumors requires the simultaneous use of a panel of biomarkers for greater accuracy. In a recent mathematical modeling study[1] it was found that a tumor could grow unnoticed for more than 10 years and reach a spherical diameter of about 25 mm, before becoming detectable by current clinical blood assays. Further complicating it, the shedding rates of most current clinical blood biomarkers are found to be 10^4-fold too low to enable detection of a developing tumor within the first decade of tumor growth. These predictions well-align with clinical observations. Thus, currently there are no biomarkers suitable for screening of healthy general populations for possible occurrence of precancerous events. Routine surveillance of cancer is currently performed through classical cancer detection technologies, such as x-ray imaging based mammography for breast cancer, visible light colonoscopy for colorectal cancer, histo-pathological evaluation of Pap smears for uterine and genital cancers, and skin lesions by microscopic pathology, etc., none of which are presently enabled via nanotechnology. Currently, several preclinical diagnostic imaging tools are going through evaluation for their suitability as adjunctive technologies to the existing contemporary cancer diagnostic approaches. Some of these technologies are magnetic nanoparticle or gadolinium chelate-functionalized nanoparticle-enabled for high resolution MRI[2–4], nanoparticle and intrinsic contrast-based photoacoustic imaging[5,6], surface enhanced Raman spectroscopy-based endoscopy[7], cancer triggered self-assembling smart optical and MRI nanoimaging agents[8–10], micro-Nuclear Magnetic Resonance imaging[11], dual (*e.g.*, PET-Near Infrared fluorescence and PET-MRI)[12,13] and nano-enabled triple modality imaging (*e.g.*, MRI-Photoacosutics and Raman)[14]. A recent review summarizes the status of nanoimaging agents and the clinical trials associated with these approaches[15].

Currently, in the field of cancer nanotechnology-focused diagnostics, two very broad groups of devices and tools are emerging and there is strong and ongoing research in both. These groups are (1) benchtop or larger scale medical diagnostic devices and (2) miniaturized nano-based or nano-enabled diagnostic assays/devices that are designed and suitable for point-of-care or for patient's use at home directly or suitable for implantable, wearable, ingestible, inhalable uses. The medical expectations from the first group of devices is that they will be extremely robust, sensitive and specific as such they are suitable for confirmatory decision making that can both inform and guide clinical management of cancer. Nanoparticle-based imaging agents (*e.g.*, paramagnetic iron oxide or gold or silica-based nanoparticles, carbon nanotubes, surface enhanced Raman nanoparticles, etc.) and their associated detection/analysis instrumentation and nanoimaging devices (*e.g.*, nanoparticle assisted MRI, photoacoustic imaging, Raman spectroscopy) are examples of this category. On the other hand, the second group of cancer nanodiagnostic tools includes: nanocantilever, nanopore, nanowire, quantum dot, plasmonic nanoparticle-enabled micro/nanofluidic

devices, among many others. The medical expectations from these second group of point-of-care devices is that they will be cheap, produce rapid and reliable results, often during the same office visit and yield actionable results for seeking further medical evaluation. The first category of nanodiagnostic tools that are typically more suitable for later stage cancer and the second category of diagnostic tools are more applicable to early stage detection of cancer, recurrence, therapeutic efficacy monitoring, as well as general surveillance. There is a continued cancer nanotechnology research need for the improvement of and innovation in both of these categories of the medical diagnostic tools, which are inherently synergistic in principle from a medical benefits perspective.

Even with the progress resulting from early detection, the long-term prognosis of cancer patients is still limited by the occurrence of distant secondary metastases via circulating tumor cells (CTCs). Clinically occult micrometastases caused by these cells cannot currently be detected at primary diagnosis even by high-resolution diagnostic imaging approaches. The presence of CTCs in blood and bone marrow has shown to have therapeutic and prognostic impact for cancer[16–20]. It is postulated that CTCs could escape from chemotherapy by maintaining a dormant non-proliferating cell state (senescence) until the conditions are optimal to start expansion to manifest metastases[21]. Thus, the detection, enumeration and characterization of CTCs and their clusters (*i.e.*, 'liquid biopsy') remains as a viable candidate to investigate its potential to increase survival benefit for cancer patients, in particular, due to its ease of access and amenability for repeat sampling. A multitude of micro- to nano-scale technologies are now available to isolate an enrich CTCs[22,23], as well as highly sensitive and specific immunological and molecular assays[24,25] to characterize these cells at the single cell level in bone marrow and peripheral blood. These studies are providing insights into the critical steps of the initiation of the metastatic cascade.

...CDs offer significant potential as replacements for toxic metal-based quantum dots that have had difficulty with clinical translation.

Similar to CTC capture and characterization, extracellular vesicles released/secreted by cancer cells and loaded with cellular signals such as microRNAs and proteins, are emerging as important oncologic clues that can be obtained from clinical cancer samples (reviewed in Zocco *et al* 2014 and Webber *et al* 2015)[26,27]. The nondestructive isolation, enrichment, enumeration and intra-vesicular content analyses of these particles via the use of nanotechnology, such as nano-mechanical filters[28,29], nanoflare-based diagnostics (reviewed in Heuer *et al*, 2013, Prigodich *et al* 2012)[30,31], nanoproteomics analysis[32], bio-barcode-based analysis (reviewed in Pritchard, *et al* 2012)[33] are emerging as important

tool for cancer diagnosis, response to therapy and for prognostic surveillance. This field is currently expanding and it is expected to play a major role in cancer medical management in the near future.

Luminescent carbon dots (CDs) are emerging as new medical diagnostic tools as alternatives to quantum dots and other carbon-based nanomaterials such carbon nano tubes and graphene. These nanoparticles have well-defined, tunable surface functionalities, and their manufacture involves simple, fast, and cheap synthetic routes. Because of good biocompatibility, hydrophilicity, non-toxicity, resistance to photobleaching and -blinking, CDs offer significant potential as replacements for toxic metal-based quantum dots that have had difficulty with clinical translation.

Another novel development in the cancer nanotechnology field is the use of mass-encoded synthetic biomarker libraries for multiplexed monitoring of cancer in bodily fluids[34]. These exogenously administered 'synthetic biomarkers' are composed of mass-encoded tandem peptides conjugated onto nanoworm nanoparticles that leverage the intrinsic features of human disease and physiology for noninvasive urinary monitoring. These protease-cleavable peptide-based cancer sensors can target sites of disease, sample dysregulated protease activity and emit mass-encoded reporters into patient urine for multiplexed detection by mass spectrometry. It was shown that these agents can noninvasively monitor disease without the need for invasive core biopsies and the respective blood biomarkers.

The Future of Nanotechnology-Based Intervention for Early-to-Late Stage Diagnosis

Nanoscience applied to cancer research is proving to be a critical and encouraging approach for the eventual elimination or at least chronic control of cancer. Nanotechnology has been making a significant impact on cancer diagnosis and therapeutic management in revolutionary ways as exemplified in the NCI's 2010 Cancer Nanotechnology Plan (http://nano.cancer.gov/about/plan). Nanotechnology will continue to advance both *in vitro* diagnostics through genomic, cellomic, transcriptomic, proteomic and circulating tumor cell enumeration as well as exosome and microRNA analysis based nanosensors and for *in vivo* diagnostics via nanoparticles for molecular imaging. Moreover, *in vitro* diagnostics used in conjunction with *in vivo* molecular imaging is expected to markedly impact future cancer patient management by providing a synergy that neither strategy alone can offer. Indeed, the areas of earlier cancer detection and the prediction and monitoring of patient response to anti-cancer therapies could be impacted by this synergetic approach. Both represent very important applications for nano-enabled diagnostics with near-term clinical translational potential.

Specifically, the earlier detection of relevant cancers that are aggressive is still a major challenge for the cancer community. Earlier intervention of potentially aggressive cancers can greatly improve patient survival, quality of life and financial outcomes. These could be achieved via the synergistic use of highly sensitive and specific *in vitro* diagnostic devices to interrogate easily accessible clinical sample sources such as blood, urine, feces, sweat, tears, and saliva for multiple biomarkers (both protein and nucleic acid-based) and verify the presence and location of the tumor with nano-/molecular imaging *in vivo* using novel nanoparticles that allow signal amplification and multiplexing. As example, a cancer patient has cancer detected at much earlier stage through use of biomarkers derived from blood or other non-invasive samples and results from these *in vitro* tests are then verified by molecular imaging that simultaneously localizes tumor(s) prior to treatment. Additionally, post-treatment and potentially during treatment, the patients' response to therapy is measured to ensure the accurate differentiation of responders from non-responders can, which could be continually evaluated by blood analysis, without necessitating another tumor biopsy and/or molecular imaging.

The application of the above two approaches (combination of *in vitro* diagnostics with nanoimaging and the combination of *in vitro* diagnostics with benchtop ultrasensitive, specific nanodiagnostic technologies) in particular to the current unsolved oncologic challenges of detection of distant micrometastases, prognostic evaluation of tumor aggressiveness and its predicted response to a given therapy, differentiation of indolent tumors from the ones that have metastatic potential, tumor border demarcation during surgery are areas where there are significant gaps in our diagnostic abilities, hence, further and significant cancer nanotechnology efforts need to be spent on these critical areas to improve cancer patient outcomes within the next 5-15 years. Ideally, nanotechnology could make a huge impact in cancer by virtue of pre-emptive interventions to detect cancer early through continuous health monitoring via wearable, ingestible and implantable nanodiagnostics to detect deviation from health to pre-neoplastic conversion as early as possible. However, being able to get there will involve not only further nanotechnological advancements, but also, further improvements in the toxicological, biocompatibility and immunological concerns related to nanoparticles' use as cancer *in vivo* diagnostics. With appropriate level and timely financial commitments for nanoscience and nanotechnology research, the future of the *Cancer Nanotechnology* field is bright and full of opportunities as well as tremendous near-term rewards for patients.

Early-to-Late Stage Diagnosis: Detecting and Analyzing Circulating Tumor Cells

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Circulating Tumor Cells (CTC)

The tissue-based evaluation of biopsy samples remains the gold standard for diagnosis and prognosis in clinical care and research. The bulk of published research focuses on tissue samples obtained by surgical excision or radiographically directed needle extractions. While these approaches have driven a tremendous amount of research, they are complicated by several issues. First, these extractions are both invasive to the patient and costly overall. Typically, serial biopsies are avoided for fear of complications from the procedure, but are essential in obtaining dynamic insight. Second, in cancers where metastatic tissue biopsies are problematic, research has relied upon historic primary tissues. Third, there is growing focus and concern for the impact of the tumor tissue's temporospatial heterogeneity.

As a measure to address these problems, circulating tumor cells (CTCs) have been proposed as they provide a means to sampling tumors across all present disease sites (they are perfused systemically in blood), including the primary tumor and metastases[35]. In addition to conventional diagnostic imaging and serum marker detection in cancer, the detection and characterization of CTCs in patients over the course of therapy creates new possibilities for personalizing cancer care by: (i) monitoring cancer progression, (ii) understanding the pathogenic mechanisms driving lethal disease and the dynamics of this evolving biology, and iii) guiding the implementation of the most effective treatment interventions and re-strategizing upon the emergence of resistance. Over the last decade, significant progress has been made in the areas of CTC detection, isolation, and characterization that has largely been driven by collaborative and interdisciplinary research efforts spanning across chemistry, materials science, bioengineering, and oncology. Recent technological advances in the field of nanotechnology offer powerful microfluidic systems and unique nanomaterials, which will enable a diversity of in-depth characterizations of CTCs with drastically reduced costs and ultimately bring the field of oncology closer to the goal of personalized care.

Conventional CTC Assays

The most widely used CTC detection assays include: (i) Immunomagnetic separation: these methods utilize capture agent-labeled magnetic beads to either positively select CTCs using a cell surface marker (*e.g.*, anti-EpCAM) or negatively deplete white blood cells (WBCs) using anti-CD45. The CellSearch™ Assay is the only FDA-cleared CTC diagnostic technology for metastatic breast, prostate, and colorectal cancers[36]. CellSearch™ Assay harvests CTCs with anti-EpCAM-coated magnetic beads, and the subsequent immunocytochemistry (ICC) process helps to identify CTCs (DAPI+/cytokeratin, CK+/CD45-) from nonspecifically captured WBCs (DAPI+/CK-/CD45+). Recently, several new systems (*e.g.*, MagSweeper, IsoFlux, Cynvenio, magnetic sifters, VerIFAST and AdnaGen/Qiagen) have been developed to further improve detection speed and efficiency. (ii) Flow cytometry: In conjunction with the use of fluorescent markers, flow cytometry is one of the most mature technologies for analyzing and sorting subpopulations of cells. However, this flow-based methodology is unable to provide the CTCs' morphological information to meet the gold standard set by pathologists. An improved method, known as ensemble-decision aliquot ranking, was developed to address this weakness[37]. (iii) Microscopy imaging. Microscopy imaging of ICC-treated blood samples allows for highly sensitive detection of CTCs, accompanied with their morphometric characteristics and protein expression. Currently, Epic Sciences is one of the leaders in the commercial sector, now providing CLIA-certified laboratory tests for both CTC enumeration and characterization. In contrast to the previous three approaches, which require the use of CTC markers, the following two approaches are recognized as label-free methods. (iv) CTC filters: Filter-based approaches have been established to trap CTCs according to their sizes. A wide collection of commercial kits/systems from Rarecells, ScreenCell, Clearbridge, and Creatv MicroTech etc. are now available to support research utility. Nevertheless, concerns regarding overlooking small-sized CTCs have been raised. (v) Dielectrophoresis: CTCs can be sorted from WBCs in the presence of a dielectrophoretic field, since the CTC's dielectric properties (depending on their diameter, membrane area, density, conductivity and volume) are different from those of WBCs. ApoCell's technology leverages these differences in a microfluidic flow channel to isolate CTCs. Silicon Biosystems' DEPArrayTM combines the use of microscopy imaging and dielectrophoresis sorting to identify and isolate pre-sorted CTCs, paving the way for downstream single-CTC molecular characterizations. (vi) Other methods: There are several outstanding review articles where side-by-side comparisons of a wide collection of CTC detection technologies are presented[38,39].

Microfluidics-enabled CTC Assays

The microfluidic affinity-capture devices demonstrated by the Massachusetts General Hospital team kicked off the research efforts devoted to the development of

nanotechnology-enabled CTC assays[40]. Their 1st-generation (gen) device (*i.e.*, CTC-Chip) featured chemically etched microposts on a silicon substrate, on which anti-EpCAM antibodies were covalently functionalized. These embedded microposts maximize the contact between the device surfaces and the flow through cells. Following CTC capture, ICC was conducted to identify CTCs from background WBCs. The CTC-Chips demonstrated significantly more gains in CTC enumeration performance than most of the conventional CTC assays. Thereafter, similar device configurations were adapted to create new microfluidic chips (*e.g.*, geometrically enhanced differential immunocapture, GEDI approach and Biocept's CTC assay), where different antibody capture agents were employed. Recently, a unique "Ephesia" approach based on microposts of capture agent- coated magnetic beads self-assembled in a microchip demonstrated combined advantages of both microfluidic and immunomagnetic cell sorting[41]. The MGH's 2nd-gen device (*i.e.*, herringbone-chip, HB-Chip) was made from an imprinted PDMS component on a glass slide[42]. Microscale herringbone patterns were engineered into the PDMS component to introduce microvortices, leading to enhanced contact between the CTCs and the antibody- coated chip surfaces. In addition to the commonly used ICC technique, the transparent nature of the HB-Chip allowed for imaging of the captured CTCs by standard clinical histopathological stains (*i.e.*, H&E stain),. Although the microfluidic setting improves CTC- capture performance, the majority of the microfluidic CTC assays suffer from depth of field issues when performing microscopy imaging due to the vertical depths of 3-dimensional device features. Time-consuming multiple cross-sectional imaging scans that generate large image files are required in order to avoid out-of-focus or superimposed micrographs. By coupling a pair of microelectrodes at the terminal of a plastic microfluidic chip, enzymatic release of the captured CTCs can be electrically counted without the issue of microscopy imaging[43]. In contrast to MGH's 1st and 2nd-gen devices, their 3rd-gen iChip represents a groundbreaking label-free approach, which combines negative immunomagnetic depletion processes with an inertial focusing setting in an integrated microchip[44]. Most importantly, this approach allowed for the recovery of unmanipulated CTCs with desired molecular integrity and viability, paving the way for downstream expressional profiling[45], as well as *ex vivo* culture and drug susceptibility tests[46]. Other microfluidic CTC assays based on unique principles, including micro-nuclear magnetic resonance (μNMR) platform[47], cell rolling[48], and Vortex technology[49] have also been developed and demonstrated. In addition to the microfluidic assays developed for the enumeration, molecular characterization, and *ex vivo* expansion of CTCs, a microfluidic device with designated sections for selectively capturing CTCs according to the amount of magnetic beads grafted on their surfaces has been created[50]. The device was employed to dissect CTCs into subpopulations according to EpCAM expression levels of individual CTCs.

Nanomaterials-enabled CTC Assays

It has long been documented that nanoscale components present in the tissue microenvironment, including extracellular matrix and cell-surface structures provide structural and biochemical support that regulates cellular behaviors and fates. Inspired by the nanoscale interactions observed in the tissue microenvironment, the UCLA team pioneered a unique concept of "NanoVelcro" cell-affinity substrates in which CTC capture agent-coated nanostructured substrates were utilized to immobilize CTCs with high efficiency[52]. The working mechanism of NanoVelcro cell-affinity substrates mimics that of Velcro™ – when the two fabric strips of a Velcro fastener are pressed together, tangling between the hairy surfaces on two strips leads to strong affinity between cell and nanosubstrates. Through continuous evolution, 3 generations of NanoVelcro CTC Chips (**Figure 2**) have been established to achieve different clinical utilities. The 1st-gen NanoVelcro Chip, composed of a silicon nanowire substrate (SiNS) and an overlaid microfluidic chaotic mixer, was created for CTC enumeration. Side-by-side analytical validation studies using clinical blood samples suggested that the sensitivity of the 1st-gen NanoVelcro Chip outperforms that of FDA-approved CellSearch™. In addition to SiNS, the general applicability of the NanoVelcro cell-affinity assay is supported by extensive research endeavors devoted to exploiting different nanomaterials, *e.g.*, polymer dots/nanotubes, TiO2 nanowires/nanoparticles, layer-by-layer-assembled nanostructures, gold clusters on silicon nanowires, Fe3O4 nanoparticles, and graphene oxide nanosheets to achieve high affinity capture of CTCs and other types of rare cells[53]. It is worth noting that NanoVelcro-like approaches allow immobilization of CTCs onto a relatively flat and small surface area, thus allowing subsequent microscopic imaging/identification of CTCs to be conducted quickly. Moving beyond CTC enumeration, UCLA's 2nd-gen NanoVelcro Chip (*i.e.*, NanoVelcro-LMD) was developed by replacing SiNS with a transparent substrate covered with polymer nanofibers[54]. The transparent NanoVelcro substrate retains the desired CTC capture performance, and allows for seamless integration with a laser microdissection (LMD) technique to isolate immobilized CTCs with single-cell resolution. The individually isolated CTCs can be subjected to single-CTC genotyping (*e.g.*, Sanger sequencing and next- generation sequencing, NGS) to verify CTC's role as a tumor liquid biopsy. Most CTC enrichment and isolation methods yield purified CTCs that are either fixed before isolation, damaged during the cell purification process, or irreversibly immobilized on an adherent matrix. Similar to MGH team's iChip, UCLA's 3rd-gen Thermoresponsive NanoVelcro Chip has demonstrated the feasibility to capture and release CTCs at 37 and 4°C, respectively[55]. By grafting thermoresponsive polymer brushes onto SiNS, the temperature-dependent conformational changes of polymer brushes can effectively alter the accessibility of the capture agent on SiNS, allowing for rapid CTC purification with desired viability and molecular integrity. The team has been exploring

the use of Thermoresponsive NanoVelcro Chips to purify viable CTCs for downstream molecular and functional analyses.

Future Scientific and Clinical Developments

Moving forward, future research endeavors in developing the Nanotechnology-enabled CTC assays will be driven by the needs of: i) acquiring a fundamental understanding of the nanointerfaces between CTCs (*e.g.*, how the underlying physical/chemical

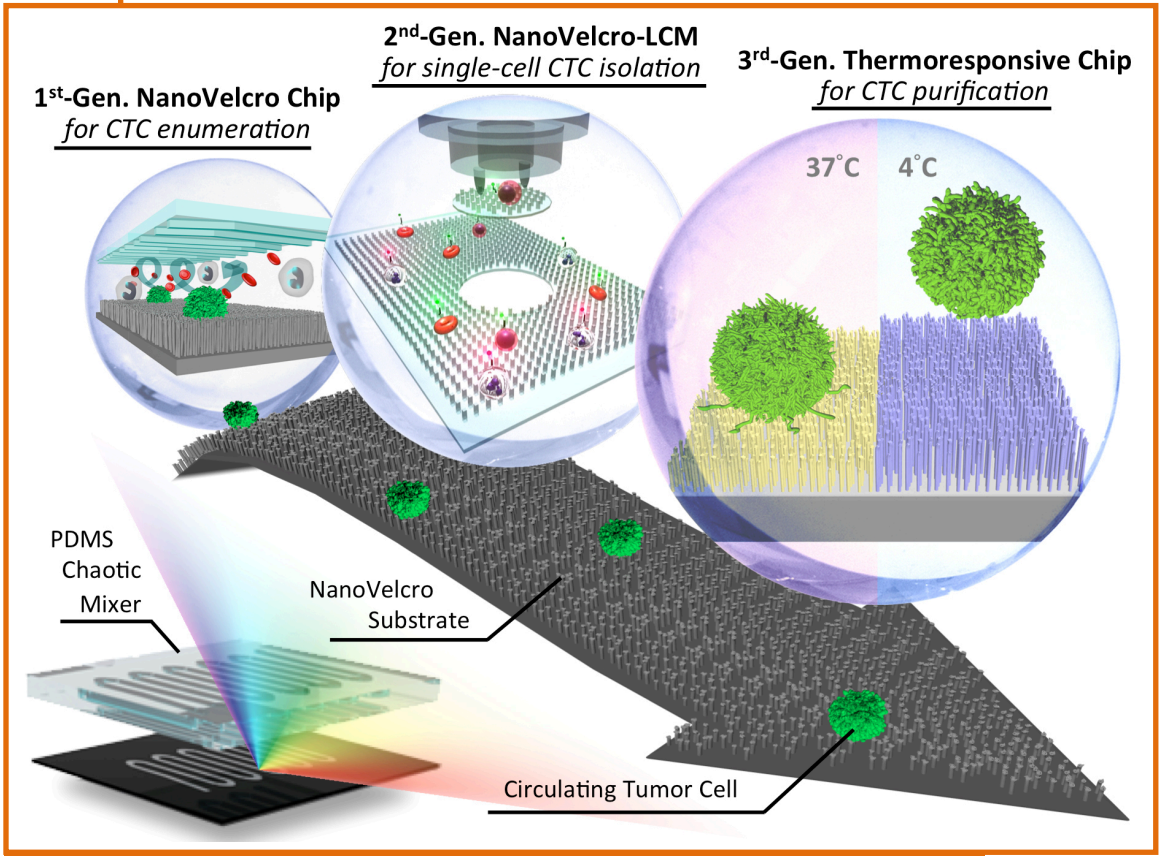

Figure 2. Conceptual illustration of the three generations of NanoVelcro CTC Assays developed by the UCLA team to achieve different clinical utilities. 1st-gen NanoVelcro Chip, composed of a silicon nanowire substrate (SiNS) and an overlaid microfluidic chaotic mixer, was created for CTC enumeration. In conjunction with the use of the laser microdissection (LMD) technique, 2nd-gen NanoVelcro-LMD technology, was developed for single-CTC isolation. The individually isolated CTCs can be subjected to single-CTC genotyping. By grafting thermoresponsive polymer brushes onto SiNS, 3rd-gen Thermoresponsive NanoVelcro CTC Chips were developed for purification of CTCs via capture and release of CTCs at 37 and 4°C, respectively. The surface-grafted polymer brushes were responsible for altering the accessibility of the capture agent on NanoVelcro substrates, allowing for rapid CTC purification with desired viability and molecular integrity. (*Reprinted with permission from Tseng et al, 2014*)[51]

properties of any given nanosubstrate affect their CTC-capture performance, as well as the viability and molecular integrity of captured CTCs); ii) developing new CTC-capture/release mechanisms governed by physiologically compatible stimulations for instant isolation/purification of CTCs with desired viability and molecular integrity in order to set the stage for conducting downstream *ex vivo* characterization, as well as molecular analysis; iii) exploiting a broad diversity of multi-omic analytical technologies (that could be from other research initiatives within NCI Nanotechnology Alliance Program) with single-cell resolution to characterize the heterogeneous CTC pool; iv) exploring the use of rare-cell culture techniques that will enable *ex vivo* expansion of purified CTCs for in-depth studies (*e.g.*, xerograph models and drug susceptibility tests); v) studying other types of circulating rare cells (*e.g.*, tumor associated macrophage and stromal cells) and non- cellular particles (*e.g.*, exosomes), which also carry information about the tumor microenvironment.

Following development of these technologic advances, challenges remain in utilizing these new assays to address unmet needs in the areas of cancer biology and, most importantly, clinical oncology. Research endeavors should be devoted to: i) performing multi-omic molecular characterizations on CTCs together with concurrent tumor tissues (including primary and metastatic sites if available) to establish CTC-tumor relationship that will become the foundation for using CTCs as liquid biopsy[35]. Consequently, CTCs can then be used as surrogate tumor tissue for providing relevant information to guide implementation of cancer treatment; ii) dissecting CTC subpopulations according to their distinct phenotypes (*e.g.*, molecular fingerprints, morphological characteristics, and behaviors) in order to address the issue of heterogeneity in tumor/CTC pool. For instance, a subpopulation of CTCs with defined small nuclei (*i.e.*, vsnCTCs) was discovered to strongly correlate with the presence of visceral metastasis in prostate cancer, offering a new way to detect the onset of the most lethal disease progression[56]; iii) conducting analyses on serial CTC samples through monitoring the dynamic change of CTC subpopulations and their multi-omic molecular signatures to better understand the evolution of cancer, which is currently limited by the difficulty of obtaining tumor tissues; iv) effectively generating and applying CTC-derived cell lines as well as xerograph models to better understand the oncogenic/resistant mechanism, and evaluate a wide range of treatment options that can poetically benefit individual patients. Validation in appropriately powered studies will be needed as these ideas translate directly into the clinical setting. Ultimately, the regulatory and commercial efforts will be required to bring these tools to the population at large.

Conclusion and Outlook

Early successes in the field of nanotechnology have shown great promise for addressing the existing unmet needs in clinical oncology. As the scientific understanding of the dynamic and complex biology of cancer evolves, it has become clear to clinical scientists and cancer biologists that characterizing this dynamic biology may add an important dimension to clinical data. Oncologists practicing cancer care in this evolving biologic environment are already accustomed to handling temporal variation of data. Monitoring the dynamic alterations of biological variables, which themselves follow a distinct and biologically relevant rhythm, is a fundamental part of clinical medicine. Given the limitations of performing serial biopsies or the limited data obtainable in single biomarker panels, to date, this type of dynamic characterization has been possible only in animal models or in limited biomarker panels. The promise that the analysis of CTCs and other circulating entities holds is in the ability to study the dynamic biology that bares the greatest relevance: that of the individual patient. In this era of molecular medicine that has brought us beyond the cell to the level of DNA, RNA, and proteins, it has become exceedingly clear that no two patients are identical and no two cancers are identical. Having a non-invasive means of dissecting these differences bridges the gap between the laboratory and the clinic. While these ideas are young, the successes seen in this field provide ample cause for continued work and fuel the enthusiasm for launching integrated transdisciplinary research in this transformative field.

The promise that the analisis of CTCs and other circulating entities holds is in the ability to study the dynamic biology that bares the greatest relevance: that of the individual patient.

Early-to-Late Stage Diagnosis: Nanoflares for Intracellular mRNA Detection

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Spherical nucleic acids (SNAs)[57] have recently emerged as a powerful tool in biomedicine with far-reaching implications in the fields of cancer research and oncology. SNAs are typically composed of nanoparticle cores (*e.g.*, gold[58], silver[59], iron oxide[60], infinite coordination polymers[61], silica[62]), densely functionalized with highly oriented oligonucleotide shells (*e.g.*, single- or double-stranded DNA[58], siRNA[63], mRNA[64], PNA[65], LNA[66], RNA/DNA hybrids[67]) (**Figure 3**). Core-less or hollow versions of these structures have also been synthesized (*e.g.*, crosslinked alkyne polymers[68], liposomes[69]), some of which are composed purely of biologically compatible components. Many of the novel chemical and physical properties that make these materials useful in cancer research and oncology stem from the unique architecture of the oligonucleotide shell and are core-independent. Indeed, SNAs are recognized by Class A scavenger receptors and enter cells (over 60 tested to date) as a single-entity without the use of ancillary transfection agents[70–72]. They also are resistant to enzymatic degradation and show no apparent toxicity or immunogenicity[73–75]. SNAs also exhibit a high affinity for complementary DNA strands (100 times higher than that of free DNA of the same sequence in solution)[76]. SNAs are highly modular and the composition of their cores as well as the sequence, length, and density of their oligonucleotide shells can be tailored; in the context of cancer research and oncology, this means that SNAs can be designed to target almost any gene, including those associated with a wide variety of cancer types, in extracellular and intracellular biodetection and therapeutic schemes. SNAs were first synthesized in the Chad Mirkin laboratory at Northwestern University in 1996, and they were first formulated as nanoflare constructs in 2007 by the same lab.

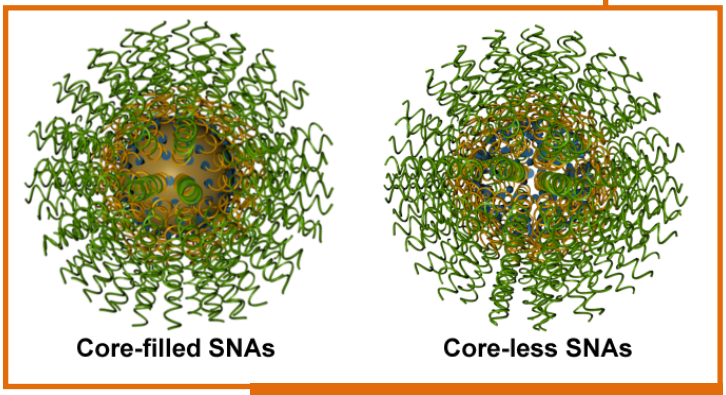

Figure 3. Gold nanoparticle-filled (left) and core-less (right) spherical nucleic acid (SNA) structures.

Based upon SNAs, these new constructs, termed NanoFlare, possess many of the

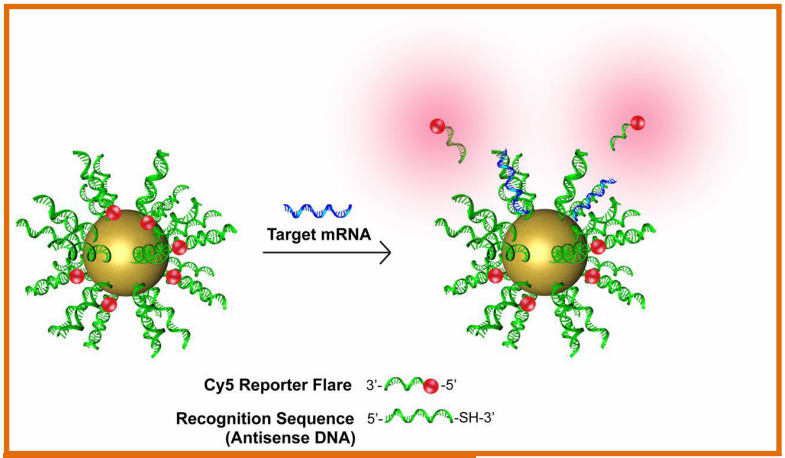

Figure 4. Schematic of Nanoflare structure and function. (*Reprinted with permission from Halo et al, 2014*)[79]

aforementioned useful chemical and physical properties[77]. Specifically, NanoFlares are gold nanoparticle-based SNAs that are hybridized with short, fluorophore-labeled complementary DNA strands (**Figure 4**). Their usefulness as a diagnostic is simple, when hybridized the fluorophores are held in close proximity to the gold nanoparticle and their respective fluorescence output is quenched. However, when a nanoflare encounters a longer, complementary target (*e.g.*, mRNA strand) in a cellular environment, it displaces one of the shorter "flare" strands and the fluorescence signal is observed. As such, these novel nanomaterials have proven to be highly useful probes for intracellular mRNA detection with exceptionally low limits of detection (*e.g.*, sub-pM). When coupled with flow cytometry, NanoFlares currently constitute the only means of interrogating the genetic content of live cells and sorting them based on such content. NanoFlares are also capable of engaging in gene regulation as potent antisense, siRNA, and microRNA delivery vehicles; indeed, these structures have been proven to have theranostic potential as they could be used to both detect and treat cancer, simultaneously[78].

In initial proof-of-concept studies, it was demonstrated that NanoFlares could be used to detect oncogenes – specifically survivin, an anti-apoptotic gene that is up-regulated in a range of cancer types – for example, in a breast cancer cell line (SKBR3) in a highly sensitive and sequence-specific manner[77]. Indeed, increased fluorescence was observed when NanoFlares targeting survivin were added to SKBR3 cells expressing survivin compared to when either NanoFlares bearing a non-complementary sequence were added or cells that did not express survivin (C166 cells) were used (**Figure 5**). These results demonstrate how researchers can use NanoFlares to distinguish cancerous cell populations based on the expression of an mRNA target of interest. Further, in the context of cancer research and oncology, it would be useful to track the up- or down-regulation of multiple genes at once. Thus, more advanced nanoflare systems have been developed that allow a single nanoflare to target multiple genes (*e.g.*, two[31], three[80], or four[81]) in cervical and breast cancer cell lines. These multiplexed NanoFlares also allow quantitative information to be obtained, the signal-to-noise level to be reduced, and to mitigate the effects of cell-to- cell variability.

More recently, NanoFlares were designed to target markers (*i.e.*, vimentin and fibronectin) of the epithelial-to-mesenchymal transition (EMT), an integral part of cancer metastasis. Coupled with flow cytometry, they also were used to capture live breast cancer circulating tumor cells (MDA-MB-231) from human whole blood samples and from an orthotopic murine model of metastatic triple negative breast cancer[79]. Furthermore, these NanoFlares were used to retrieve GFP-positive cells in a HER2+ mouse model of breast cancer and subsequently cultured into mammospheres (**Figure 6**), which are spherical clusters formed only from cancer stem cells. These results suggest that it may be possible to isolate and further culture live CTCs from human patients *ex vivo*, providing the opportunity to study cancer cell heterogeneity and its relation to patient outcomes. Simultaneously, these results demonstrate the ability of NanoFlares to survey the metastatic potential of cells in the blood stream. This approach provides an unprecedented opportunity to isolate cancer stem cells based on the presence of genetic markers and may improve cancer diagnosis and prognosis.

In 2012, nanoflares were commercialized by AuraSense, LLC, a company founded by Chad Mirkin. Two years ago, AuraSense entered into a multi-million dollar partnership with EMD Millipore to commercialize them under the trade name SmartFlares™ for use in *in vitro* cell assays. SmartFlares™ are now available as research tools to investigators with over 1,700 different versions sold in over 230 countries. Over the next 5-15 years, the number of flares available through EMD Millipore is expected to increase, and subsequently nanoflares will move beyond the research setting to the clinic to be used for medical diagnostic purposes. Concurrently, there is an initiative to quantify and track the spatial location of mRNA in cells, as this is highly related to cellular function. As such, it is anticipated that drugs coupled to nanoflare systems

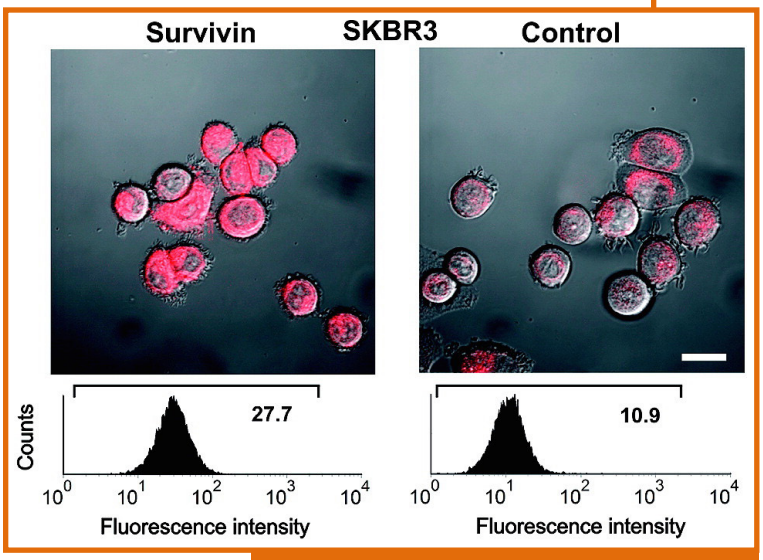

Figure 5. Intracellular testing of nano-flares. Differential contrast and fluorescence image of survivin-expressing SKBR3 cells treated with survivin-specific nano-flares (top left panel) and noncomplementary nano-flares (top right panel). Scale bar is 20 µm. Flow cytometry data are shown below each image. The bold numbers to the right of the histogram are the total mean fluorescence of the cell populations. (*Reprinted with permission from Seferos et al, 2007*)[77]

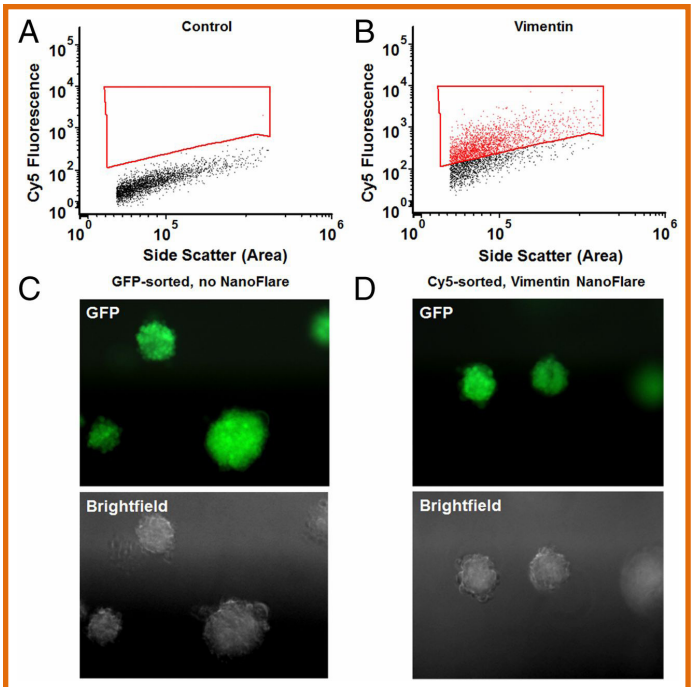

Figure 6. Cell isolation and mammosphere formation post NanoFlare treatment and flow cytometry analysis. Representative scatter plots show Cy5 fluorescence (NanoFlare) of GFP recurrent cells spiked into (A) untreated human whole blood or (B) Vimentin NanoFlare-treated blood. Upon treatment with NanoFlares, Cy5 fluorescence of GFP-positive cells increases 5.4-fold. Cells in the red gate in the Vimentin sample were sorted for mammosphere culture. Cells retrieved from blood form mammospheres (C) untreated or (D) Vimentin NanoFlare-treated. (*Reprinted with permission from Halo et al, 2014*)[79]

will allow therapy to be administered based on the genetic content of the cell, in a highly targeted manner. These research directions are already underway and will have significant implications for the field of cancer research and oncology.

Intraoperative Imaging

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Introduction

In the operating theatre, there is an urgent need for implementing new image-directed visualization tools that will enhance surgical vision, facilitate minimally invasive surgical procedures, and dramatically alter surgical outcomes of oncological patients. Early detection, staging, and treatment of cancer are essential to minimizing morbidity and mortality. Each year, nearly 13 million new cancer cases and 7.6 million cancer deaths occur worldwide[82]. The cornerstone of clinical cancer care rests on surgical management. However, intervention is often limited to tumors diagnosed in an early stage as outcomes are notably poorer when surgery is no longer a treatment option[83]. Adjuvant radiation and/or chemotherapy are typically added for specific indications including locally invasive tumors and/or spread to regional lymph nodes. The challenge has been in the lack of clear 'surgical vision,' which impacts the ability of the operating surgeon to accurately and specifically identify the extent of malignancy[83,84], macroscopic/microscopic tumor burden[85–88], or remnant disease, notably at the site of surgical removal (*i.e.* surgical margin). Complete assessment of surgical margins will be based upon the quality and extent of tissue sampling[89]. Collectively, these factors will affect therapeutic outcome, prognosis, and treatment management. Moreover, despite technical advances that have enabled large-scale imaging instruments, such as PET-CT and MRI, to meaningfully impact preoperative cancer diagnostics and staging, they are either not practical for intraoperative settings or offer limited utility in terms of achievable spatial resolution and/or sensitivity. Alternatively, newer molecular imaging probe designs (*i.e.*, engineered optically- active nanomaterials), coupled with state-of-the-art device technologies, may enhance cancer care, provide real-time imaging guidance, and lead to new, more efficient approaches for early-stage detection and treatment.

A key goal of cancer surgery is to reliably distinguish cancer from normal tissues at an early stage to pursue a surgical cure while maximizing safety, limiting damage to vital structures, preserving cosmesis, and increasing throughput. The current standard of care relies upon palpation and visual inspection[90]. Although anatomic structures can be efficiently identified, such evaluations depend on successful discrimination of a narrow range of spectral features (*i.e.*, contrast) or subtle textural differences, rather than elucidating molecular processes

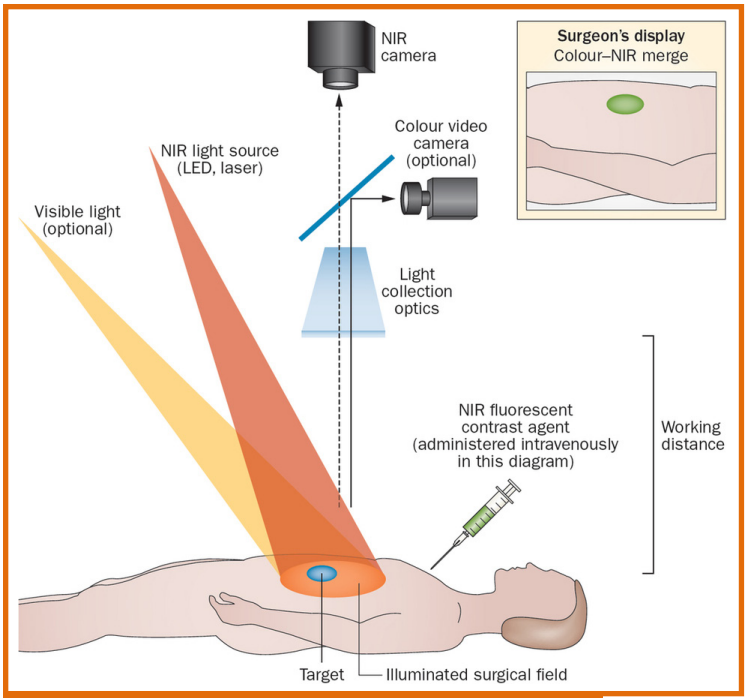

Figure 7. Mechanics of NIR fluorescence imaging. During surgery, an NIR optically-active agent is visualized using a fluorescence camera system. All systems must have adequate NIR excitation light, collection optics, filtration and a camera sensitive to NIR optical emissions. Optimal imaging systems include simultaneous visible (white) light illumination of the surgical field, which can be merged with NIR optical images. The display can be a standard computer monitor, goggles, or a projector. Current imaging systems operate at working distances that enable illumination of a sizable surgical field. LED, light-emitting diode (*Reprinted with permission from Vahrmeijer et al, 2013*).

defining a given disease stage[91]. This leads to a higher risk of incomplete surgical resection and/or soft tissue injury.

These limitations may be overcome by the application of improved intraoperative optical imaging approaches, which have traditionally been hampered by (1) the small number of imaging agents available in the near-infrared (NIR) spectrum, (2) high background autofluorescence that restricts depth and detection sensitivity, (3) large spectral overlap between optical agents preventing concurrent detection of multiple targets (*i.e.*, multiplexing), and (4) rapid photobleaching that reduces the imaging duration[15]. However, significant progress is being made on a number of fronts. Fueled by the emergence of an increasing number of new, diverse, and clinically promising NIR fluorescence probes, including particle-based agents, that can enhance soft tissue contrast, detection sensitivity, and depth penetration, some of these key drawbacks are being addressed, noting that these probes require an intraoperative optical imaging system with clinical grade accuracy (**Figure 7**). In addition to offering exquisitely sensitive real-time detection sensitivities, the higher resolution offered by these systems has enabled lesions to be detected down to sizes smaller than 10 µm, which truly revolutionizes imaging capabilities by dramatically increasing the sensitivity and specificity of detection over human vision[92]. Such tools can be seamlessly integrated with minimally invasive, robotic-assisted surgical equipment to enable navigation to target sites deep within the body. Unlike other imaging modalities, the combination of optically-active, disease-targeting probes and state-of-the-art multichannel camera systems offers

the possibility of interrogating real-time biological processes and identifying one or more novel biomarkers for (1) imaging (*i.e.*, cancerous nodes, surgical margins, remnant tumor); (2) staging; and (3) treatment response (**Figure 8**). Such markers can be further validated in the clinical trials setting. Collectively, the potential of these technologies to improve patient outcomes, minimize surgical risk, promote clinical throughput, and lower health care costs represents a significant clinical advance, and promises to transform the current practice of surgical oncology.

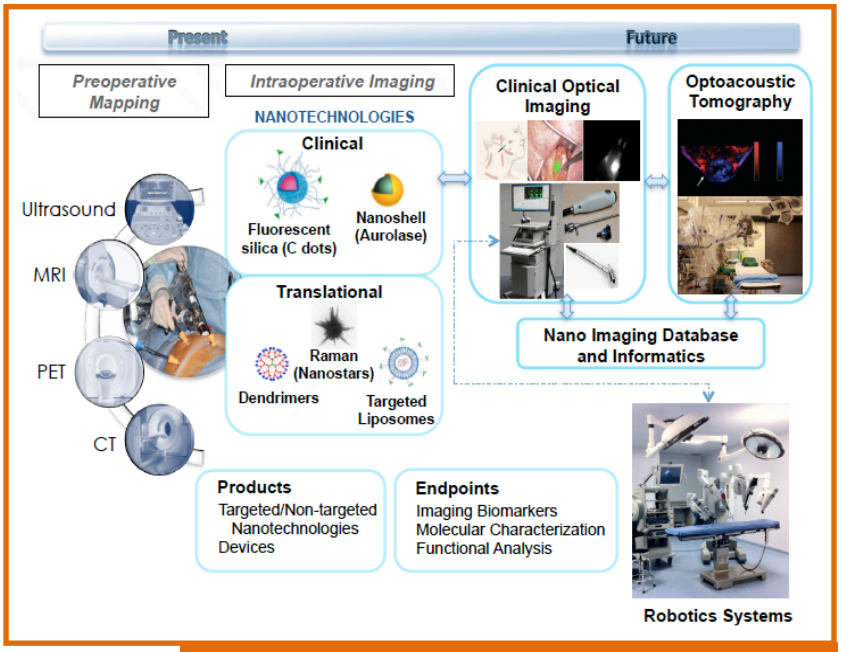

Figure 8. Present and future of NanoOncology Image-guided Surgical Suite. Preoperative conventional imaging tools are used to screen for disease and inform optically-driven minimally-invasive and open surgical procedures. Clinically available particle platforms can be monitored in real-time using portable multichannel camera systems. Representative translational probes and devices for future clinical use are also shown. In the future, the operating surgeon will select suitable probe-device combinations for specific indications, and be provided with structural, functional, and/or molecular-level data regarding tissue status for further treatment management.

Intraoperative Imaging Via Nanotechnology

A significant volume of work, however, has been performed utilizing endogenous tissue contrast, which is restricted to examination of only very small fields-of-view, or by administering non-specific optical agents[93,94]. The latter class of agents have included particle-based probes (*i.e.*, quantum dots)[95] and fluorescent dyes, such as indocyanine green (ICG)[96,97], an FDA-approved NIR dye for selected clinical indications. However, the lack of selective targeting found with these agents limits their utility for many applications aimed at detection of strictly cancer-bearing tissues. Thus, to enhance surgical vision during image-guided procedures, as well as impart labeling specificity, NIR optical probes targeting tumor-selective biomolecules are desired. Towards this end, a number of targeted molecular products, including dye-bound antibodies and peptides, can be applied as visualization tools for improving examination of tumor borders or localization of tumor deposits by attaching to upregulated cancer receptors[98–100]. Although not yet reaching full potential in surgical

practice, early potential benefits of optical imaging have been shown in clinical studies utilizing targeted molecular probes, albeit conjugated to visible dyes. However, such dyes reduce contrast resolution and depth penetration due to higher absorption and scatter in this part of the light spectrum[101,102].

More recently, the emergence of diverse classes of NIR fluorescent nanoparticle platforms, designed to improve the sensitivity, accuracy, and reliability of lesion detection over that of organic dyes, has revealed exciting new possibilities for probing and characterizing new molecular targets and novel biomarkers within human subjects[15]. The ability to tailor and refine the physicochemical and photophysical properties of these materials in a well-controlled and iterative fashion can favorably modulate their biological activities, resulting in one or more characteristics that improve upon those exhibited by simple molecular agents. These characteristics include multivalency enhancement (potency) as a consequence of simultaneous interactions of multiple targeting ligands with cell surface receptors, improved target retention, extended plasma residence time, bulk renal clearance, and improved pharmacokinetic profiles. Moreover, in some cases, the encapsulation of dyes within the particle structure has led to significantly enhanced brightness and photostability relative to the native dye, in addition to increasing tissue penetration depths (up to several centimeters)[103]. Collectively, these adaptations can improve target-to-background ratios and *in vivo* detection sensitivities following particle administration, the ultimate goal being to identify and remove all cancer cells. Finally, the ability to create multimodality platforms by incorporating more than one contrast-producing moiety into the particle design can yield multiparametric imaging data that validates potential biomarkers, potentially altering current standard of care.

Given these diverse, highly versatile, and integrated particle surface designs, coupled with improved state-of-the-art optical clinical camera systems, key surgical indications can be performed more reliably and accurately. Current applications have mainly focused on (1) selective mapping of cancerous lymph nodes, (2) precise identification of surgical borders (crucial landmarks), (3) accurate detection and treatment of remnant disease, and (4) reliable assessment of tissue function (*i.e.*, perfusion). For SLN mapping, the principal aim is to map the lymphatic drainage of exogenous agents and highlight only cancer- bearing nodes for selective resection. The primary factor controlling lymphatic transport is the agent size. An optimal size is one that is small enough to exhibit rapid lymphatic transport to the SLNs and other downstream nodes, yet large enough to be retained, typically around 5–10 nm[87,104]. One such sub-10 nm hybrid (PET-optical) cancer-targeting imaging platform is shown in **Figure 9**. A second surgical indication, the mapping of surgical margins, involves precise delineation of the tumor extent. The presence or absence of tumor cells at the site of resection is a key determinant of treatment success or failure, and is often used

to determine the need for adjuvant therapy. Positive margins are a negative prognostic indicator for many solid cancers[83]. Furthermore, surgical margins are often evaluated by immediate intraoperative analysis of the specimen, which can lengthen operating time and/or lead to incomplete readouts due to suboptimal specimen quality or inadequate sampling, the result being a positive surgical margin and poor outcome[89]. One such triple-modality (*i.e.*, MR-photoacoustic-Raman imaging, MPR) particle has sought to address this issue by efficiently and accurately delineating brain tumor margins (**Figure 10**)[14].

In addition, newer higher resolution whole-body optical imaging strategies, such as multispectral optoacoustic tomography (MSOT) (**Figure 8**), which detects optical absorption by means of ultrasound, have grown in popularity due to the concurrent development of clinical imaging systems[91,95]. These methods utilize multiple optical wavelengths and spectral demixing algorithms to permit imaging at depths greater than those typically achievable with fluorescence imaging. In addition, these methods can detect a broad range of novel light-absorbing nanoparticles (gold nanorods)[105], among other entities (*i.e.*, endogenous chromophores, organic dyes)[91], to yield high resolution optical assessments of targets deep to the tissue surface, as well as provide functional measures of viability and/or perfusion.

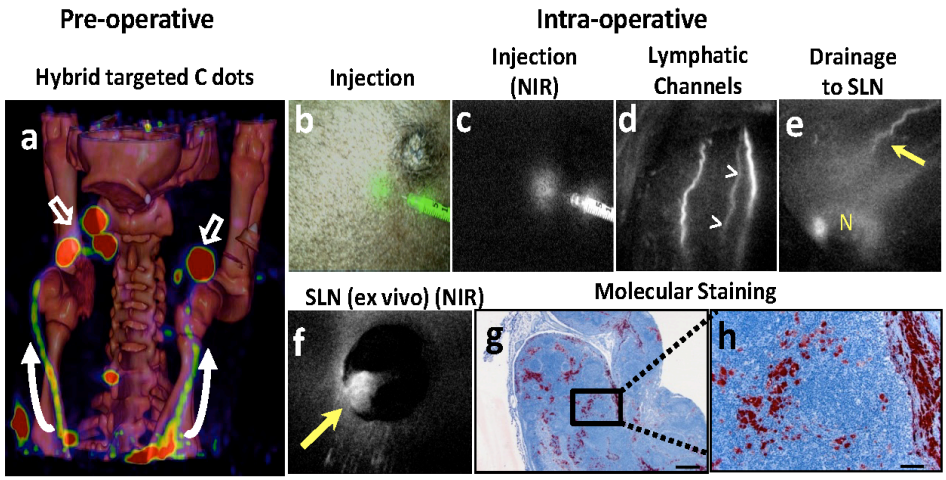

Figure 9. Mapping of Metastatic Lymph Nodes Using a Clinically Translated Hybrid PET-Optical Silica Nanoparticle (C dots). (a) Volume-rendered pre-operative PET-CT fusion images of the neck shows metastatic lymph nodes (red) bilaterally and lymphatic channels after injection of ultrasmall (6 nm diameter) integrin-targeting C dots into melanoma miniswine. (b,c) Intraoperative SLN mapping with two-channel NIR optical imaging of the exposed nodal basin. Local injection of fluorescent C dots displayed in dual-channel model (b) RGB color (green) and (c) NIR fluorescent channels (white). (d,e) Draining lymphatics (arrowheads) distal to the injection site extending toward the node (N). (f) Image of excised SLN in the NIR channel. (g) Low-power view of HMB45-stained (red) SLN confirms the presence of metastases (black box, bar = 500 µm). (h) Higher magnification reveals HMB-45+ expressing melanoma cells (bar = 100 µm) (*Reprinted with permission Bradbury et al, 2013*).

Future of Intraoperative Imaging Via Nanotechnology

It is anticipated that fluorescence-enhanced surgical vision, despite its limitations, will significantly impact and likely transform conventional surgical practice in oncology over the next 5 to 15 years by increasing the sensitivity and accuracy of surgical procedures, such as evaluation of surgical margins, mapping of local and distant cancerous lymph nodes, and detection of microscopic disease. Rather than relying on visual and tactile cues for guiding disease assessment and therapeutic management, the surgeon will utilize a growing array of dedicated intraoperative treatment tools in the form of targeted optically-active particle probes and portable multichannel optical devices. Nanoparticle surface versatility and their unique physicochemical and biological properties will play a key role in this field, providing new opportunities to probe critical cancer targets and identify potential biomarkers that can be validated in clinical trials. Although in its infancy, a variety of particle therapeutic strategies are currently being developed for effectively treating disease in the intraoperative setting. The future implementation of such tools in clinical practice should lead to improved patient outcomes and reduced surgical risks. The foregoing developments are also expected to promote acceptance of optical technologies and, as a consequence, accelerate the growth of minimally invasive surgical procedures, with the intent of maximizing functional outcomes and limiting treatment-related morbidity. Identification of normal tissue markers may also enable particles to be engineered with specific ligands and fluorescent labels for highlighting poorly visualized vital structures (*i.e.*, nerves). In addition to their expected utility for real-time intraoperative procedures, the application of these optical technologies

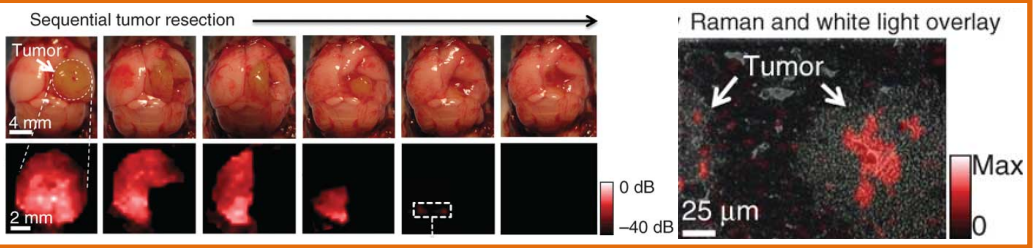

Figure 10. Raman-guided intraoperative surgery using Raman imaging nanoparticles (MPR). (a,b) Living tumor-bearing mice underwent craniotomy. Quarters of the tumor were sequentially removed (photographs, a), and intraoperative Raman imaging was performed after each resection step (b) until the entire tumor had been removed, as assessed by visual inspection. After gross tumor removal, small foci of Raman signal were found in the resection bed (dashed white square). Raman microscopy image (right) of dashed white square depicts Raman signal within an infiltrative tumor, indicating the selective presence of MPRs. Raman color scale (red): –40 dB to 0 dB (*Reprinted with permission from Kircher et al, 2012*).

may additionally aid inspection of resected tissue specimens, leading to less time-intensive evaluations and improved clinical throughput.

Despite the significant data generated to support the translational developments of new, optically-active particle probes for intraoperative cancer treatment, advancing such agents into the clinic has been challenging, particularly those exhibiting molecular specificity[106-108]. Importantly, FDA-IND approvals have been issued for both targeted particle drug[106] and device[109] technologies, and such developments are paving the way for translating additional targeted optically-active technologies to the clinic for use in image-guided surgeries. Furthermore, as tumor heterogeneity is an important consideration for selecting a targeting ligand, 'cocktails' of multiple cancer-targeting particle probes will be increasingly utilized, each probe incorporating a different ligand and optical dye for improving detection and staging accuracy. Enabling simultaneous visualization of these cocktails will require implementation of state-of-the-art multichannel fluorescence camera systems that can detect fluorescence from multiple wavelengths. Several of these camera systems are already in clinical use. As additional novel particle probes are developed and camera systems continually evolved to permit both structural and functional assessments, the true clinical value of these combined technologies will ultimately be realized. Promising higher resolution techniques, such as optoacoustic imaging, may be increasingly implemented to overcome instances where degradation of the emitted fluorescence signal is observed, notably when interrogating complex tissue compositions.

Nanoparticle surface versatility and their unique physicochemical and biological properties will play a key role...

Finally, the need to establish standardized quantitative metrics for intraoperative decision-making is paramount, and is at a very early stage of development. Often these assessments are of a qualitative nature, and the chosen endpoints may depend on many factors, including the nanomaterials probe selected and the device providing the measurements. It is expected that the optical imaging community will address these issues in the near future, as they will significantly hamper efforts to make effective comparisons among different probe-device combinations for a specific indication. Implementation of well-designed outcomes studies will also be critically important for widespread dissemination and acceptance of image-guided optical technologies in standard surgical practice.

Targeting the Tumor Microenvironment

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The Big Picture

Personalized medicine, or precision medicine, relies on the selection of the correct drugs, or drug combinations, based on the disease-specific genetic traits. Selecting the proper drugs is the first step toward precision medicine, but its completion needs effective delivery of the selected drugs to the target (*e.g.*, tumor). Recent progress in nanotechnology has made drug delivery more efficient compared with the control solution formulation, but subsequent effectiveness of the drugs delivered is still in question. Nanoparticulate drug delivery systems are designed and tested for the ultimate goal of developing clinically useful formulations to treat various cancers. Thus, the usefulness of nanoparticle formulations needs to be considered in the context of treating cancers (*i.e.*, improving efficacy and safety) in human patients.

Benefits of Nanoparticle Formulations

Over the last few decades, various nanoparticles have been prepared for treating cancers. One large benefit to using nanoparticle formulations is in the ability to avoid non-aqueous solvents when administering hydrophobic drugs to patients, resulting in fewer side effects, even if the efficacy remains the same. This has been exemplified by the success of Abraxane® (based on nanoalbumin particles) and Doxil® (PEGylated liposome formulation), which in large part, rely on delivering anticancer drugs without using organic solvents. Although, nanoparticle formulations, or for that matter any formulation, can deliver drugs to the area near target tumors, but the subsequent delivery to the tumor cells is hindered by the complex microenvironment of tumors. Drug efficacy occurs only after the drug is absorbed into target tumor cells. Thus, it is important to understand the tumor microenvironment (TME) to achieve or improve upon the desired drug efficacy.

Understanding the Tumor Microenvironment (TME)

The tumor microenvironment comprises a highly heterogeneous mixture of tumor and stromal cells embedded in an extracellular matrix with many cytokines, growth factors, inflammatory cells and macrophages[109]. The current difficulty of developing new anticancer

drugs and drug delivery systems partly stems from the lack of a clear understanding of the delicate interplay between tumor and stromal cells in the complex TME[111]. Here, pancreatic ductal adenocarcinoma (PDAC) is used as the fundamental, albeit extreme, example of this in order to portray the importance of improved targeting to TME.

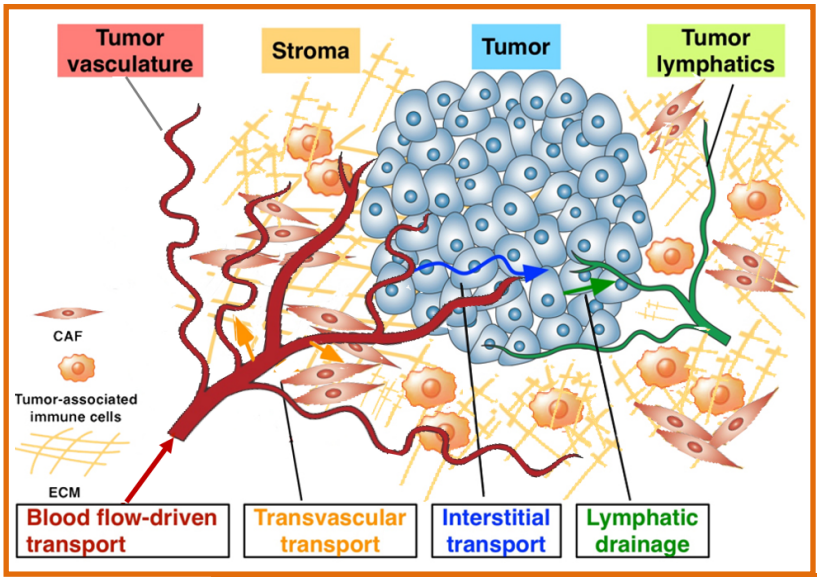

Figure 11. Transport of drug molecules and nanoparticles in the TME of PDAC. Drugs and nanoparticles can only reach the target tumors via multiple transport processes in the TME. PDAC has a very complex TME with dense stroma composed of cancer-associated fibroblasts (CAFs), tumor-associated immune cells, and dense ECM structure.

PDAC consists of two components, the malignant epithelial cell population and a complex, large stromal compartment. **Figure 11** describes a highly desmoplastic PDAC tumor which is infiltrated with activated cancer-associated fibroblasts (CAFs) and inflammatory cells. CAFs release collagens, laminin, and fibronectin. The complex extracellular matrix (ECM) includes dense collagen types I and III bundles, hyaluronic acid (HA), fibronectin, desmin, cytokines, growth factors, and the matrix metalloproteinase family of proteases. The exact roles of the stromal compartment are still not clearly established, but it certainly provides an immense physical barrier to the multiple transport steps for effective drug delivery. Overcoming the transport barriers presented by both stroma and tumor for effective delivery requires ingenious design of nanoparticles, at least beyond the nanoparticle design paradigms currently in clinical use due to their size and surface functionalities. Moreover, interactions between tumor cells and various cell types in the stroma may affect the drug response of tumor cells. The outcome of these interactions is highly context-dependent, and further understanding of dynamic cancer biology and oncology is critical. The current idea of targeted drug delivery using nanoparticles addresses only a very small portion of this complexity. As such, any new paradigm should comprise tools for overcoming the enormous complexities of the TME.

Future Needs to Efficient Delivery of Anticancer Drugs Through Priming of the TME

The TME has enhanced stiffness, increased HA content, and elevated hydrostatic pressure, all of which are known to reduce effective intratumoral drug delivery. For drugs to be effective, they must reach the target tumor cells through the TME or the stromal surrounding. Thus, solid tumor priming, *i.e.*, modulating the abnormal TME, is promising idea for enhancing the antitumor efficacy. The strategies of solid tumor priming includes vascular normalization using anti-angiogenic treatment, solid stress alleviation by induced apoptosis and stromal normalization, and using tumor-penetrating peptides[112]. Of these stromal normalization is attractive because it can be achieved by using relatively benign components.

Stromal HA is known to be a key factor making the too TME dense for proper diffusion of drug molecules, not to mention nanoparticles. This provides a means to enhance the permeation of nanoparticles through TME by treating PDAC first with hyaluronidase[113]. Calcipotriol, a synthetic, highly potent derivative of vitamin D that does not cause hypercalcemia, was recently reported to reduce the activation of pancreatic stellate cells and their conversion to CAFs by activating the vitamin D receptors that are expressed in these cells, thereby decreasing desmoplasia[114]. When used in combination with gemcitabine, calcipotriol prolonged survival in a genetically engineered mouse model (GEMM) of PDAC by decreasing fibrosis, increasing intra-tumoral vasculature, and enhancing gemcitabine delivery into the tumor. Importantly, Calcipotriol has been shown to exert anti-proliferative and pro-differentiation effects, as well as immune-modulating effects[114]. Interpretation of these results is complicated by a very recent finding that vitamin D may also promote tumor chemoresistance to gemcitabine, *underscoring the need to improve our knowledge on how to target the stroma*[115].

While the stroma-targeting approach has been successful in GEMMs of PDAC, it did not work in clinical trials. The successful treatments observed in mouse models seldom translate into clinical success. There may be several reasons for this discordance between findings in humans and in GEMMs of PDAC. The TME in mouse is likely to be very different from that in human. In addition, the amount of a drug delivered after HA priming was simply not adequate in clinical trials. Disrupting stromal layer alone may not be sufficient to kill tumor cells without delivering sufficient drugs. Since tumors are highly heterogeneous, delivering a single drug might have not been effective. Indeed, the heterogeneity of gene alterations in the cancer cells and the complexity of the stromal components mandate the design of novel multi-targeted and multi-drug dosing approaches.

Future Needs for New In Vitro Test Methods

Effective tumor treatment requires testing various priming agents in combination with delivery of multiple drugs, either simultaneously or sequentially. This involves a very large number of studies, and it makes animal testing expensive and time consuming. Moreover, small animal data may not be good predictors of clinical outcome. Thus, it is essential to develop *in vitro* test methods that can represent the microenvironment of human tumors.

Recent advances in tissue engineering and microfluidic technologies present an opportunity to realize *in vitro* platforms alternative to animal testing.

Recent advances in tissue engineering and microfluidic technologies present an opportunity to realize *in vitro* platforms alternative to animal testing. These platforms enable mimicking complex and multiple transport processes of drug delivery systems including circulation in the blood, extravasation from blood vessels to the tumor region, and diffusion of drug to the target tumor[116]. Tumor cells can be grown in 3D matrices with other relevant stromal cells to more closely recapitulate the complexity of solid tumors in patients. The current ability of forming 3D perfused tumor tissue needs to be advanced further to create an accurate TME, which accurately represents that of human tumors. This requires the design of 3D co-culture systems in which cancer cells, CAFs, and other stromal cells are grown within the necessary ECM components, yielding a delicate balance of biological, chemical and physical parameters relevant to human tumors.

Exact duplication of the human TME in microfluidic systems may not be feasible in the near future, but the TME-on-Chip can be used to systematically study the significance of given biological, chemical and physical parameters on the efficacy of nanotechnology-based drug delivery system and priming agents. Eventually, it should serve as a useful screening system for testing a large number of priming agents and drug combinations for personalized medicine.

Overcoming Specific Biological Barriers: Stromal

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related deaths in the United States and its 5-year survival rate has remained unchanged (6%) over the past decades (*Cancer Facts & Figures 2014*, www.cancer.org). Due to the inevitable late diagnosis and early metastasis, chemotherapy is the only approved option for the majority of PDAC patients, with the standard of care involving the use of nucleoside analog gemcitabine or a more potent (but more toxic) four-drug regimen, oxaliplatin, irinotecan, 5-fluorouracil, and leucovorin (a.k.a FOLFIRINOX). Chemotherapy failure can be partly explained by the presence of an abundant dysplastic stroma, serving as a physical and biological barrier for drug access and unfavorable pharmacokinetics. It is appropriate, therefore, to consider the important stromal contribution to drug delivery and chemoresistance and sidestepping this barrier to improve survival outcomes[117]. This short overview will address the inhibitory role of the stroma in the treatment of PDAC, including the consideration for the use of nanocarriers to potentially engineer past this obstacle. We provide a perspective and guidance towards the implementation of nanotherapeutic approaches that could prove useful to improve therapeutic delivery and efficacy of gemcitabine and FOLFIRINOX.

Overcoming Tumor Stroma is Important to Cancer Nanotherapeutics

Because the stromal volume in PDAC is the highest among solid tumors (~70% of the total tumor volume), this requires special consideration in the treatment of this deadly disease[117]. Not only is the stroma poorly vascularized, but the existing vessels exhibit low permeability due to a high pericyte coverage, which blocks the extravasation of drugs, molecular therapeutics, and even nanocarriers to the tumor site (**Figure 12A**)[118]. The stroma also contributes to chemo-resistance and an unfavorable pharmacokinetic/pharmacodynamic (PK/PD) profile[117], including the expression of a high content of cytidine deaminase (CDA), which leads to gemcitabine inactivation, limiting its half-life to as little as 0.28 hours

(**Figure 12A**)[119]. Moreover, the intracellular activation of gemcitabine is dependent on phosphorylation by the rate-limiting kinase, deoxycytidine kinase (dCK) to generate the active metabolites, dFdCDP and dFdCTP (**Figure 12A**)[120]. It is believed that chemo-resistance to gemcitabine in PDAC is due in part to decreased expression of dCK. Another important stromal contribution is its pro-tumorigenic effect through supportive cell types that promote cancer cells proliferation and metastasis via complicated cross-talk mechanisms. Given this background, it is important to consider overcoming the challenges of the stromal barrier to address drug delivery and unfavorable PK/PD to the cancer site, including the improvement of intratumoral distribution, bioavailability, and overcoming drug resistance.

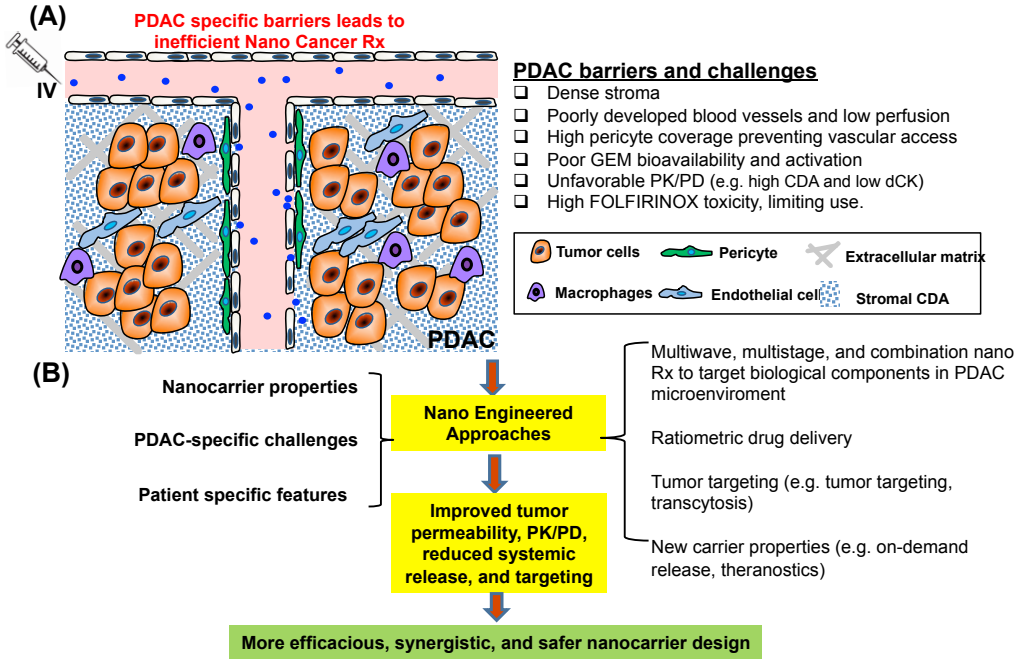

Figure 12. (A) Schematic to show the barriers and challenges that are responsible for failed chemotherapy in PDAC, including as a result of an abundant dysplastic stroma, which serves as a physical and biological barrier. This includes interference in vascular access and the presence of a high local concentration of deaminase activity, which leads to in activation of GEM. **(B).** We propose an engineered approach using nanocarriers, which can overcome stromal vascular gate or suppress the stromal abundance by the delivery of drugs that suppress pericyte coverage or decreases the stromal volume and abundance of deaminase activity. Moreover, a combination of these features could be used in synergistic designed nanocarriers. It is also a possible to include tumor targeting or the use of peptides that induce transcytosis across the stromal barrier.

The Current State of Overcoming Stromal Barriers in Cancer Nanotherapy

A number of stromal treatment strategies are currently being considered to improve PDAC treatment. These efforts have involved the use of enzymatic degradation, pharmacological suppression, tumor vasculature modification/intervention, and stromal targeting peptides. The first approach is the introduction of stromal-directed agents that obliterate the dense stromal microenvironment to improve drug delivery[113]. An ongoing clinical trial has demonstrated that the combination of gemcitabine with PEGylated hyaluronidase (PEGPH20) can ablate hyaluronan and overcome the stromal barrier, allowing chemotherapeutic drug access to the cancer site[121]. While PEGPH20 showed promising results pre-clinically and in some clinical studies, success is dependent on the dosing schedule as well as the specificity of this treatment[122]. In April 2014, FDA announced a clinical hold due to dosing and safety (*e.g.*, induction of thromboembolic event) concerns about the use of PEGPH20 in a Phase II clinical trial (www.halozyme.com). Although the clinical study resumed in September 2014, no update is available at this time. The second approach is to consider the use of pharmacokinetic suppression, as illustrated by the FDA granting approval for the use of the albumin-bound paclitaxel nano-complex, Abraxane®, in PDAC; co-administration of this therapy promotes gemcitabine survival outcome by 1.8 months. The proposed mechanism of Abraxane® action is the suppression of stromal density and reduced expression of CDA at the tumor site[123,124]. While the efficacy of this treatment is premised on using conventional therapeutic doses of each drug, it is not designed to deliver a ratio-dependent drug combination, which is an important consideration due to differences in the PK, distribution and elimination of the synergistic drug combination. This provides the opportunity to consider the ratiometric design of a single gemcitabine/Abraxane carrier to achieve *in vivo* synergy. The third approach is to use vasculature modification to improve drug delivery. In this category, there are a number of options, including targeting of the transforming growth factor beta (TGF-β) pathway, which promotes pericyte coverage of vascular fenestrations, among its pluripotent biological effects[125]. Intervention in the TGF-β signaling pathway using receptor kinase inhibitors or monoclonal antibodies have shown promising results to enhance vascular access and delivery of cancer drugs and nanocarriers to the tumor site[126,127]. However, the use of free inhibitor or antibody may require relatively high-dose/frequency and/or "off-target" effects due to the limited tumor targeting of these agents. Vasculature access can also be improved by stromal depletion through the use of antifibrogenic drugs, such as losartan (a clinically approved angiotensin II receptor antagonist)[128] and Hedgehog inhibitors[129], leading to decreased contractile elements, lowering of the interstitial fluid pressure[130] or a transient increase in intratumoral vascular density. While it has been shown that small 30 nm drug-loaded polymeric micelles can

permeate the stromal barrier to deliver antitumor drugs in PDAC without the need for targeting, the use of small particles may come at the expense of a reduced drug loading capacity[131]. The last approach is to develop stromal targeting therapy. This includes the recent discovery that iRGD peptides can increase PDAC vasculature access[132]. The exposed "CendR" motif, upon cleavage from the iRGD peptide, interacts with NRP-1 kinase receptor, which is capable of triggering transcytosis of macromolecules and liposomes, without the need of covalent conjugation of the peptide to the nanocarrier. This pathway is likely analogous to the vesiculo-vacuolar organelle, which has been observed in tumor vasculature during performance of electron microcopy[133].

Future Perspective in Overcoming Stromal Barriers

Because of the challenges of conventional chemotherapy for PDAC and the realistic expectation that there are no imminent changes in the treatments for metastatic disease, there is a unique opportunity for the use of nanotechnology in the treatment of this disease over the next 5-15 years. This is evidenced by the introduction of classic (*e.g.*, liposome and polymer) as well as novel (*e.g.*, inorganic-based) nanocarriers for this purpose. Although the use of small particles that rely on size-exclusion principles has shown promising results, nanotherapeutics are poised to make an even bigger impact because nanocarriers can be designed to deliver single or synergistic drug combinations, target, image and deliver, as well as allowing for engineered approaches to treatment. We define an "engineered approach" as the dynamic integration of the drug delivery properties with additional nanocarrier properties that address tumor-specific challenges, such as the stromal barrier (**Figure 12B**). Such an engineered approach could be particularly relevant to stroma-rich cancers in which the tumor stroma and other inferring biological components result in heterogeneous treatment effects in the tumor microenvironment. It is possible to design stromal targeting nanocarriers to enhance the efficacy of existing cancer drugs such as small molecules, peptides and proteins. One example is the introduction of a proof-of-principle "two-wave" platform in which a small molecule inhibitor of the TGF-β receptor kinase was used to decrease pericyte coverage at PDAC vascular fenestrations, allowing 2nd wave access of gemcitabine-laden liposomes, which could enter the tumor site to enhance gemcitabine tumor killing[134]. We postulate that the use of multiwave, multistage, and combination nanotherapeutics could have a translational impact on PDAC therapeutics in the clinic[135–137]. Another approach would be to design nanocarriers that can deliver synergistic drug combinations in a ratiometric fashion. In this sense 'ratiometric delivery' is defined as the *in vivo* release of a drug combination from a nanocarrier, with the purpose of providing a fixed drug ratio at the target site[138]. One example is the combination of a drug that exerts therapeutic effects on the suppression of the stroma (*e.g.*, paclitaxel) and a drug that kills PDAC cancer cells (*e.g.*, gemcitabine). In this regard, we have recently demonstrated

the design of a lipid bilayer supported mesoporous silica nanoparticle that can achieve ratiometric delivery of gemcitabine (trapped in the porous interior) with a sub-cytotoxic dose of paclitaxel incorporated into the lipid bilayer[139]. This synergistic combination resulted in the suppression of the tumor stroma and CDA expression in subcutaneous and orthotopic PDAC models in mice, providing more effective tumor shrinkage than free gemcitabine plus Abraxane. This type of nanocarrier could also be useful for treatment of other cancers with the same drug combination. Moreover, we envisage that this carrier can be further improved through the addition of incremental design features, such as on-demand release, theranostics, and promotion of transcytosis with iRGD peptides[132]. It is important, however, to consider the design complexity against the cost of each component and the ability to achieve GMP level manufacturing production volumes.

...nanocarriers could prove useful for addressing the toxicity of FOLFIRINOX.

It is possible to develop nanocarriers for precision medicine and addressing patient-specific response differences for treatment with gemcitabine and FOLFIRINOX. This could include the use of drug profiling, PK, drug uptake and metabolic effects in treatment design (*e.g.*, consideration of the delivery of a diphosphorylated version of gemcitabine to patients that have a relative low expression of dCK enzyme) leading to intracellular gemcitabine activation. To achieve this integration of nanotherapeutics with clinical-based approaches for PDAC, we have assembled a multidisciplinary team to advance the clinical tools, infrastructure and imaging approaches for delineating gemcitabine-responsiveness in PDAC patients (*e.g.*, PET scanning and intratumoral drug profiling)[120]. This could constitute the basis of future translational studies that build on the development of nanocarriers that can address patient-specific disease characteristics in orthotopic implant models in animals.

In addition to influencing the stromal barrier, nanocarriers could prove useful for addressing the toxicity of FOLFIRINOX. While this regimen has an increased response rate compared to gemcitabine (31.6% *versus* 9.4%), FOLFIRINOX is far more toxic and therefore restricted to patients with good performance status[140]. Encouraged by the promising results of MM-398 (an irinotecan liposomal formulation in Phase III trials)[141], single and multi-drug nano formulations are being developed to provide toxicity reduction, while maintaining efficacy. This could lead to FOLFIRINOX usage in more patients, with the ability to enhance the efficacy by combining this treatment with the “engineered approaches” described in the foregoing section. It is possible to envisage the use of engineered and targeted approaches (**Figure 12B**) to stromal therapy in preclinical studies over the next 5 years, assisted by the use of the transgenic KPC model and patient-derived orthotopic tumors. GMP-level

manufacturing, quality control and initiation of Phase I into clinical studies are achievable within 10 years. FDA approval and the introduction of at least one nanocarrier platform are envisaged after 15 years.

Overcoming Specific Biological Barriers: The Blood-Brain Barrier to Target Primary and Metastatic Brain Tumors

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Clinical Problems in Glioma Treatment

Gliomas are the most common primary brain tumors; grades III (*anaplastic astrocytoma*) and IV (*glioblastoma multiforme, GBM*) are characterized by increased cell and vessel density, cellular atypia and high mitotic activity. Malignancy grade is directly related to endothelial proliferation[142]. Despite considerable clinical and scientific efforts, patient survival still remains at 15.8 months on average. Little progress in pharmacological brain cancer treatment is due to the inability of many drugs to cross the blood-brain barrier (BBB) mostly formed by brain vascular endothelium. The BBB was discovered by Edwin E. Goldman more than 100 years ago. It protects the brain from environmental "noise", but, when the pharmacological treatment is needed, the same barrier prevents the brain influx of most drugs useful for the brain cancer treatment. Over a century-long scientific effort to circumvent the BBB has failed to answer many questions about drug delivery through the most powerful biological barrier in the body.

Nanomedicine Advances in Overcoming the Blood Brain Barrier

Glioma-derived signals triggering an intense angiogenesis in the tumor are not completely understood. Importantly, GBM and BBB interactions occur via extracellular proteins. For instance, the imbalance of tenascin and fibronectin in the tumor contributes to vessel formation[143]. We have described a switch of vascular basement membrane protein laminin isoforms in GBM from laminin-421 detected in normal brain to laminin-411, which may lead to higher rate of recurrences and shorter patient survival (Ljubimova *et al.* 2004, Cedars-Sinai Medical Center, clinical trial). The overexpression of laminin-411 in gliomas may contribute to increased glioma invasion (**Figure 13**). One clinical complication is the development of vasogenic brain edema, which dramatically increases the intracranial pressure (ICP) due to the BBB leakage[144]. Brain tumor-related edema can be a life-threatening complication of glioma growth, and so far, its treatment has relied on the use of corticosteroids.

Using systemically administered novel nanobiopolymer, Polycefin, anti-laminin drugs were delivered through the BBB, which dramatically reduced GBM size and normalized

brain cancer vasculature[145]. After the BBB crossing, polymeric nanobioconjugate release molecular inhibitors into the cytoplasm of glioma cells *in vivo* preventing the syntheses of laminin-411. Inhibition of this ECM protein decreased the tumor size by 90%. It has further been shown that the molecular mechanism of action of the endosomal drug releasing unit trileucine peptide (Leu-Leu-Leu) is based on pH sensitivity[146]; nano drug toxicity was found to be negligible and scale-up production has already begun. These nano drug treatments may significantly protect the brain from edema developing (**Figure 13**).

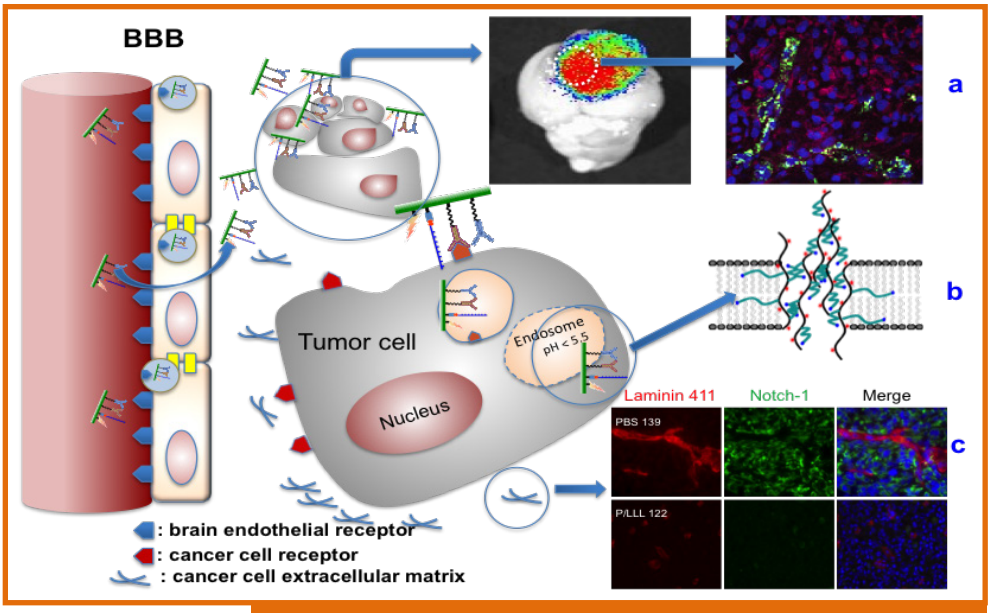

Figure 13. Multifunctional nanoconjugates for drug delivery into brain tumors. a, The nanoconjugates specifically target and accumulate in brain tumor (left), and cross BBB through receptor mediated transcytosis confirmed by confocal microscopy (right); b, Nanoconjugates are delivered into the cytoplasm by pH-dependent endosome membrane disruption and antisense oligonucleotide drugs are released; c, Successful inhibition of brain cancer stem cell marker Notch-1 as a result of inhibition of glioma-overexpressed vascular laminin-411.

Recently, the combination treatment of glioma-bearing animals with polymeric nano drugs showed significant life prolongation[147]. The polymeric nanoparticles were used for convection-enhanced intratumoral delivery of herpes simplex virus type I thymidine kinase DNA combined with the prodrug ganciclovir. An obstacle in brain tumor treatment is the limited ability for the delivery of a number of therapeutic and immunoregulatory molecules. For instance, therapeutic monoclonal antibodies, such as trastuzumab for breast and ovarian cancer, cetuximab for lung and breast cancer, and rituximab for lymphoma are effective for primary tumor treatment however cannot penetrate the BBB to reach the brain, and thus fail to treat their respective metastases in the brain. However, these antibodies can be used for brain drug delivery when they are part of 'nano-vehicles' capable of crossing

the BBB. Nanotechnology can master these problems with nanomedicines designed to cross the BBB and deliver drugs and/or immunostimulatory agents directly to a brain tumor and the respective immune cells in its microenvironment. Taking these possibilities into consideration Polycefin nano drug variants were engineered to treat human EGFR-positive triple negative breast cancer[148] and HER-2/neu positive breast tumors[149] in nude mice. The same nano drugs were similarly used to treat brain metastases from triple negative and HER2/neu positive breast cancer metastases to the brain). Furthermore, primary HER2/neu positive breast cancer has been successfully treated with a combination nanodrug that blocked HER2/neu synthesis and provided an immune system boost by directly targeted IL-2 at the same time. In this case, IL-2 was delivered as part of fusion monoclonal antibody against HER2/neu positive breast cancer[150].

Overall, the development of versatile biodegradable and non-toxic nanobioconjugate based on naturally derived polymalic acid[151] with its ability of targeting brain and breast human tumors in preclinical cancer models, inhibiting the expression of tumor-specific markers, normalizing vasculature, reducing invasion, and blocking their growth, resulted in significantly increased tumor-bearing animal survival. Additional recent nanodelivery systems/methods studied to deliver drugs across the BBB, include: focused ultrasound (FUS) disruption, SR-mediated endocytosis, and targeted adsorptive-mediated transcytosis among several others[152–158].

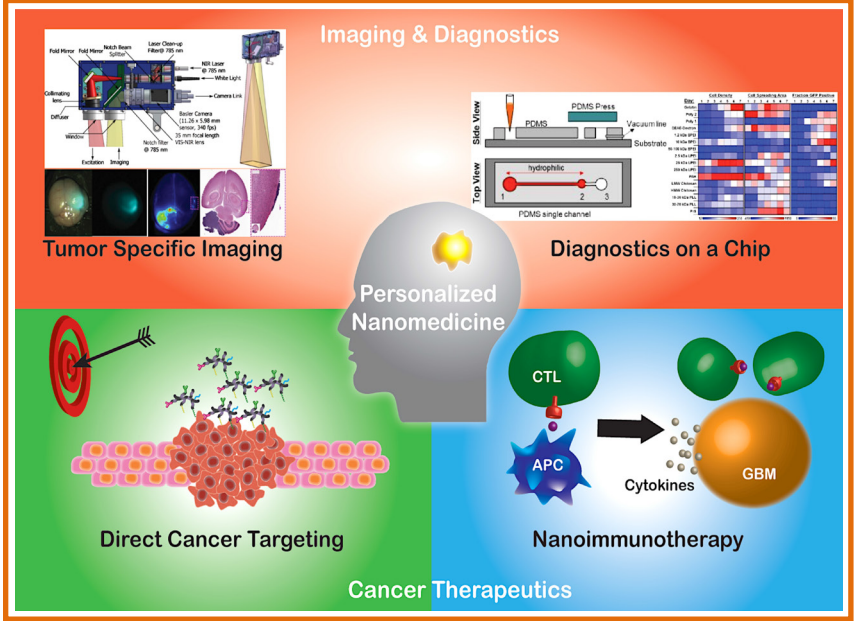

Figure 14. Brain tumor diagnostics and treatment.

Future Scientific and Clinical Developments

Treatment of brain metastases

Progress in treatment of primary cancers has led to improved patients' survival but has also increased the chance of residual tumor cells to metastasize, in particular to the brain. Melanoma, breast and lung cancer form brain metastases in up to 50% of cancer cases, with 3 to 6

months median survival. Therefore, brain metastasis treatment becomes a major issue for brain cancer management.

Personalized nanomedicine

During the last two decades, the dominant model of cancer based on genetic changes has been the chief conceptual foundation for developing targeted therapies. However, cancer immunology is currently coming back and may soon provide new mainstream cancer therapies[159]. We believe that tumor-targeted nano drugs can combine cancer genetics providing tumor cell markers, and immunotherapy providing anti-cancer immune response to treat each cancer patient individually (**Figure 14**).

Diagnostic and targeting

Current targeting strategies of nano drugs and imaging agents are based on monoclonal antibodies that will be substituted by peptides in the future to reduce immunogenicity and production costs. Significant advances of nanotechnology in cancer treatment give hope for the use of its achievements to treat a variety of other human diseases. Notable examples include neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, which are on the rise due to the aging of the world population.

Significant advances of nanotechnology in cancer treatment give hope for the use of its achievements to treat a variety of other human diseases.

Non-Intravenous Routes of Delivery: Aerosol Therapy for Cancer Management

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Nanoparticle-based inhalation drug delivery holds several advantages over intravenous drug delivery. First, inhalation is less invasive and drug administration is more rapid than intravenous. Second, inhaled therapeutics enter circulation directly and avoid the first pass through hepatic clearance. Lastly, nanoparticles allow for tunable drug release in the lung that can provide long-term treatment with fewer administrations[160]. Additionally, nanoparticles can be used to program the local mucosal immune response and re-purpose resident immune cells for tumor immunotherapy[161,162]. Historically, aerosol delivery of nanoparticles has been considered inefficient due to the low particle mass impacting aerodynamic properties and airway deposition. However, recent advances in particle fabrication and inhaler designs are changing this outlook[163]. This document will discuss the existing science and future directions for aerosol cancer treatment using nanoparticle chemotherapy, chemopreventatives, and cancer vaccines (**Figure 15**).

Aerosol Chemotherapy

Inhalation chemotherapy offers the potential for higher drug concentrations in the lung[163–166]. Additionally, aerosol delivery allows for enhanced access to the intra-thoracic lymphatic system either through direct drainage or intra-cellular transport. Preclinical studies have suggested that there may be benefits to aerosol chemotherapy. Inhaled liposomal formulations of chemotherapies have demonstrated superior efficacy over traditional routes for the treatment of lung metastases in preclinical models[167]. Other formulations such as aerosol particles of 5-fluorourcil (5-FU), paclitaxel, carboplatin, and gemcitabine have also been studied preclinically[164,168–174]. Clinically, chemotherapeutic drugs have been delivered to the lungs through the use of nebulizers for both free drug and liposome formulations. The liposome formulations have encapsulated 9-nitrocamptothecin, doxorubicin, and cisplatin[175–177]; however, clinical trial results to date are inconclusive and suggest utilizing caution with this approach.

Delivery of Chemopreventatives to the Lung

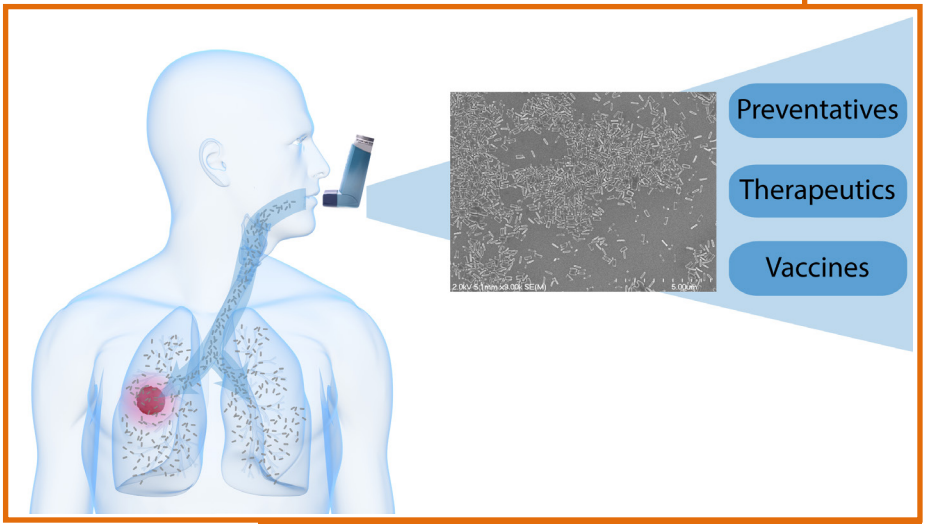

Figure 15. Depiction of aerosol based delivery of chemopreventitives, chemotherapeutics, or cancer vaccines via nanotechnology delivery with SEM image (inset) of nanoparticles designed for aerosol delivery route.

While chemotherapeutics are intended to alter disease progression following tumor establishment, chemopreventative agents are pharmaceutical interventions aimed at halting, or reversing disease progression[178–181]. Chemopreventatives can be given at a tumors' primary stage to high-risk patients, a secondary stage to patients with an identified pre-malignancy state, or a tertiary stage to prevent a secondary occurrence of the tumor[178]. To date, there have been numerous clinical trials targeting lung cancer, with minimal, or even negative, impact on disease progression. These trials have included mainly dietary supplements including various anti-oxidants, vitamins, and retinoids. Pre-clinical studies administering inhaled corticosteroids as a chemopreventative reduced cancer formation in mouse models; however, these findings did not translate to humans[182–185]. Despite these negative data, there is cause for optimism in this approach. There have been considerable successes in preclinical models involving aerosol delivery of selenium and cyclooxygenase inhibitors delivered at the primary stage[178–181]. Aerosol liposomal formulations of interleukin-2 (IL-2) have resulted in disease remission or maintenance in canine cancer models, and a number of clinical trials using nebulized IL-2 show slightly decreased tumor occurrence in humans[166,182]. Inhaled delivery of interferon, granulocyte-macrophage colony-stimulating factor (GM-CSF), and cyclosporine have also demonstrated efficacy in pre-clinical studies and, to some extent, in humans with no adverse systemic effects. Furthermore, use of oral iloprost in a randomized Phase II, placebo controlled trial for heavy smokers, has demonstrated the ability to decrease endobronchial dysplasia[186].

Lung Targeted Nano-Based Cancer Vaccines

Modulating the local immune environment of the tumor and surrounding tissue to enhance tumor eradication may be further achieved through a cancer vaccine. An ideal cancer vaccine would direct the power and precision of the patient's own immune system toward tumor elimination while providing immunological memory for rapid elimination of subsequent malignancies. The biggest challenge for cancer vaccine development is convincing the immune system that the tumor is harmful and needs to be eliminated while minimizing collateral damage in healthy tissues[183]. Achieving tumor specific immune responses requires immune targets that are exclusively (or at least preferentially) expressed by tumors, termed tumor associated antigens (TAA). The hope is that vaccines combining TAAs and immune modulating adjuvants will instruct the immune system to eliminate tumor cells.

Recent clinical trials for lung cancer vaccines incorporating non-small cell lung cancer (NSCLC) TAAs and strong immune modulators have shown measurable increases in patient survival (~3 month increase OS versus placebo control); however, none were curative[183]. Potential explanations for modest efficacy include patient selection and vaccination timing; however, another major consideration is the route of vaccine delivery. Some vaccines required multiple injections via parenteral routes[184]; however, recent pre-clinical studies using lung targeted nano-based vaccines suggest that pulmonary vaccine delivery may provide more robust immune responses with implications for targeting cancer[162,185].

Pre-clinical infectious disease models using a variety of nano-based vaccines provide protection from subsequent pathogen challenge[162,185–189]. Two of these studies directly compared pulmonary and parenteral vaccine administration and found that direct immunization of the lung provided better protection than injection at distal sites[162,188]. Part of the protective immune mechanism works through activation of cytotoxic T cells (CTLs) that seek out and eliminate cancerous cells. In addition to CTL activation, several of these vaccines also promoted TNFα and IFN-γ cytokine production, which are known to promote an anti-tumor environment by inhibiting suppressive tumor associated macrophages[162,185,190]. The added benefit of an efficacious cancer vaccine is that these immune cells roam the body and have the capacity to target sites away from the primary tumor, which has major implications for metastatic control. Support for this hypothesis includes a study in which a nano-vaccine delivered to the lung was able to eliminate melanoma in the flank and establish long-term tumor rejection and survival[162].

Future Directions for Aerosol Delivery of Nanoparticles in Cancer Management

Nanoparticle therapeutics in the lung represent an area of great potential, especially for treating cancer. To date, most aerosol therapies have involved delivery of 1-5 µm sized particles, due to their aerodynamic properties and their assumed deposition in the lung[191]. Indeed, even the chemotherapy liposome formulations evaluated in clinical trials were on the order of ~1 µm[164,167,192]. More recent nanoparticle formulations (<200 nm) could offer tremendous benefits to the three aspects of cancer management mentioned here: drug delivery (including enhanced tumor uptake), mucosal diffusion, and lymph trafficking[160]. However, delivery concerns will need to be addressed in order for nanoparticles to deliver and deposit at high efficiencies in the airways. Controlled aggregation or a "Trojan horse" approach may be required for effective delivery, with independently tunable aerodynamic properties for controlled deposition in the region of interest within the lung[173]. Additionally, advancement of particle-based lung therapies will require continued optimization of inhaled delivery devices[165,193].

Of the potential applications for aerosol cancer management, nanoparticle delivery of cancer vaccines may be best situated to make the greatest impact within the next decade. The extensive research and success in particle formulations for intravenous nanoparticle therapies can be readily translated to lung administration with minimal reformulation, while current clinical evaluations of aerosol liposome formulations establish precedence for use of a particle approach for direct vaccine delivery. The biggest challenges moving forward will be choosing the most specific TAA's, overcoming immune tolerance mechanisms and avoiding immune pathology in an already vulnerable patient population. Overcoming immune tolerance may require co-administration of therapeutic antibodies to disrupt normal lymphatic checkpoint mechanisms (anti-CTLA4, anti-PD1, anti-PDL1) and allow the vaccine to establish an immune response[194]. Another challenge will be establishing the safety of the nanoparticle platforms, especially in combination with immune adjuvants with a goal of inducing strong immune responses without damaging lung tissue. Ultimately, studies assessing patient tolerance to pulmonary-targeted nano-vaccines will be critical to the use of safe adjuvant combinations.

Aerosol chemotherapy faces a steep uphill battle to fruition. There are two deeply rooted schools of thought regarding inhaled chemotherapeutics and it is likely to remain a controversial issue. Most clinicians believe the direct delivery of highly toxic chemotherapeutics to the lungs exposes the patient to unacceptable risk, and could inflict further damage to an already susceptible tissue. The opposing argument points to the urgent need for alternative approaches for lung cancer treatment. Thus moving forward,

nanoparticle aerosol delivery of chemotherapeutics will require substantial and strategic preclinical and clinical research to discern the practical application of these therapies.

Chemopreventative agents have demonstrated success in preclinical models...

Chemopreventative agents have demonstrated success in preclinical models, but the difficulties in identifying target patient populations makes widespread chemoprevention in a primary stage cancer challenging. Evaluation of lung specific biomarkers and further characterization of the lung cancer progression will help identify patient populations likely to benefit from chemoprevention; however, dosing at a secondary or tertiary stage following the identification of pre-malignant lesions or prevention of a secondary occurrence may be more tractable. Winterhalder *et al.* suggest that cell surface receptors, such as EGFR and HER2, may be important targets to halt progression of epithelial lung cancer; given the history of systemic nanoparticle formulations targeting these pathways, this may be a tractable first nanoparticle approach[181]. Finally, there are many genetic factors in lung cancer that could be potential targets for gene therapy that are considered "undruggable" using conventional approaches, which are also ideally suited for nanoparticle formulations[195,196].

The nanoparticle approaches discussed here represent novel lung cancer management strategies that may also apply to other cancers. Additionally, topics discussed here may be better suited as combination therapies with more traditional approaches including surgical resection, chemotherapy, and radiation. We anticipate that many of these approaches will be first investigated in recurrent or late-stage disease following alternative interventions. Success in these situations may ultimately lead to a paradigm shift that utilizes aerosol-only based approaches.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will conduct further preclinical studies on direct lung chemotherapeutics use and efficacy; develop chemopreventatives to better establish effects on lung cancer progression; and identify and validate drug targets for local lung cancer vaccine therapy. Looking further ahead over the next 5 years, researchers will identify tumor associated antigens and adjuvant combinations that target lung related tumors for nano-based cancer vaccines; and carry out perspective studies on effects of direct lung therapy, positive or negative. In the next 10 years, researchers will establish a clinical development program for aerosol treatment of lung cancer, utilizing chemotherapy, chemopreventatives, and nano-based cancer vaccines.

Non-Intravenous Routes of Delivery: Oral

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Introduction

Nanoparticles (NPs) have the potential to make a tremendous impact on the treatment of cancer. Combining biological understanding with engineering and materials science principles has led to the development of nanomedicines for the treatment of cancer that are now entering clinical trials[197–199]. However, NPs are currently limited to parenteral methods of administration. In addition, many chemotherapeutic agents and biological therapeutics are limited to parenteral administration because of low bioavailability. Injection-based therapies can suffer from poor patient compliance and reduced efficacy due to the pain and inconvenience associated with the treatment regimens. Therefore, alternate routes of administration, such as transdermal, nasal, buccal, pulmonary, and oral, are under investigation as a means to improve these therapies. Of these alternate routes, oral is considered the most desirable, especially for long-term treatment of diseases, because of the convenience and improved compliance[200].

In clinical studies with cancer patients, most favored oral over intravenous chemotherapy because of the increased convenience as long as efficacy was not compromised[201–203]. The convenience of taking medications at home was especially convenient for patients that lived far from hospitals and clinics[204]. Several trials have demonstrated that oral-based therapies can be as efficacious as parenteral administration, but offered additional advantages. In one trial, oral administration of Tegafur-uracil (UFT) was compared with intravenous administration of 5-fluorouracil (5-FU) for the treatment of metastatic colorectal cancer[205]. The oral administration was associated with decreased incidence of drug-related adverse effects without compromising efficacy. Other studies have shown that intravenous methods required more frequent hospitalizations that were expensive, time intensive, and required intravenous access[206]. Oral formulations have advantages for physicians as well, providing flexibility and adaptability to tune dosing schedules to individual patients based on efficacy and toxicity[204]. Without the intensive demands on staff required by intravenous administration, studies in the United Kingdom showed that switching from intravenous to oral chemotherapy allowed a 7-fold increase in patients treated[207]. Finally, reducing hospital or clinic visits as well as costs associated by using oral formulations could reduce overall costs for cancer treatments[208–210]. Indeed, cost-benefit studies conducted in Europe and Canada examining oral versus standard intravenous regimens for colorectal cancer suggested

significant savings with the oral route despite the higher cost of the orally formulated therapies[211].

While oral delivery is highly desirable, it presents many challenges due to the number of barriers presented by the gastrointestinal tract before therapeutics are absorbed and enter the bloodstream. These barriers include extreme pH environments ranging from 1 to 8[212] and enzymatic degradation, which limit the absorption of biologic therapeutics such as proteins and nucleic acids. In addition, there is a transport barrier presented by the intestinal epithelium, which is a polarized cell monolayer that tightly regulates the transport of material from the external environment (intestinal lumen) to the *lamina propria*[213]. This intestinal epithelium is covered by a mucus layer, which protects the epithelial surface by trapping pathogens and foreign particulates and rapidly clearing them[214]. Therapeutics that reach the intestinal cell surface and enter the cells must than bypass the cells metabolic systems and P-glycoprotein (P-gp) drug efflux pumps, which can cause low bioavailability for many small molecule drugs such as chemotherapeutic agents[215]. Finally, if the therapeutics cross the intestinal transport barrier, they must avoid immune cells that patrol the *lamina propria* in order to reach the bloodstream and the mononuclear phagocyte system of the liver in order to reach other organs in the body.

Polymeric NPs are a well-studied option for oral delivery that can aid in overcoming many of the intestinal barriers

Polymeric NPs are a well-studied option for oral delivery that can aid in overcoming many of the intestinal barriers. The NPs are stable in the GI environment and can protect encapsulated therapeutics from the pH environment, enzyme degradation, and drug efflux pumps[200,216]. However, intestinal absorption of NPs is highly inefficient because the physicochemical parameters, particularly size, of NPs prevent their transport across cellular barriers such as the intestinal epithelium. To improve the absorption efficiency of NPs and make oral administration practical in the clinic, additional strategies are necessary to overcome the intestinal epithelial barrier.

Oral Delivery Strategies

There are several pathways across the intestinal epithelial barrier that could be used for oral delivery[217]. One option is the paracellular pathway, which is a major passive permeation pathway across the intestines and allows diffusion of small molecules in the space between epithelial cells. The tight junctions between epithelial cells regulate the permeability of this pathway based on the size and charge of the molecules[218,219]. Another option is the

transcytosis pathway, which is an active transport pathway that relies on receptors specific for a molecule to guide the molecule through the cell in endosomes without entering a degradation pathway. Because of their large size, NPs are restricted to this pathway.

One approach for oral delivery that has been extensively evaluated is the use mucoadhesive materials (**Figure 16A**). These are polymers such as chitosan[220], polyacrylic acid (PAA)[221], and poly(fumaric-co-sebacic) anhydride[222] that interact with the mucus layer covering the epithelial cells. Adherence to the mucus layer increases the residence time and contact of released drug with the underlying epithelium, resulting in increased drug concentrations at the site of absorption[223]. In addition to increasing the concentration of therapeutics near the epithelium, many mucoadhesive polymers increase intestinal absorption by acting as permeation enhancers, reversibly opening tight junctions between epithelial cells to allow enhanced paracellular transport[224]. Since the tight junctions are less than 20 nm in diameter, NPs are unable to pass through this pathway, but small molecule therapeutics can cross the epithelium[225]. One disadvantage of this approach is that the permeation enhancer activity

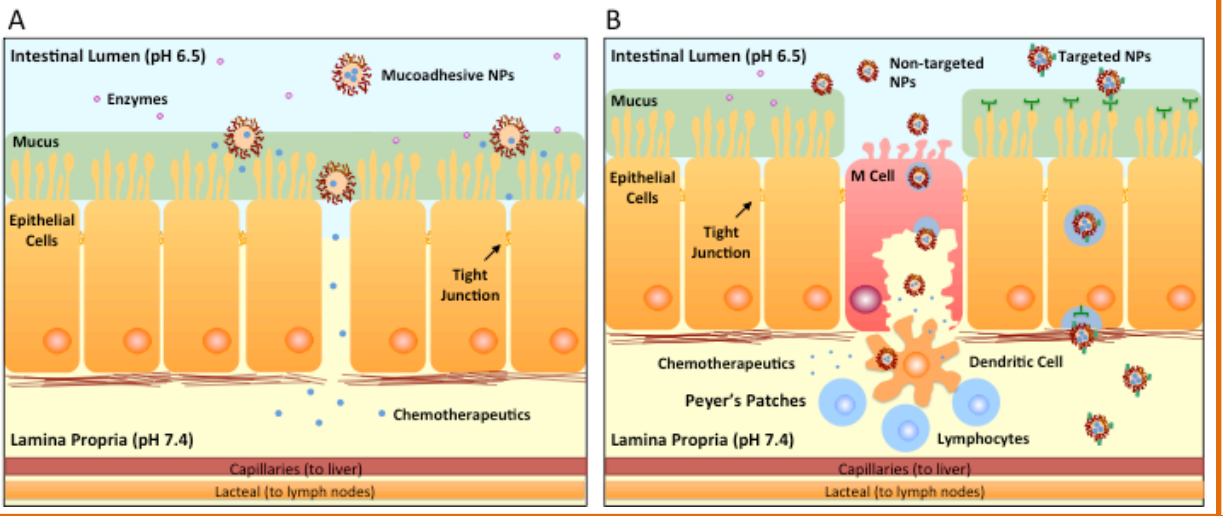

Figure 16. Schematic illustration of strategies for oral delivery. (A) Mucoadhesive materials used to form NPs adhere to the mucus layer above the epithelial cells and release therapeutics at high concentrations near the surface of the epithelial cells. In addition, they are able to reversibly open tight junctions to allow paracellular transport of therapeutics between the cells and across the epithelial barrier into the *lamina propria*. (B) The transcytosis pathway is an active transport pathway that transports material across cells in endosomes while evading degradation pathways in the cell. Examples of transcytosis pathways include M cells, which are responsible for transporting antigens across the intestines for immune surveillance and are associated with Peyer's Patches. Other examples include the vitamin B12 receptor pathway and the FcRn pathway, where NPs targeted to the specific receptors are trafficked across the epithelial cells and released in the *lamina propria*.

is non-specific, potentially allowing toxins and other pathogens present in the intestines to cross the intestinal barrier once the tight junctions are open[226,227]. Another limitation is that the surface area for absorption through the paracellular pathway is less than 0.1% of the total intestinal epithelium surface area, which could limit the capacity for absorption of therapeutics[228].

Targeting NPs to natural transcytosis pathways is another approach used for oral delivery (**Figure 16B**). It offers a way to cross the intestinal barrier without affecting the intestinal epithelium barrier integrity. There are several mechanisms that have been studied for transcytosis of NPs. The most extensively studied is the M cell transcytosis pathway. M cells are associated with Peyer's Patches, which are organized components of the gut-associated lymphoid tissue (GALT). The role of M cells is to transport antigens across the intestines through a non-degradative pathway for immune surveillance[229,230]. This pathway is attractive because M cells have reduced protease activity, lack mucus secretion, and have a sparse glycocalyx[231]. One potential problem with this approach is that since M cells are closely associated with immune cells in the *lamina propria*, NPs crossing the intestines through this pathway may be engulfed by immune cells before reaching the bloodstream and releasing their cargo[232]. Absorption by M cells may also be limited because M cells only make up a small percentage (5-10%) of the non-absorptive epithelium in humans[233,234].

Other strategies have focused on targeting NPs to receptor-mediated transcytosis pathways that are not associated with the GALT, which may help NPs evade immune cells after crossing the epithelium. One example is the vitamin B12 receptor, which traffics vitamin B12 across the intestinal epithelium[235]. NPs targeted to this pathway have been shown to successfully deliver biologic payloads to the bloodstream, although transport of NPs has not been demonstrated yet[236,237]. One potential drawback of this approach is that vitamin B12 absorption does not occur until the distal section of the ileum, requiring NPs to maintain stability and not release their cargo while traveling through most of the small intestine. Another example is the neonatal Fc receptor (FcRn), which transports IgG antibodies across the intestinal epithelium[238,239]. This receptor is expressed throughout the intestines. NPs targeted to the FcRn were able to cross the epithelium and circulate in the bloodstream to several different organs, including the liver, spleen, lungs, and kidneys, along with releasing a therapeutic payload[240].

Clinical Impact

While oral delivery has been extensively studied and many strategies have had success in animal models, there has not been much success translating the research into practical clinical solutions. Most of the effort has focused on developing technologies for oral delivery

of insulin. However, NPs are flexible in terms of the molecules that can be encapsulated and changes to formulations could easily result in NPs capable of delivering chemotherapeutic molecules. In addition, NPs can encapsulate protein therapeutics and small interfering RNA (siRNA), which are emerging treatment modalities for cancer. The major limitation to translation is that the technologies developed are not efficient enough to make them practical for the clinic. More recent technologies such as NPs targeting the B12 receptor and FcRn have demonstrated higher efficiencies, but only in animal models at this point.

There are currently several technologies that are entering early-stage clinical trials for oral delivery of therapeutics. These include Oramed's oral formulation consisting of permeation enhancers that is now entering Phase II clinical trials. Novo Nordisk is developing an absorption enhancer technology that is entering Phase I trials. Entrega is developing a mucoadhesive technology that is still in early stage development. Each of these technologies is focused on enhancing transport through paracellular pathways, which would enable drugs, but not NPs, to cross the intestinal epithelium.

As nanomedicines are shown to be effective for cancer therapy in clinical trials, future efforts should focus on translating technologies to the clinic that utilize the transcytosis pathway. These technologies could enable the NPs carrying chemotherapeutics to cross the intestinal epithelium and reach circulation. In this case, the advantages of NPs in the bloodstream could be utilized for the treatment of cancer, such as passive or active targeting of tumor cells, delivery of multiple therapeutics in a controlled or triggered release manner, and selective biodistribution of the therapeutics to the tumor to reduce side effects. Future research should also focus on discovering other natural transcytosis pathways that could be used to transport NPs across the intestines. This could include studying how some bacteria are able to cross the intestines and the subsequent rational design of NPs that could mimic those processes. In addition, new technologies such as microneedle-based pills have shown promise in improving bioavailability of biologics in initial animal studies, but need further study to determine clinical feasibility[241].

...researchers will develop NP delivery vehicles targeted to transcytosis pathways that specifically encapsulate and deliver chemotherapeutic agents...

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers

will optimize the physicochemical parameters of NPs targeted to transcytosis pathways to maximize bioavailability after oral administration; and conduct research into alternate transcytosis pathway receptors and alternative technologies such as microneedle-based pills. Looking further ahead over the next 5 years, researchers will develop NP delivery vehicles targeted to transcytosis pathways that specifically encapsulate and deliver chemotherapeutic agents; and evaluate the performance of permeation enhancer and mucoadhesive technologies currently entering clinical trials. In the next 10 years, researchers will gain FDA approval for permeation enhancer and mucoadhesive technologies that are successful in clinical trials; conduct clinical trials on NP delivery vehicles targeted to transcytosis pathways for cancer treatments; and study how patient-to-patient variability, diet, fasting states, and disease states affect the performance of these technologies in humans in order to determine the robustness of these technologies.

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