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Molecular Imaging Probe Development using Microfluidics

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Abstract

In this manuscript, we review the latest advancement of microfluidics in molecular imaging probe development. Due to increasing needs for medical imaging, high demand for many types of molecular imaging probes will have to be met by exploiting novel chemistry/radiochemistry and engineering technologies to improve the production and development of suitable probes. The microfluidic-based probe synthesis is currently attracting a great deal of interest because of their potential to deliver many advantages over conventional systems. Numerous chemical reactions have been successfully performed in micro-reactors and the results convincingly demonstrate with great benefits to aid synthetic procedures, such as purer products, higher yields, shorter reaction times compared to the corresponding batch/macroscale reactions, and more benign reaction conditions. Several ‘proof-of-principle’ examples of molecular imaging probe syntheses using microfluidics, along with basics of device architecture and operation, and their potential limitations are discussed here.

Keywords

microfluidics; molecular imaging

1. Introduction

1.1. Molecular Imaging

Molecular imaging is the “visualization” of biochemical processes from simple reactions, e.g., phosphorylation, to complex biological phenomena, e.g., cell metabolism. It allows the

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detection, localization and monitoring of such processes in cells, tissue, and living subjects at the subcellular, cellular and organ level. [1–3]. In contrast to “conventional” diagnostic imaging, molecular imaging sets forth to probe detailed molecular mechanisms that underlie diseases based upon abnormal biochemical pathways, rather than to image the end anatomical changes from these molecular alterations [2, 4]. The ability to visualizing certain biological events associated with a specific disease in real time not only provides a more meaningful understanding of the molecular basis of disease but also leads to earlier detection, accurate evaluation of the state or degree of disease, and the opportunity to evaluate therapeutic effects (Figure 1) [5, 6]. Molecular imaging allows direct biological measurement(s) *in vivo* of specific molecular states, gene function, metabolic processes and protein-protein interactions in a repetitive, non-invasive manner, helping clinicians to select the appropriate treatment according to patient’s molecular phenotypes [7, 8].

Without exerting perturbation of the system under study, molecular imaging seeks to understand the dynamic interactions between biological components during the normal processes as well as the progression of disease from a molecular perspective by various imaging technologies and contrast agents [3, 9, 10]. The field of molecular imaging is a truly multidisciplinary research involving the efforts from radiolabeling and bioconjugation chemistry to develop suitable molecular probes, cell and molecular biology to identify the relevant target/biomarker for given specific biochemical process, pharmacology to optimize the probes for improving targeting efficacy and *in vivo* kinetics, and image-capture techniques to non-invasively visualize the fate of imaging probes. Great advances in imaging instrument technologies, molecular biology, chemistry, and computing have enabled the recent emergence of molecular imaging.

Molecular imaging research promises to bring significant impacts in disease prevention and treatment for the next decade. Several of the prominent advantages include: (1) early detection and diagnosis of disease; (2) direct molecular assessment of therapeutic outcome during the initial treatment cycles (Figure 2); (3) enhanced fundamental understanding of specific biological events and signatures of many human diseases [11–16]. Especially, in the preclinical setting, it allows a repetitive, non-invasive, rather identical and uniform study on the same living subject in a relatively automated fashion with identical or different imaging probes at different time points, thus enabling longitudinal studies and reducing the cost and the number of animals. Nowadays, researchers are able to investigate dynamic biochemical processes such as metabolic activity, cell proliferation, apoptosis, receptor occupancy, reporter gene expression, and antigen modulation within living animals. In addition, as many preclinical animal models (e.g., transgenic mouse models) now become available, during drug or molecular probe development, it is particularly critical to determine the biodistribution and pharmacokinetics of lead candidates in those diseased models using molecular imaging techniques before human trials.

1.2. Imaging Modalities

Recent advances in molecular imaging techniques and rapid development of various modalities permit the non-invasive study of human anatomy and physiology in great molecular details. The potential of molecular imaging to stratify patients and discern disease states has triggered a revolution of much deeper understanding of the corresponding diseases and will hopefully translate such information into improved diagnosis and treatment. Currently, widely used molecular imaging techniques, including positron emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), ultrasound (US), and optical bioluminescence and fluorescence imaging, play critical roles in clinical and preclinical settings [3, 17–20]. Traditional cross-sectional molecular imaging techniques, such as MRI, CT and US, are based on the measurement of change on physical properties (e.g., proton density, relaxation rates,

absorption/scattering) and physiologic states (e.g., blood flow) as the main source of contrast to detect and characterize abnormal regions caused by diseases [14, 21–25]. On the contrary, currently available molecular imaging modalities, which are built on nuclear and optical imaging principles (e.g., SPECT, PET, MRI, various optical imaging techniques, and US variations), are based on specific molecules and their interactions with targets in human body as the source of image contrast [26–33]. This paradigm shift from nonspecific physical interactions to target-specific molecular sources is the very focus of current research efforts in the field of molecular imaging.

Since current modalities have their unique advantages as well as disadvantages, the suitable imaging method(s) for the biological problem or clinical condition at hand must be carefully selected and evaluated. Although every modality differs in their sensitivity, resolution (e.g., spatial, temporal, or due to contrast) and complexity (e.g., the degree of expertise required to interpret the images, or the cost of performing the studies), they are all designed to offer high specificity. Thus, efforts are currently under intensive research to perform dual or multi-modality detections, such as dual gamma/X-ray imaging (PET/CT), PET/MRI, and other combinations of modalities to compensate the issues of sensitivity and specificity.

1.3. Molecular Imaging Probe

Molecular imaging probes are a special class of pharmaceuticals that are used in conjunction with medical imaging scanners, such as PET, SPECT, MRI, CT, ultrasound etc., allowing clinicians to visualize disease in a non-invasive fashion. A probe typically consists of a *ligand* and a signaling component and is used to visualize a specific target, organ or tissue *in vivo*. After the ligand carries the signaling component to the site of interest by binding to or interacting with a chosen target, the signaling component then provides the information about its location by emitting a signal (light, radiation, etc.), which can be detected by certain molecular imaging modalities. For example, in optical imaging methods, the signaling component can be a fluorophore; in nuclear imaging, it can be a small isotopic substitution (e.g., replacing C-12 with C-11) or a non-isotopic substitution (e.g., replacing hydrogen atom with F-18) of a ligand, or chelating approaches using metal radioisotopes (e.g., Cu-64, Tc-99m); and in MRI, it can be a paramagnetic atom (e.g., gadolinium) or superparamagnetic iron oxide (SPIO) nanoparticles.

In the post genomics era, there is a great opportunity to design and develop probes to target specific biochemical signatures associated with diseases. Often the alternations in biological and biochemical processes occur before diseases reach an advanced stage. Therefore, molecular imaging could foster earlier and more personalized diagnosis of disease, allowing clinicians to visualize disease and injuries sites non-invasively. Since such information-rich images could not to be generated without a specific contrast agent, the imaging probe development is of paramount importance in the field of molecular imaging, which aims to support the molecular analyses of biological information and the diagnosis of diseases. Most available imaging probes and contrast agents are chemical or radioactive agents, which have been the workhorses for current biomedical and clinical imaging [14, 61]. In light of tremendous advances in the field of nanotechnology, many new types of nanoparticle-based probe are now available [54, 62]. The properties of such nanoscale materials, especially those smaller than 100 nm, are very different from their bulk counterparts due to the increased reactive surface area and the dominant quantum confinement effects. The dramatic increase in the surface area also increases the material's chemical reactivity and ability to connect with additional functional materials. Moreover, quantum-confinement effects, derived from the nanometer particle size, can exert size-dependent electronic, magnetic, and optical properties. In this review, some of examples will be highlighted.

In general, implementation of new imaging modalities (or even device upgrades) usually requires a significant amount of cost, effort and resources before they can be widely applied and adopted in laboratories or hospitals [14, 61]. Hence, in economical and practical points of views, the implementation of novel molecular imaging probes that can maximize the ability of the current imaging modalities might be more practical. Conducting molecular imaging research is a multidisciplinary endeavor that requires the expertise from many fields of biology, engineering, chemistry and medicine. Chemists/radiochemists, in particular, play a vital role in this effort of developing smart molecular imaging probes that can produce detectable signals that arise from low concentrations of target biomolecules associated with disease progression in cells and tissue.

To gather such detailed in vivo molecular information, the ideal molecular imaging probe should possess: (1) highly-specific ligand toward target of choice, (2) signaling component for sensitive detection at low concentration, and (3) appropriate amplification strategies if possible. Widely utilizing in nuclear imaging, most radiolabeled probes are given in very low doses [often in nanogram levels (i.e. non-therapeutic dose)] as compared with molecular imaging probes for MRI and optical techniques that are usually given in relatively large amount (typically micrograms to milligrams) (Table 1). In addition, the short half-lives (due to the rapid decay of radioisotopes) of those tracers necessitate rapid production and, perhaps, in a single dose on demand for an individual patient. Novel technology breakthrough will certainly bring great impact in the field of molecular imaging.

2. Microfluidics

In recent years, microfluidics has been a rapidly emerging field with broad advances in technology [63–69]. There are numerous microfluidic-based applications in pharmaceuticals, biotechnology, life sciences, and clinical diagnoses and becomes the technology of choice for lab-on-chip platforms [70–73]. Microfluidic devices are commonly made of a network of micron-sized channels (typically 10–500 μm) embedded in a solid substrate (e.g. glass, metal or plastic) [74, 75]. A wide variety of designs and fabrication methods are available so that the corresponding microfluidic chips can be tailored to their purpose, such as the nature of reaction processes, functions to be performed, and consideration of materials, cost, reliability and accessibility [78, 79]. For example, glass is often the material of choice for organic chemists because it is mechanically strong, compatible with a wide range of chemicals and solvents, optically transparent and supports different operation modes. Alternatively, plastic- and polymer-based microfluidic chips, especially those made of poly(dimethylsiloxane) (PDMS), provide easy fabrication, flexible design and great controllability using microvalves [74, 75, 80]. But applications in such devices are restricted to those using aqueous media since their organic solvent compatibility is low and prompt to swelling or damage [76]. To resolve these issues, the same device architecture could be fabricated with a more inert material such as perfluoropolyether polymers [77] to provide greater generality in chemical applications. Now there are several companies, e.g., Surface Logix, Fluidigm, Dolomite Centre, Rain Dance Technologies, and Advion, which can supply microfluidic chips capable of carrying out many basic functions and also offer the service for custom design, fabrication and chip-control system integration to fulfill the need of customers. Therefore, whether using commercial or home-made chips, it is highly feasible for any academic and industry research laboratories to develop their own functional microfluidic-based platform to conduct operations required in their research projects.

Many common organic reactions have been successfully performed in micro-reactors and convincingly demonstrated with tremendous benefits to aid synthetic procedures [71, 81]. Purer products, higher yields, shorter reaction times compared to the corresponding batch

reactions, and more benign reaction conditions are some of the major advantages of this technology [82, 83]. In some cases, enantioselectivity and regioselectivity are also enhanced [84]. These improvements are directly due to the effects of increasing the surface area-to-volume ratio of the microreactor [85]. Besides the shortened reaction times due to the rapid heat transfer, the microenvironment with a precisely-controlled temperature inside microfluidic devices can provide more homogenous reaction condition, which increases reproducibility and significantly improves many chemical and biological reactions [86, 87]. Compared to those reactions performed in the bench-scale apparatus, the reagent/sample consumption is greatly reduced by microfluidic-based reactors [88–91]. Moreover, despite the small amount of material produced per microfluidic chip, scale-out procedures can be implemented using a massive paralleled array of chips to increase the amount of final product thereby retaining and exceeding the original capacity [92]. The microfluidic-based chemistry has great potential as a novel technology platform for making molecular imaging probes [93, 94].

2.1. Different Designs of Microfluidic Devices

There are two major types of microfluidic reactors; those based on continuous flow or discrete droplets [63, 65, 88, 93, 95–97]. Continuous-flow devices have evolved from a simple single channel device made of a micro-sized tubing to a complicated multi-channel device sometimes integrated with heaters, fluid control mechanisms, and reagent reservoirs. The intrinsic characteristics of microfluidics serve to enhance heat transfer and a more accurate control of temperature, usually resulting in higher product yield and better selectivity. On the other hand, by using the microfluidic droplet generators, individually-isolated microdroplets can be produced to compartmentalize reagents/samples and reactions, and thus used as discrete microreactors. Capable of producing microdroplets in a rapid and reproducible fashion, microfluidic droplet generators are versatile platforms for high-throughput screening. For example, the reagent or sample ratio in each microdroplet can be fine-tuned to the desired composition and used to construct a product library for further downstream screening. Since only very small volumes are used, screening using microdroplet format can potentially conserve a significant amount of reagents and samples. The general comparisons between these two designs are listed in Table 2. Lastly, despite only a few examples, the third category named integrated microfluidics exists. These are equipped with microscaled pumps, valves, mixer, separation components, etc., and are capable for carrying out complicated processes on-chip [64, 98–101]. For example, PDMS-based integrated microfluidics offers great flexibility and capability for easy handling of extremely small-volume fluids and performing essential operations for chemical reactions, such as metering, mixing, heating, filtering, separation, etc. [102–105] These types of devices are gaining more attention due to its versatility and potential to be fully automated.

The main purpose of this review is to provide a general overview and summarize examples of applying microfluidic approaches in advancing molecular imaging probe syntheses. Especially with the rapidly growing field of microfluidics and molecular imaging probe syntheses, some great works could have been inadvertently omitted when we attempt to choose representative examples from recent highlights of microfluidic technology in molecular imaging development. For that, the authors must apologize in advance. The potential benefit of applying microfluidics-based syntheses to increase the availability and accessibility of a wide variety of molecular imaging probes could be enormous. For example, the concept of “personalized medicine”, which relies on many advanced molecular imaging techniques to detect disease and individualize/personalize treatments on the basis of a “molecular phenotype” at the time of diagnosis and throughout the course of therapy, could become practical to be carried out routinely.

3. Microfluidics for Imaging Probe Syntheses

Recently, the microfluidics-based molecular imaging probes synthesis has emerged as a new and exciting methodology [112]. It would greatly shorten the period of development, process optimization as well as the production time of imaging contrast agents; reduce reagent consumption; improve the efficiency and reproducibility of synthesis. The additional advantages of less space requirements, improved safety, facile automation, and real-time online analysis of reactions, can also be realized with microfluidic reactor systems [102, 113]. As a result, microfluidic platforms can be an ideal technology for good manufacturing practices (GMP) in the production of imaging contrast agents. Presently, many research groups produced different imaging contrast agents for PET, MRI, ultrasound, and optical imaging by employing microfluidic-based platforms. In the proceeding sections we will outline several examples of molecular imaging probes synthesized by microfluidics for different imaging modalities.

3.1. Radiolabeled Tracers for Positron Emission Tomography (PET)

Molecular imaging using PET provides unprecedented opportunities for the visualization and quantification of normal biochemical processes as well as the onset, extent, and kinetics of pathological events in vivo [114]. Consequently, PET and/or PET/CT have been established as a unique and versatile tool for a broad spectrum of preclinical and clinical applications. Despite being powerful, PET imaging is limited by the complexity of radiochemistry that produces probes labeled with positron-emitting radioisotopes of short half-lives [e.g., carbon-11 ($T_{1/2}$ = 20.4 min) or fluorine-18 ($T_{1/2}$ = 109.8 min)] [115]. In addition, as the need for PET imaging increases, high demands for a wide variety of radiolabeled compounds keep driving the exploration of novel radiochemistry and engineering technologies to improve the production and development of PET probes.

In many aspects, microfluidic devices could be the ideal platform for performing the rapid radiolabeling reactions required for PET [102, 113, 116–124]. The enhanced mass and heat transfer in such microreactors could shorten reaction times and provide better radiochemical yields (RCY) and radiochemical purities (RCP). The microreactors are ideally suited for handling and reacting small amounts of reagents (typically nanogram to microgram quantities), which is especially advantageous when the reagents are in limited supply. Easy to be shielded and isolated, using small-footage microfluidic devices to produce PET tracers is an obvious safety advantage. Additionally, automation of microfluidics has great potential to meet current GMP standards for tracer production. Furthermore the ability to manipulate reagent concentrations, reaction interfaces, and rapid mixing in both space and time within the microchannel network of a microfluidic device provide the fine level of control that is highly desired in PET probe production processes. Therefore, synthesizing PET probes using microfluidic platforms is very attractive and yet practical.

In 2004, *Lu and Pike et al.* [125] reported the first microfluidic-based radiochemical synthesis. Using a simple hydrodynamically-driven, T-shaped glass microfluidic chip, a ^{11}C -labeled methyl 3-(3-pyridinyl)propionic ester was synthesized via a direct ^{11}C -methylation. The chip (illustrated in Figure 3) is composed of two inlet ports for reagent delivery, one outlet port for product collection, and a microchannel [dimensions: 220 μm (width) $\times$ 60 μm (height) $\times$ 14mm (length) and total volume of approximately 0.2 μL] for mixing and radiolabeling. Precision flow control was accomplished by syringe pumps that accurately delivered ^{11}C -labeled methyl iodide and 3-(3-pyridinyl)propionic acid into the same microchannel. The best RCY (decay-corrected) of the corresponding ^{11}C -methyl ester was obtained (ca. 88%) at the infusion rate of 1 $\mu\text{L}/\text{min}$ at room temperature. Several ^{11}C -labeled compounds, such as a ligand for brain peripheral benzodiazepine receptor, were also synthesized via ^{11}C -methylation reactions using the microfluidic chip. The highest RCY was

65% at the infusion rate of 1 $\mu\text{L}/\text{min}$. This simple device demonstrated the unique advantages and promises of using a microfluidic for PET imaging probe synthesis.

The same T-junction microfluidic chip illustrated in Figure 3 was also applied to produce ^{18}F -labeled PET tracers using 2- ^{18}F fluoroethyl tosylate [125]. 3-(3-Pyridinyl)propionic acid and 2- ^{18}F fluoroethyl tosylate were injected by syringe pumps into the microchannels via port A and B, respectively. It was found that initial ^{18}F -fluoroethylation did not proceed at ambient temperature. After heated to 80°C , the desired product, ^{18}F -labeled ester (collected in port C), was obtained in 10% RCY. At the flow rate of 1 $\mu\text{L}/\text{min}$, the average residence time is about 12 seconds inside the microfluidic chip and total batch processing time is about 10 minutes. This further demonstrated the flexibility and diversity of microfluidic reactors to perform the radiosynthesis of PET imaging probes.

In contrast to the continuous-flow microfluidic platform, *Lee and Tseng et al.* developed an integrated microfluidic platform utilizing microvalved PDMS chips (Figure 4) to perform complicated, multi-step syntheses [102]. It uniquely integrates several functional modules with simple microchannel networks, which are controlled by microvalves. Such types of integrated devices possess essential components required for carrying out sequential radiofluorination processes: (1) microvalves providing precise control of liquid movement, isolate reagents, and control reaction mixtures; (2) peristaltic pumps facilitating mixing; (3) high gas permeability of PDMS matrix allowing solvent evaporation/exchange directly within the microfluidic channel upon heating. During the proof-of-concept synthesis of the most widely used PET tracer, 2- ^{18}F fluoro-2-deoxy-D-glucose (^{18}F FDG), four sequential processes occur: (1) F-18 is concentrated via miniaturized anion exchange column located in a square-shaped F-18 concentration loop (orange box; Figure 4): the diluted ^{18}F fluoride from a cyclotron is trapped by an on-chip anion exchange column, before being released by an aqueous potassium carbonate solution into a ring-shaped reaction loop (blue box; Figure 4); (2) F-18 drying and solvent solvent into CH_3CN to produce $[\text{K}^{2.2.2}][^{18}\text{F}]\text{F}$; (3) radiofluorination of the precursor D-mannose triflate (MT) with the $[\text{K}^{2.2.2}][^{18}\text{F}]\text{F}$ affords tetraacetylated- ^{18}F fluorodeoxyglucose (^{18}F FTAG) by heating the reaction mixture in the reaction loop and (4) hydrolytic deprotection of ^{18}F FTAG using aqueous HCl solution is accomplished in the reaction loop to yield ^{18}F FDG. The unique operation mechanism relies on the on-and-off combinations of several on-chip pressure-driven microvalves, including regular microvalves for chamber isolation, pump microvalves in peristaltic pumps for fluidic metering/circulation, and sieve microvalves in the on-chip F-18 concentration module for trapping anion exchange beads. The entire operation is highly automated by a computer and takes about 14 minutes from loading the diluted ^{18}F fluoride solution. It produced 272 μCi ^{18}F FDG with a RCY of 38% and a RCP of 97.6%. Recently, *Elizarov et al.* reported an improved coin-shaped microfluidic chip which can produce higher dose [$>100\text{ MBq}$ (3 mCi)] of ^{18}F FDG (RCY is ca 96% based on the starting activity of ^{18}F fluoride) in 14 minutes. The RCP of the product was 99.3% after passing through the off-chip alumina column. The ^{18}F FDG produced was sufficiently pure and can provide several doses for microPET scans using mice [126], demonstrating the feasibility to utilize microfluidic platforms for PET probe syntheses.

ImmunoPET, based on radiolabeled biomolecules (e.g. antibodies and their fragments), is emerging as an important means in cancer detection and monitoring therapeutic efficacy [127, 128]. Reports of radiolabeling proteins in the literature often require large amounts of samples and generally have sub-optimal labeling efficiency [6]. This has been a major obstacle preventing their more wide-spread and routine use in biological research and, ultimately, their clinical translation for imaging specific biomarkers. Optimization of reaction parameters (e.g. pH, concentration, temperature) for radiolabeling biomolecules can bring about significant improvements, but is considered impractical due to substantial

amounts of protein required for carrying out such a screening process using current bench-scale methods [121, 129, 130]. An ideal solution would be to create a miniaturized reaction platform for screening a range of conditions with minimal consumption of biomolecules and radiolabeling agents to identify optimal labeling parameters. Recently *Liu and Shen et al.* introduced a microfluidic-based method for optimizing ^{18}F -labeling of biomolecules using N-succinimidyl-4- ^{18}F fluorobenzoate (^{18}F SFB), a very versatile prosthetic group to tag a wide spectrum of biological entities [131]. The PDMS-based digital microfluidic droplet generation (DMDG) chip utilizes integrated microvalves for producing composition-specific droplets digitally in an oil-free environment. Harnessing the ability to manipulate nanoliter volumes, it enables operators to fine-tune and explore various reaction conditions in a droplet-by-droplet fashion. Using information gathered from chip-based screening, a detailed reaction profile based on variables being tested can be established and the optimal conditions can be selected in a fast, sensitive and reagent-economic fashion (Figure 5).

As a proof-of-concept, a novel immunoPET agent, ^{18}F -labeled anti-PSCA (Prostate Stem Cell Antigen) diabody which is an engineered antibody fragment specific to prostate cancer, was produced using the optimal condition obtained from chip screening [131]. During the optimization stage, compared to conventional methods, this microfluidic-based approach can reduce the protein consumption by 200- to 2000-fold. Furthermore, it took only 20 minutes to screen 10–12 different reaction conditions, mainly limited by the readout speed. In the case of anti-PSCA diabody, 25–30% radiolabeling yield can be typically obtained under the optimized conditions. Under the chip-optimized condition, the ^{18}F -labeling reaction was also successfully scaled up in batch-scale, resulting in sufficient quality and quantity for imaging mice with prostate tumor xenografts using microPET. This versatile microfluidic platform can be employed broadly for optimization of radiolabeling reactions of a diverse spectrum of biomolecules, including intact antibodies, their engineered fragments, other proteins and peptides, etc. This approach can ultimately accelerate the development and investigations using novel biologic-based PET tracers by providing optimized facile radiolabeling of biomolecules.

3.2. Microbubbles for Ultrasound Imaging

Ultrasound (US)-based imaging technology is widely used in current clinical practice, because of its safety, low-cost, and easy-to-use [20, 132, 133]. In order to enhance the images, contrast agents which are acoustically active can be used. Although some consist of liquid-based particles, the majority of contrast agents are microbubbles filled with innocuous fluorinated gases, such as sulfur hexafluoride, octafluoropropane, or decafluorobutane, which exhibit low solubility in aqueous media. As a result, the contrast of US imaging could be substantially enhanced by detecting the different echogenicity between the encapsulated gas in the microbubbles and the surrounding soft tissues [134]. In addition, microbubbles can be target-specific through conjugation of targeting ligands onto their surface or tailoring the desired chemical coating of the microbubble shell. For example, such types of microbubbles can specifically recognize abnormal endothelial cells at the disease sites, which can be identified by an intensified ultrasound signal [10, 59, 135–137]. Because of their great sensitivity, microbubble-based contrast-enhanced US scan is becoming one of the preferred modalities for molecular imaging of diseases. In fact, US imaging is currently undergoing major revolution since the commercial availability of microbubble-based contrast agents. However, due to the large size of these microbubbles (in the range of μm), they are limited to image intravascular targets. Even so, a number of specific biomarkers are upregulated on the vascular endothelium surface around various disease sites, such as angiogenesis, inflammation, atherosclerosis, and other endothelial dysfunctions. Those are important targets to be imaged and investigated with targeted microbubbles by US imaging [138].

The level of imaging enhancement is strongly related to the microbubble diameter and its size distribution [139]. Due to bandwidth limitation (2–10 MHz) in US imaging, the optimal size of the microbubbles should range between 1–5 μm [138]. The current production of microbubbles uses mechanical agitation techniques by which gas at the ambient environment is introduced into the aqueous solution. Despite this easy and convenient method of production, the microbubbles usually have large size variance. Since only a certain size of microbubble can be detected by the frequency bandwidth of ultrasound imaging system, the polydispersity of microbubbles reduces the sensitivity and quality of ultrasonic molecular imaging. For instance, in scheme of the ultra-sensitive detection, only a small amount of targeted microbubbles are retained at target sites of pathology (on the order of 5 to 25 per mm^2) [140, 141]. Therefore, it is crucial to optimize production process to obtain size-controlled monodisperse microbubbles, especially for targeted ones.

To resolve issues related to the size variation of microbubbles, *Lee et al.* developed a practical microfluidic-based method to produce size- and shape-controlled, monodispersed microbubbles, which can be modified with targeting ligands [139, 142, 143]. Utilizing microfluidic flow-focusing system, a stream of gas can be sandwiched between flows of liquid and focused at the orifice of the flow-focusing geometry. Then the flow thread breaks into a continuous stream of uniform microbubbles due to a combination effect of shear force and surface tension. Within this system (Figure 6), the size of the microbubbles primarily depends on the orifice size, gas pressure, and liquid flow rate. By adjusting the gas and liquid flow rates, the microbubble size can be precisely controlled and, therefore, it greatly eliminates the size disparity issue. For example, the polydispersity (i.e. size variation) of the resulting microbubbles produced by this microfluidic-based method is much less than that of commercially available contrast agent Definity[®] ($3.7 \pm 0.2 \mu\text{m}$ v.s. $2.7 \pm 2.0 \mu\text{m}$, see Figure 6). For targeted US molecular imaging, they succeeded to apply two hydrodynamic flow-focusing regions to generate the mono-dispersed specific lipid-coated microbubbles, whose size range is suitable for US imaging [139, 143]. They are encapsulated by a shell of lipid and an emulsifier containing a hydrophilic polymer moiety and a lipid anchor. The lipids are pre-conjugated to a cyclic Arginine-Glycine-Aspartic Acid (RGD) peptide for molecular recognition. The resulting microbubbles are stable for several hours in solution. As a molecular contrast agent for US, this size-controlled, lipid-stabilized, multilayer gas lipospheres conjugated with RGD could specifically target $\alpha_v\beta_3$ integrin-expressing cells [139].

The microfluidic platform has been shown to be a very efficient tool to produce uniform microbubbles as US contrast agents. These microfluidic-produced microbubbles give signal significantly greater than that from polydispersed ones. Several-fold of increase in sensitivity was reported due to the narrowed size distribution of microbubbles. From the data of acoustic testing, it was found that higher correlation between echoes of nearly monodispersed microbubbles compared with that of polydispersed ones. It implies that optimizing the size distribution of microbubbles has great potential to enhance the sensitivity of US imaging. In summary, through the size-controlled production using the microfluidic chips, monodispersed, targeting ligand-conjugated US contrast agents (microbubbles) could provide higher tissue/organ contrast and better imaging sensitivity and target specificity.

3.3. Fluorescent Tags for Optical Imaging

3.3.1. Quantum dots—Quantum dots (QDs), a novel class of fluorescent nanoparticles, exhibit distinct narrow luminescence characteristics compared to those of traditional small organic molecule-based fluorophores [17, 144, 145]. They are inorganic semiconductor nanostructures with physical dimensions that are smaller than the exciton Bohr radius [145].

In most cases, the sizes of the QDs are below 100 nm; within this size range, these nanometer-sized structures have tunable properties. QDs exhibit many advantages over conventional organic fluorophores, such as continuous absorption wavelength from UV to near-infrared, large molar extinction coefficients, high tolerance to chemical/biological/photobleaching, long fluorescence life-times, narrow emission band width, and high quantum yields. Therefore, they could be ideal signaling components for molecular probes used for imaging.

To be clinically useful, QDs have to be able to effectively, specifically, and reliably target a specific organ or disease site without non-specific binding [146, 147]. Although specific targeting ligands can be decorated onto QD's surface, in vivo molecular imaging using QD bioconjugates is still challenging due to their relatively large overall hydrodynamic size (typically >20 nm), non-specific binding, and short circulation half-lives in blood. In addition, despite many synthesis methods exist, several challenges still remain in the QD production procedures. For instance, it is very difficult to produce large amounts of QDs (>1 g) with a high chemical yield (>95 %) and a narrow fluorescence emission [full width at half maximum (<30 nm)]. Reactions to make QDs are extremely sensitive to synthetic conditions, such as temperature ranges, heating/cooling speed, and mixing kinetics, etc. Under non-uniform reaction conditions, products tend to be continuously distributed in size. To obtain desired high-quality, uniform-sized QDs with optimal chemical, physical, and optical properties, microfluidic devices have been employed for such endeavors [148–153]. The microfluidic systems with highly controllable microenvironment, where the temperature, reaction time, mixing speed can be precisely adjusted, is very suitable for QD syntheses. At the same time, concentration of precursor, and types of ligands can be screened rapidly and reproducibly within microfluidics as well [154–156]. Furthermore it is possible to integrate several micro-reactors together to perform parallel or multi-step QD syntheses to increase output for industrial applications without the conventional scale-up issues. By fine-tuning physical properties and screening the condition with high reproducibility, the microfluidic reactors show the great promise to accelerate the development and production of novel QD-labeled molecular imaging probes.

There are two major methods for microfluidic-assisted nanoparticle syntheses: single-phase continuous flow synthesis and two-phase emulsion-based microdroplets/segmented flow synthesis [88, 157–159]. Although there are many reports demonstrating the utilization of continuous-flow micro-reactor to prepare QDs [148, 160–162], most of them use a single-phase continuous flow synthesis in which precursor solutions are premixed and then delivered into a pre-heated reaction area within the microreactor. The precise control of residence time can alleviate some of reproducibility issues. However, in many cases, more than two reagents are required to prepare certain QDs, such as those with multi-core-shell structures. For example, to overcome this limitation and make core-shell QDs (Zinc Sulfide (ZnS)-coated Cadmium Selenide (CdSe)), *Maeda's et al.* utilized a simple micro-reactor composed of a mixer and a capillary tube to carry out two reactions in sequence in a continuous-flow fashion (Figure 7) [163]. By adjusting the length of the capillary tube and the flow rate, the reaction time (i.e. the residence time in heating channel) can be well-controlled so that the size and layer thickness of ZnS-coated CdSe can be fine-tuned. In order to facilitate efficient mixing, the residence time of CdSe nanoparticles in the mixer must be greater than or equal to the diffusion equilibration time, which represents the time required for ZnS raw material to mix with the CdSe nanoparticles completely, before the final coating process at 220 degree. Therefore, although the QD size and layer thickness of ZnS coating can be directly adjusted by the flow rate, the importance of the mixing efficiency of the micromixer cannot be underestimated.

However, the continuous flow reactor design still suffers from slow mixing and broad residence time distributions, which are detrimental to the QD size distribution. QDs which are just produced upon reagent mixing can also nucleate and deposit on the walls, leading to runaway growth and channel clogging. This promotes unstable reactor conditions and thus lowering reproducibility and increased size polydispersity. Aiming to improve the mixing efficiency as well as the dispersion and deposition challenges inherent to QD synthesis, droplets/segmented flow microfluidic devices were developed [164]. Several research groups have reported successfully using droplet-based/segmented flow microfluidic device to perform high-temperature QD synthesis (Fig. 8) [164–168]. The general strategy is to inject precursors of QDs into microfluidic mixing channels with individual flow rate control and then split the stream by the second immiscible phase (oil/air) to generate nanoliter droplets/segments. Compared to that obtained from continuous flow method, the enhanced mixing efficiency and narrow residence time distribution improved the reaction yield and narrowed the QD size distributions [149, 168].

The ability to apply tunable, biocompatible, and efficient coatings on QDs to make them probes for molecular imaging and reagents for biological applications is critical. *Sinton and Moffitt et al.* utilized the microfluidic-based droplet generator to self-assemble of an amphiphilic polystyrene-*block*-poly(acrylic acid) block copolymers and polystyrene-coated QDs into composite micelles [149]. Even though mixing time was greatly reduced by chaotic advection within the sinusoidal mixing channel using droplet-based approach (Figure 8C), it was later discovered that this enhanced mixing alone is not sufficient to improve QD size distributions. The mean composite QD sizes and polydispersities are both significantly decreased in the post-formation channel by tunable shear-induced processing, implying the significant effects from shear-induced particle breakup within the recirculating flow fields of droplets. Along with new insights on size and polydispersity control from self-assembly of polymeric coatings, and other nanoscale building blocks onto QDs (under the equilibrium or non-equilibrium conditions), these results showed general strategies to fine-tune the formation of micro- and nanoemulsions via microfluidic-based QD syntheses [169].

3.3.2. In situ Probe Synthesis for In vitro Fluorescence Imaging using an Integrated Microfluidic Platform—Fluorescently-labeled probes are common reagents for cell-based binding assays used by microscopic evaluation. However, considerable amount of fluorescent tag and biomolecules (e.g. antibody, hormone, growth factor, etc.) are required since the experiments are often carried out on a macroscopic scale. In addition, the syntheses and purification of fluorophore-labeled probes are also a time-consuming task. To conserve precious reagents, Tseng *et al.* developed an integrated microfluidic platform, which can automatically perform fluorophore labeling of biomolecules on a nanogram scale [170]. This microfluidic device is composed of two functional modules: (i) a circular mixer powered by an on-chip peristaltic pump as a reaction loop for fluorophore-labeling reactions and (ii) four individually addressable on-chip cell incubation chambers for examining cell binding using fluorophore-labeled probes under microscope (see Figure 9). As an example, epidermal growth factor (EGF) (3 ng) was first loaded into the reaction loop and then a fluorescent tag, Alexa Fluor 594 NHS ester (0.73 ng), was added. After 2 minutes, the excess (unreacted) tag was quenched by oligo-PEGylation (with 2-[2-(2-aminoethoxy)ethoxy]ethanol). The resulting Alexa Fluor 594-labeled EGF solution was directly applied to cells cultured in the microfluidic chambers to perform binding assays. The entire process can be completed within 30 minutes. When compared to studies using traditional Petri dish or multi-microwell formatted slides, microfluidic-assisted experiments are much more time-efficient, consume much less reagents, and are programmable can be fully automated, making these platforms a very promising new tool for biological researches. This microfluidic platform is very useful for labeling extremely small volume of

protein with fluorophore of choice to conduct molecular imaging studies even with as few as hundreds of cells.

3.4. Probes for Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is very useful molecular imaging technique in biomedical research and clinical applications [54, 171, 172]. It provides high-resolution anatomic images of all body organs, including soft tissues and can be used for visualizing functions of cardiac, brain, and central nervous system, as well as detecting tumor. Commonly available MRI contrast agents come from paramagnetic complexes: gadolinium (Gd^{3+}) chelates and superparamagnetic iron oxide (SPIO) conjugates, a nanoparticulate MRI probe [173–175]. For Gd-based contrast agents, they enhance the signal in T1-weighted images [54]. And for SPIO conjugates, they provides a strong contrast effect in T2-weighted images. From many recent developments in molecular and cellular imaging, nanoparticles as MRI contrast agents have been increasingly applied as molecular imaging probes [176–178]. The nanoparticulate MRI contrast agents are often composed of three parts: (1) nanoparticle cores for contrast enhancement; (2) water-dispersible shells for prevention of non-specific binding as well as increment of biocompatibility and (3) bioactive materials for target recognition. It is well known that the size, shape and size distribution of core nanoparticles affect the contrast resolution of MRI [179]. Therefore, in the application of nanoparticle-based MRI contrast agents, it is highly desirable to have uniform size of core material, which is one of the most important parameters for molecular imaging [54]. Conventionally, the nanoparticles were produced by the solution-phase chemical reduction of ionic iron precursors, which often results in the formation of polydispersed and poorly crystalline iron oxide nanoparticles with inferior magnetic properties [172, 176]. To circumvent this issue to obtain high-quality MRI imaging, different procedures for producing size- and shape-controlled nanoparticles are being explored.

As indicated in the previous sections, microfluidic technology has demonstrated its ability to improve size- and composition- control in the field of nanoparticle synthesis [152, 165, 180]. SPIO nanoparticles, a versatile multifunctional material, have been widely used for MRI imaging, magnetic separation, drug delivery, and even hyperthermia cancer treatment. As dominantly used core materials in MRI contrast agents, SIPO nanoparticles have been prepared by continuous flow microfluidic devices [181–185]. For example, Abou-Hassan and co-workers reported the use of millimetric coaxial continuous flow microreactor to perform such syntheses (Figure 10A) [186–188]. The iron “precursor” solution (a mixture of $FeCl_3$ and $FeCl_2 \cdot 4H_2O$) was injected into the inner capillary and the alkaline solution [tetramethylammonium hydroxide (TMAOH)] was introduced into the outer capillary by two syringe pumps. The use of three-dimensional geometry coaxial flow microreactor can avoid adsorption/deposition of any precipitate species onto the PDMS walls that might promote channel clogging. The flow rates of reagents and reaction residence time can be adjusted precisely so that the stable magnetic colloidal iron oxide particles with a size less than 7 nm can be prepared. In addition, the multistep syntheses can be carried out by this device, in which several sets of the coaxial flow microreactors are connected together to synthesize multi-layered nanoparticles, such as magnetic/fluorescent $\gamma-Fe_2O_3@SiO_2$ core/shell nanoparticles (Figure 10B) [188]. Encapsulating the $\gamma-Fe_2O_3$ nanoparticles in silica shell provides a protective, biocompatible, inert, and hydrophilic surface. It is possible to perform further on-line modifications of the silica surface and conjugation of specific ligands and fluorophores for targeted MRI/optical imaging.

On the other hand, droplet-based microfluidic systems are also being utilized to make magnetic iron oxide nanoparticles as MRI contrast agents [183, 189]. In Griffiths’ system, before synchronously fused by electrocoalescence, droplet pairs containing iron salt (Fe^{2+} and Fe^{3+}) and alkaline solution (NH_4OH) were reliably generated based on hydrodynamic

coupling of two spatially separated nozzles (Figure 11) [183]. SPIO nanoparticles are formed immediately upon the first contact with base after fusion, indicating that reaction required fast mixing (*ca.* 2 ms). Although hydrophilic nanoparticles are formed very fast and precipitate, the oil-based droplet approach can prevent particles from blocking the channels due to the hydrophobic interactions between SPIO nanoparticles and oil. As a result, the nanoparticle sizes (*ca.* 4 nm) produced by this microfluidic-based method are similar to that of the smallest particles synthesized by other processes. In addition, the average size distribution of particles obtained from the fast microfluidic mixing is smaller (4 ± 1 nm) than that from bulk mixing (9 ± 3 nm). Furthermore, the droplet-fusion step to modify core-shell nanostructures provides the microfluidic-produced nanoparticles the additional flexibility needed for extracellular targeting, enhancing their biocompatibility, improving drug loading, and increasing signal-to-noise ratio. Such microfluidic platforms could have the potential to profoundly improve development of MRI-based molecular imaging contrast agents.

Summary and Outlook

Molecular imaging allows one to assay and identify important and key alterations in molecular pathways, signal transduction, and receptor expressions from molecular disorders in a non-invasive fashion. It is a multidisciplinary endeavor of which is set to revolutionize our understanding and treatment of human diseases. The essential tools of molecular imaging are the imaging modalities and their corresponding probes. With continuous improvements in instrumentation, identification of better imaging targets by genome-based approaches, and design of optimal imaging probes by innovative chemistry, molecular imaging fueled with these scientific and technical advancement promises to have great impacts in disease diagnosis and therapy.

However, most molecular imaging techniques cannot proceed without proper imaging probes. The issues encountered in designing molecular-imaging probes are similar to those encountered in designing new drugs, although the criteria for imaging probes are often more stringent due to the consideration of their pharmacokinetic, half-lives, QC/QA (quality control/quality assurance) within limited period of time, etc. As the result, the continuous development and discovery of novel molecular imaging probes specific to disease signature and biochemical processes are indeed challenging. Currently, the field of molecular imaging probe chemistry is still largely based on the traditional organic synthesis of labeled drug analogs, and inorganic or polymer composites. High-throughput screening of relevant chemical/biological libraries that could cover extensive chemical/biological space and be generated very rapidly is the key for advancing the field of probe discovery. On the other hand, to move forward into the clinic, the production of molecular imaging probes will require more innovative chemistry for optimization and reliable technologies suitable for scaling up. In our opinion, microfluidic technology could be the answer for both. For many chemical reactions it is highly desirable that reagents are rapidly and intimately mixed. Within the microfluidic environment, increased surface area-to-volume ratio is the key enhancement of rapid mass transport (i.e. improved mixing) and significantly improved heat transfer. Thus many chemical reactions, performed in microfluidic devices, have been ameliorated in terms of reaction rates, product yields and/or selectivities compared with their corresponding bulk reaction systems. In addition, the massive parallel processing using microfluidic chips can overcome the scale-up issues.

In summary, although the field of microfluidic is still in its early adolescence, as demonstrated in all of the abovementioned examples, the use of microfluidic reactors for synthesizing molecular imaging probes can harness those unprecedented advantages as a means of improving their yields, quality and availability. What emerges from those examples of the integration of microfluidic-based probe syntheses into molecular imaging is

the vision of the future, a presentation of great opportunities for exploiting benefits from microfluidics, including superior reaction control, enhanced product yields, enhanced reaction kinetics, production reliability, safety and facile automation and integration with downstream processing, all of which are essential for good manufacturing practices (GMP) in the molecular imaging probe production for routine *in vivo* use. With specific molecular imaging probes, molecular imaging turns early stage diagnosis and treatment into possibility, and is indispensable in the coming era of individualized therapy and therapeutic monitoring.

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Figure 1.

Concept of molecular imaging and personalized medicine. (A) A patient diagnosed with a certain disease (orange arrow: existing disease site) (B) A molecular imaging probe specific to this disease administered to the patient; (C) The location(s) of disease visualized by the accumulation of molecular imaging probes; (D) Molecular imaging modalities used to locate disease site (e.g. PET and MRI); (E) According to the result revealed from molecular imaging, appropriate therapeutic or surgical procedures can be applied to eradicate disease.

Figure 2. Molecular imaging used to evaluate tumor response to therapy. (A) Combined 2- $[^{18}\text{F}]$ fluoro-2-deoxy-D-glucose ($[^{18}\text{F}]$ FDG) PET/CT scan of left lower extremity tumor (sarcoma) prior to initiating chemotherapy. (B) Combined FDG-PET/CT scan after 4 weeks of chemotherapy. Although it is difficult to compare the tumor size (before and after treatment) visually, the tumor is much less metabolically active demonstrating that the chemotherapy is having an effect on the tumor cells.

Figure 3.
A T-shaped glass microfluidic chip for synthesis of ¹¹C-labeled carboxylic ester via [¹¹C]methylation.

Figure 4. Schematic illustration of a microvalve-based integrated microfluidic platform for $[^{18}\text{F}]\text{FDG}$ production.

Figure 5. Workflow of scouting ^{18}F -labeling condition of biomolecules using a digital microfluidic droplet generator (DMDG) before scaled-up production used for microPET imaging. (A) The design and picture of a DMDG chip. In the detailed architecture (bottom) of a DMDG chip, food dyes were used to aid visualization of different components. (B) Schematic illustration of screening a reaction condition (pH) in each droplet. The final labeling yield (%) is used as a read-out. (C) Under the optimized reaction condition, ^{18}F -labeled biomolecule can be produced efficiently for *in vitro* and *in vivo* studies.

Figure 6.

(A) Schematic illustration of a microfluidic flow-focusing system for generating microbubbles. (B) Enlarged photo of the core area for microbubble generation. (C) A size distribution plot to show the comparison between the microbubbles generated by the flow-focusing microfluidic device and the commercial US imaging agent (Definity®).

Figure 7. Schematic illustration of continuous flow microreactor for multi-step synthesis of ZnS-coated CdSe QDs.

Figure 8. Various designs of droplet/segment-based microreactors. (A) A gas-liquid segmented flow reactor with multiple temperature zones [168]. The reactor is made by silicon and glass, therefore can be operated at high temperatures, while maintaining excellent mixing and control of residence time distributions. (B) A multifunctional and high-efficient microfluidic device for droplet generation and fusion. Well-controlled CdS nanoparticle syntheses were carried out using this design [167]. (C) A two-phase gas-segmented microfluidic reactor to perform the self-assembly of multi-component micelles with block copolymers and polymer-coated QDs [149]. The size distributions of nanoparticles were further narrowed by the enhanced recirculating flow fields within the liquid plugs in the post-formation channel.

Figure 9.

(A) Schematic illustration of microfluidic chip used for labeling EGF and the subsequent on-chip cell binding studies. The two functional modules of the microfluidic chip: (1) the circular reaction loop (top) and (2) four cell incubation chambers (bottom). The operation of the microfluidic chip is controlled by pressure-driven valves; delegated responsibilities are illustrated by their colors. Red identifies regular valves for controlling flow of solutions containing reagents and cells. Green identifies circulation pump valves for active mixing inside the reaction loop. Blue sections represent cell culture chambers. (B) The photograph of the entire microfluidic chip. The various channels are loaded with food dyes to help visualize the different layers of the microfluidic chip: green for fluidic channels and red for control channels. (C) Fluorescent micrograph of on-chip cultured A431 cells after incubating with Alexa Fluor 594-labeled EGF.

Figure 10.

(A) Schematic illustration of a coaxial flow millichannel reactor for preparing SPIO nanoparticles. (B) A tandem multistep microfluidic-based method for making magnetic/fluorescent $\gamma\text{-Fe}_2\text{O}_3 @\text{SiO}_2$ core/shell nanoparticles. Abbreviations: TMAOH: tetramethylammonium hydroxide; APTES: (3-aminopropyl)triethoxysilane; TEOS: tetraethyl orthosilicate; RITC: Rhodamine B isothiocyanate.

Figure 11.

Two-step synthesis of magnetic iron oxide nanoparticles by using a droplet-based microfluidic device: (A) hydrodynamic coupling of two spatially separated nozzles to generate droplet pairs. (B) Synchronous fusion of droplet pairs by electrocoalescence.

Table 1

Imaging Modality	PET [6, 34–39]	SPECT [40–44]	Fluorescence Imaging [45–49]	Bioluminescence Imaging [50, 51]	MRI [52–57]	US [10, 58, 59]	CT [60]
Tracer Energy source	Radionuclide; positron emitter and annihilation reaction –photons	Radionuclide Gamma rays	Fluorescent dye/ Quantum dot Visible to near-infrared light	Luciferase-luciferin Visible to near-infrared light	Paramagnetic atom (Gd) Radiofrequency waves	Microbubbles Sound waves	Iodine based intravenous contrast X-rays
Spatial resolution (mm)	1–10	1–15	2–10	3–10	0.01–0.1	0.1–1	0.5–1
Tissue depth (mm)	>300	>300	<20	<10	>300	<200	>300
Molecular probe quantity (ng)	1–100	1–100	$10^3 - 10^6$	$10^3 - 10^6$	$10^3 - 10^6$	$10^3 - 10^6$	N/A

Table 2

Design	Pros	Cons	Ref.
Continuous-flow	high-throughput, easy to operate, high reproducibility, easy for multistep reactions	Easy blockage of the microfluidic channel, low mixing efficiency (Laminar flow), significant sample consumption	[63] [82, 84, 106–108]
Droplet-based	precise control of reaction parameters, rapid mixing (more homogeneous), sample economy, without contacting microfluidic channel (oil)	Oil removal after reaction, complicated setup for multistep reactions	[92, 93, 95, 109–111]