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Peptides for optical medical imaging and steps towards therapy

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Abstract

Optical medical imaging is a rapidly growing area of research and development that offers a multitude of healthcare solutions both diagnostically and therapeutically. In this review, some of the most recently described peptide-based optical probes are reviewed with a special emphasis on their in vivo use and potential application in a clinical setting.

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1. Introduction

Over last decade, optical medical imaging technologies have received growing attention as non-invasive methods to allow the visualization and monitoring of biological processes at a molecular level, with the potential to monitor patients response to therapy in real-time.1–15

These techniques make use of fluorescent probes that typically constitute a recognition moiety that targets a disease biomarker conjugated to a fluorophore thereby providing a signal at the target site. Among the myriad of fluorescent probes generated those based on peptide-based recognition elements are of particular interest with multiple applications reported within a clinical setting for early disease diagnosis or directing surgery.

Fluorescent peptide probes rely on a labelled-peptidic sequence that is able to bind selectively or become modified to/by the molecular target overexpressed at the disease site. Compared to antibodies, peptides offer a multitude of advantages including rapid distribution, reduced or absent immunogenicity, ease and scalability of synthesis and affordable labelling.6 In addition, an increasing number of novel potential molecular ligands for cancer, bacterial infections and other pathological targets are peptide-based and these have begun to be widely employed for disease imaging in vivo.7 This review will focus on peptide-based optical imaging agents. Due to the complex regulatory issues, possible toxicity, tissue localization concerns and the now documented lack of an enhanced permeability and retention (EPR) effect in humans,116 nanoparticle-based probes have been specifically excluded.

1.1. Applications

In oncology, the identification of tumour boundaries is critical for appropriate surgical resection and surgery remains one of the most effective ways to treat cancer,8 with the precise removal of the tumour, a dominant factor in reducing the frequency of local disease recurrence alongside improving the subsequent efficacy of chemotherapy and radiotherapy.9,10 However, during surgery, it is often challenging for surgeons to confidently distinguish between malignant and normal tissues based on visual inspection or palpation. MRI, PET and CT can be employed, but direct use during surgery is instrumentally complex (e.g. MRI), or hazardous (e.g. radioactivity for PET)11,12 and even despite their potential application, the molecular resolution of these platforms maybe unable to identify microscopic tumour deposits.

Intraoperative optical imaging, using fluorescent imaging, is a relatively novel approach that relies on fluorescent contrast agents, offering clear advantages such as non-invasive real-time imaging, at high resolution, in the absence of ionizing radiation and at reasonably low cost.13 Furthermore, these systems have other potential applications including detection of proximal metastases and assessment of therapeutic response to treatment.14 Nevertheless, the use of light presents some issues, such as light scattering and tissue absorbance limiting the ability to quantify fluorescent signals and detect targets deeper than a few mm (even with NIR dyes).15,16 Despite this, optical imaging systems have been shown

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to be suitable for translation into clinical settings. Image guided surgery has been used on patients with ovarian cancer using a folate-fluorescein probe and other sensors are undergoing clinical trials as will be described in the next sections.

In the case of microbial infections, direct in situ imaging of bacteria and fungi would allow rapid and accurate diagnosis of infection, improving therapy and facilitating specific antimicrobial treatment.

Optical medical imaging of inflammation is a major area of research and clinical development. The pathways of acute inflammation, resolution, repair and regeneration are crying out for molecular imaging biomarkers to guide therapeutic intervention and disease stratification.

Thus, several fluorescent probes are available for studying such biological processes in small animal models, and probes have begun to be used in human trials with applications for human diagnosis within a clinical setting. Molecular optical imaging is still in its infancy and over the last few years there have been much progresses in this field in terms of probe design and device technology to overcome many of the limiting factors and improve disease detection in vivo. In support of this, the range of commercial targeted fluorescent probes has expanded significantly in the past decade. Several companies provide optical probes for small animal in vivo imaging (PerkinElmer – ProSense, MMSense and BacteriSense. Licor – IR800CW RGD, Vergent Bioscience – probes for caspases and cathepsin imaging) although to our knowledge none of them have made the full transition to licensed clinical use. In this review, we will focus on the most promising peptide-probe candidates for tumour and bacterial infection imaging in vivo reported recently. We will discuss the probe designs and modes of action that has led some of these probes entering into clinical trials. We will classify the probes according to their mechanism of action/emission of light namely affinity and activity probes while the potential of advances such as fluorescent assisted robotic surgery will also be mentioned.

2. Affinity probes

Peptide based affinity probes typically consist of a peptidic recognition moiety attached to a fluorescent dye with the peptide moiety driving binding and accumulation of the fluorescent tag at the biological target. The dye is usually passive in nature but it can be “switched-on” upon binding due to environmental changes.

There are many examples such as the in vivo imaging of nerves with the probe NP-41, (Ac-SHSNTQTLAKAPEHTGK[Dye]-NH2) that binds preferentially to peripheral nerve tissue after systemic administration. Investigations in mouse models have shown this could become a unique tool to facilitate nerve repair surgeries and an aid to prevent accidental transection.

Another example is a probe that binds bacteria (Fig. 4), but that only becomes fluorescent due to the switch-on upon binding. In addition, there are many examples of probes that have an amplification effect based on the application of a multi-valent scaffold.

In this review, we have focused on affinity probes for oncolgy applications based on receptor binding (mainly c-Met, Cholecystokinin-2 (CCK2), gastrin-releasing peptide receptor (GRPR)α,β, integrins, somatostatin and EGFR; and affinity probes for bacterial detection based on binding ligands (mainly Vancomycin, Ubiquicidin, Apidaecin and Polymyxin).

2.1. Affinity probes in cancer detection

2.1.1. c-Met

The over-expression of the transmembrane human growth factor receptor c-Met is known to be associated with tumour growth and correlates with a poor clinical prognosis in many cancer types. A high affinity optical probe targeting the human c-Met protein, EMI-137 (1), consists of a sulfonated Cy5-tagged 26 aminoacid peptide with two disulphide bridges (see Fig. 2). Since c-MET is expressed in early stage colon cancer lesions, that often are not readily visible during white-light endoscopic procedures, a first-in-human pilot study demonstrated that molecular imaging with the probe was feasible and safe, and enabled the detection of early-stage lesions. Fifteen patients at high risk of colorectal neoplasia were included in the study. Fluorescence colonoscopy with EMI-137 was able to identify additional polyps that were not visible with white light. EMI-137 (1) is currently in phase IIb for colorectal cancer (Fig. 1) (NCT2016-002827-27). Other pilot studies in phase I/II will evaluate the probe and fluorescence imaging to improve the accuracy of surgical excision in patients with breast cancer or oral cancer (NCT2014-003354-13) and in conjunction with another optical probe, identify potentially malignant pulmonary lesions during bronchoscopy (NCT20167050).

2.1.2. Cholecystokinin-2 (CCK2)

The cholecystokinin-2 (CCK2) receptor has been identified as a potential therapeutic and diagnostic target for cancer. Indeed, it has been well established that this receptor is overexpressed in different types of tumours including gastrointestinal, pancreatic, lung and medullary thyroid cancers. Furthermore, it plays an important role in tumorigenesis and cancer progression. The CCK receptor is a G protein-coupled receptor (GPCR) that upon activation by gastrin or CCK (two peptide hormones) induces cell proliferation. Interestingly, among the gastrin derivatives, minigastrin (a peptide sequence of 13 amino acids) has been widely used for radionuclide imaging of CCK2 receptors. Laabs replaced the radioactive moiety of minigastrin with a fluorophore (2, see Fig. 2) for in vivo imaging of cancer in mice. DY-minigastrin (2) was able to bind to CCK2/gastrin receptors in vitro, and was suitable for real time imaging of mice with implanted colorectal carcinoma cells (HT-29) (selectivity was confirmed using the unlabelled peptide in competitive binding assays).

Following on from the c-Met probe (above) and the highly sulfonated Cy5 fluorophore used in EMI-137 (1), Kossatz developed a modified minigastrin probe with a hemicyanine dye (DY-754) bearing four sulfonate groups and showed that it too had low unspecific binding to healthy tissues of the gastrointestinal tract. This probe exhibited a strong affinity and specificity to CCK2 receptors in vitro and the ability of the probe to image tumours in mice from 2 to 24 h after injection was confirmed with selective distribution in cancer areas confirmed by histological analysis.

2.1.3. Gastrin-releasing peptide receptor (GRPR)

GRPR is overexpressed in prostate, pancreatic, gastric, and small cell lung cancers. Bombesin (BBN) is a 14-amino acid peptide able to bind selectively to GRPR and was linked to Alexa Fluor 680 to generate the affinity probe (AF680-BBN, 3) that was tested on human T-47D breast cancer cells. The probe showed high affinity and specificity to the receptor both in vitro and in vivo. AF680-BBN (3) was used to image lymph nodes and metastasis in prostate cancers in mice, and was able to monitor the spreading of cancer into the lymph nodes.

2.1.4. αββ Integrins

Integrins are a family of cell surface receptors involved in the attachment of cells to the extracellular matrix. They are heterodimeric transmembrane glycoproteins constituted by different subunits (α and β). The integrin αββ has been shown to be critical for tumour regulation and metastasis, playing a pivotal role in angiogenesis, proliferation and migration of cancer cells. The high expression of αββ on endothelial cells during cancer metastasis...
makes this integrin a valuable biomarker for tumour imaging. Many optical probes are based on the tripeptide RGD in its linear or (better) cyclic version as targeting moieties. Thus, Liu used the dye 800CW coupled to cRGD (800CW-RGD, Fig. 2) that was shown to specifically bind to integrin receptors and selectively accumulate in glioblastoma tissues in vivo in mice allowing fluorescence-guided tumour resection. Building on this approach, Liu generated a similar probe for imaging of tumour vasculature during tumour angiogenesis. Upon injection in mice bearing pancreatic cancer, the probe was able to visualize cancer endothelial cells over 24 h.

An important point for all probes is the contrast between tumour and normal tissues. Therefore, multiple efforts have been made to strengthen the emission signal and reduce the background. Thus, Choi reported a novel optical probe based on cRGD attached to a zwitterionic fluorophore (ZW800-1) that had a low nonspecific tissue background. In vivo analysis in mice bearing human melanoma cells showed that the optical probe had enhanced signal to non-specific background compared to dyes such as non-sulfonated (hydrophobic) Cy5.5 and 800-CW. The specificity of this probe was confirmed by further in vivo experiments in mice with colorectal, breast, pancreatic, and oral tumour xenografts.

Multi-valent probes have also been developed based on cRGD. This includes a cRGD dimer based probe labelled with 800CW that was investigated in a U87MG tumour model. Cal conjugated a fluorophore (ICG-Der-02) to c(RGDyK), and used it for in vivo integrin imaging in mice bearing subcutaneous MDA-MB-231 and U87MG tumours. In this case within 4 h post-injection the probe accumulated selectively in the cancer tissue and showed good signal over background.

Cheng carried out a comparison study using monomeric, dimeric and tetrameric Cy5.5 c(RGDyK) in a subcutaneous U87MG glioblastoma xenograft model. The tetramer provided the clearest images of the tumour in vivo with the highest contrast 0.5–4 h post-injection. In the light of this, a further probe was developed by linking a tetrameric RGD peptide to a cyclic decapeptide scaffold (RAFT) and labelled with Cy5. This probe (Cy5-RAFT-c(RGDfK)₄) was successfully used for early detection of deep ovarian metastases in mice.

Tumour targeting cyclic (RGDyK) peptides have also been used to functionalize a nanoprobe together with a fluorescent dye (Cy5) and a blood brain barrier (BBB) permeable peptide (Angiopep-2). First, the nanoprobe targets and accumulates in the peripheral tumour neovasculature, allowing the interaction between the BBB permeable peptide and a specific receptor on the vascular endothelial cells. This process facilitates the crossing of the BBB of the nanoprobe via receptor-mediated transcytosis, enabling brain tumour detection. The G5-PAMAM dendrimer was selected as platform due to its globular architecture, and the resulting conjugated nanoprobe was investigated in mice bearing U87MG human glioblastoma xenograft cancer. In vivo imaging demonstrated the ability of the probe to visualize tumours in the brain with high contrast. In a related study, Liu developed a probe by conjugating Cy5.5 to c(RGDfK) on a G5-PAMAM dendrimer via PEG spacers in order to improve biocompatibility and extend the blood circulation time. In mice, the probe was able to visualize esophageal adenocarcinoma in vivo (again confirmed histologically).

3. Somatostatin receptor

Overexpression of the somatostatin receptor has been found in many tumours and radiolabelled somatostatin (SST) derivatives are commonly used in clinic for the treatment of gastroenteropancreatic cancer. With this in mind, Becker developed an optical probe based on octreotate (a SST analog) labelled with the fluorescent dye indotricarbocyanine (ITCC) that was investigated for in vivo tumour targeting. Three hours post injection into mice bearing a RIN38/SSTR2 tumour, fluorescence was clearly observed in the region of the cancer.

4. Epidermal growth factor receptor (EGFR)

The epidermal growth factor receptor (EGFR) is a cell-surface receptor overexpressed in many cancer types including glioma. The ability to non-invasively observe EGFR levels may be of significant utility in the diagnosis of cancer and monitoring of anticancer drugs. Two very similar preclinical studies have been published where the 12-mer peptides, YHWYGYPQNV (GE-11) or FPMFNHWEQWP, selected by phage display methods were labelled with Cy5.5 and applied in vitro and in vivo in mouse models to detect tumours.

5. Other receptors

There are many other examples, but space precludes full details. One example is so-called Tumour Paint (BLZ-100) – a
peptide-based probe deriving from chlorotoxin venom (CTX), a 36 amino-acid peptide, which is able to bind selectively to several types of cancer cells including glioma, while having negligible toxicity in humans. These favorable properties make CTX a promising scaffold for imaging a broad variety of cancers. Thus, a modified form of CTX was conjugated to indocyanine green to yield BLZ-100, a fluorescent agent that was extremely efficient at imaging brain tumours in mouse models. After preclinical validation in dogs with spontaneously occurring solid tumours, BLZ-100 was translated into clinic. It is currently under Phase I clinical trials for treatment of adult skin cancer, sarcoma, pediatric tumours of the CNS, glioma, and other solid tumours (http://www.clinicaltrials.gov identifiers: NCT02097875, NCT02464332, NCT02462629, NCT0234297, NCT02496065).

Another cell-surface receptor of interest as a diagnostic target is the zinc metalloenzyme prostate-specific membrane antigen (PSMA) or glutamate carboxypeptidase II. The only FDA approved imaging agent-targeting PSMA in prostate cancer is ProstaScint, a radiolabelled antibody. In this context, optical probe design has tended to relay on the structure of urea based-peptide inhibitors of PSMA, e.g. the fluorophore 800CW conjugated to a lysine-glutamate urea inhibitor through a spacer of three D-Glu residues to give PSMA-1-IR800 (7, see Fig. 2). The molecule was investigated in vitro and in vivo proving high binding affinity and selectivity.
for PSMA on PC3pip tumours, allowing the visualization of the boundaries of the solid cancer in mouse models and distinguishing between PSMA-expressing and non-expressing tumours and other tissues. These data suggest that PSMA targeting molecules have the potential for clinical applications in intraoperative fluorescence-guided surgery.

Lysosomal protein transmembrane 4 beta (LAPTM4B) shows high expression in several solid tumours and has been the target for the generation of a number of peptide-based probes based on an environmental “switch-on” dye and dual valency (8, see Fig. 2). The ability of this probe to target LAPTM4B was evaluated in vitro using human hepatocellular carcinoma cells (HepG2) and in vivo in tumour bearing mouse models. After 1 h it was possible to clearly identify the edges of the tumour.

In the field of glioma detection, Burden-Gulley et al. described a probe that labels extracellular cleaved fragments of the receptor PTP (protein tyrosine phosphatase) that accumulates in the tumour microenvironment, but is not present in normal brain tissue. The peptide GECDDFNWQVNTLKPTSD (identified using crystallographic data from the extracellular segment of the receptor), capable of binding to the PTP receptor, was labelled with Cy5 and the resultant probe was able to label not only the main tumour mass and cells at the margin, but also cells that had moved away from the main tumour mass in an intracranial animal model of glioma. Dispersed cells, detected up to 3.5 mm away from the tumour provided high-resolution detail of local tumour cell migration.

5.1. Affinity probes to detect bacterial and fungal infection

Some of the most promising probes for imaging bacteria and fungi in situ are based on antimicrobial peptides with the vast majority of work carried out on preclinical infection models. However, the long-term clinical potential for detecting bacteria in vivo is huge with possible applications such as the analysis of the infection of prosthetic materials (which are common sites for bacterial colonisation) to determination of infective aetiology to direct therapy. The indiscriminate and widespread use of antimicrobials fuels antimicrobial resistance (AMR) and optical medical imaging offers potential for inherent fluorophore quenching upon aggregation.

Substantial efforts are being made to target vancomycin-resistant bacteria. This includes the preparation of dimers of vancomycin linked by photosensitizers. With the aid of light, photosensitizers are able to generate ROS and cause irreversible damage to the bacteria. Based on this principle, Xing et al. constructed an adduct with a porphyrin bridging moiety as a photosensitizer meanwhile Feng et al. took advantage of the Aggregation-Induced Emission properties (AIE) of tetraphenylethene to achieve turn on fluorescence upon binding to Gram-positive bacteria. The resulting probes act as fluorescent agents to monitor bacteria with photodynamic antimicrobial activity.

7. Ubiquicidin-based probes

Ubiquicidin (UBI) is a well-studied antimicrobial peptide (AMP). This cationic 59-aminoacid peptide displayed high accumulation in bacterial infections targeting the negatively charged membranes of Gram-positive and Gram-negative bacteria. The 13-mer fragment UBI29–41 [TGRAKRRMQYNRR] has been radiolabelled for bacterial imaging. In the field of fluorescent imaging recent examples of the use of UBI29–41 include the NIR labelled peptide ICG-02-UBI29–41 (10, see Fig. 3) which was used for bacteria detection in infected mouse models. A dual-modality tracer was generated by the conjugation of UBI29–41 to a radioisotope and a fluorophore (111In-DTPA-Cy5-UBI29–41) and was able to detect S. aureus and K. pneumoniae infections in vivo in mice. A more clinically relevant scenario is offered by bacterially infected human lung tissues. Thus, UBI29–41 was labelled with the environmentally sensitive fluorophore NBD, and subjected to a number of structural modifications to improve its stability (unmodified it was found to be rapidly degraded in vivo with the methionine residue a clear point of concern). A cyclic variant was able to detect bacteria in ex vivo human lung tissue using fibre-based fluorescence microscopy (Fig. 4).23

8. Apidaecin-based probes

Apidaecine-peptides are a family of small peptides (18- to 20-residues, rich in proline) produced by insects and predominantly active against Gram-negative bacteria while non-toxic to animal or human cells. Dosselli et al. attached a photosensitizer (11, see Fig. 3) to apidaecin which thus offers a method for improving both the water solubility of the photosensitizer. Although the porphyrins used by Dosselli were poorly fluorescent for imaging they allowed PDT.
**9. Polymyxin-based probes**

An optical imaging probe have been developed based on the antimicrobial peptide polymyxin conjugated to the environmentally sensitive fluorophore NBD (Fig. 3) with the first in-human clinical trials of bacteria-targeted optical imaging now in progress (NCT02491164). This probe aims to label Gram-negative bacteria in vivo in situ within the distal lung in the intensive care unit.

**10. Probes for fungal infection**

Although many studies have been carried out with imaging modalities such as PET, which cannot differentiate between fungal infections, bacterial infections, malignancy, or inflammatory lesions) the field of fungal optical imaging is very unexplored.

A recent example of optical imaging probes for Aspergillus fumigatus, the pathogen that causes the fatal disease Invasive Pulmonary Aspergillosis was recently reported. Here the authors used Trp-BODIPY in a cyclized version of the antimicrobial hexapeptide Peptide Antifungal 26 (PAF26, Fig. 3).

**10.1. Affinity probes to detect inflammation**

Affinity probes developed for fluorescence imaging of neutrophils have used the neutrophil-binding peptide cinnamoyl-(D)LF(D)LF (a FPR1 antagonist) targeting the formyl peptide receptor expressed on neutrophils, attached to some form of Cy7. As this hexapeptide is highly hydrophobic a PEG moiety was used to enhance its water solubility with results achieved in a mouse model of ear inflammation.

**10.2. Activity based probes**

Peptide-based activity probes have long been used to analyse a variety of enzymes, with the affinity probe endowed with a recognition moiety (i.e. a peptidic sequence that targets a specific enzymatic activity overexpressed in a pathological process), a reactive war-head (that binds to the targeted protein – historically this used to be a potent alkylating agent, but more subtle variants are now used) and a reporter group (here a fluorophore). The majority of activatable peptide-based probes have targeted proteases and most classes of proteases have been explored (Fig. 5A). Some of the most recent probes used for in vivo imaging are described below listed by their target enzyme/class.

In the case of the probes based on Activatable Cell-Penetrating Peptides (ACPPs) (Fig. 5B), probe activation and cargo uptake require proteolysis of a peptide linker sequence that connects anionic and cationic domains – cleavage then allowing the cationic domain to enter cells. This method provides detection of spatially localized enzymatic activity in living tissues through the accumulation of cleaved probe, targeting the cargos to sites of protease activity.

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**Fig. 3.** Structure of some of the antimicrobial peptides used to prepare optical imaging probes to detect bacteria and fungi.
activity in vivo. A related strategy is to retain the cleaved substrate by locating the fluorophore on a peptide bearing amino groups produced upon cleavage of the probe.

Quenched Activity Based Probes are small molecules that become covalently bound to a target enzyme in an activity-dependent manner and have a long history in biochemistry. These probes typically have a short peptide sequence that is targeted by the protease, an electrophilic moiety that facilitates binding to the active site and a fluorophore and quencher. Protease activation leads to fluorescent protein labelling with release of the quencher (Fig. 5C). An alternative strategy is to retain the cleaved substrate by locating the fluorophore in a peptide bearing amino groups produced upon cleavage of the probe. Ofori et al. reported an example of this kind of probe (Fig. 6) that was successfully used for imaging of breast cancer in mouse models.

10.3. Cathepsins

Cathepsins are a family of lysosomal cysteine proteases encompassing some eleven different members. Numerous studies have indicated that they play a key role in the degradation of the extracellular matrix (ECM) promoting angiogenesis and tumour progression. Cathepsins have been reported to have increased expression in cancer cells and altered localization in the ECM during metastasis. Bogyo has led this area by the development of a number of fluorescent (Cy5) activity probes (Fig. 5C). It is important to remark that the main advantages of the covalent labelling are the increased durability of the signal and the localization of the proteolytic activity site. An example of such a probe is BMV-109 (see Fig. 7) based on the surprisingly short peptide recognition sequence (Phe-Lys) coupled with a tetrafluoro phenoxymethyl ketone (PKM). This probe gave good target labelling and showed in vivo stability and was used in murine studies for noninvasive optical imaging to visualize the shape of breast and colon tumours with impressive contrast at 1 h post-injection. BMV-109 was able to label tissues when administered topically providing support for further development as a tool for fluorescence-guided cancer surgery.

However, the covalent labelling strategy has a drawback, namely stoichiometric labelling (which can be quite weak) with no possibility of amplifying the signal. Thus, probes have been generated that undergo turnover, thereby generating an amplified fluorescent signal with signal retention within the cleavage site achieved by a variety of strategies. This includes a protease-activated fluorescent probe (LUM015) based on a long PEG spacer (MW = 22 kDa) that remained attached to the fluorophore Cy5 upon enzymatic cleavage thus limiting its diffusion. LUM015 was first tested in mice bearing soft tissue sarcoma and breast cancer and provided good contrast between tumour and normal tissues, while preclinical toxicity studies of the probe in rats and dogs revealed no adverse effects. Furthermore, histological experiments indicated the ability of the probe to selectively label canine tumour tissue. In the light of these findings, LUM015 was translated to humans and it is currently in Phase 1 clinical trials. In fifteen patients with soft tissue sarcoma and breast cancer, the probe was well tolerated and subsequent fluorescent imaging of human tissue samples displayed selective distribution of the probe to tumours with high contrast compared to normal tissue.

Nevertheless, the use of such a large probe could lead to a slow uptake into tumours together with unwanted background signals coming from other tissues, while the peptide sequence could be prone to activation by other proteases.

A further approach concerning probe design consists of the introduction of caged fluorophores that become fluorescent upon enzymatic decaging. As an example the probe Z-Phe-Arg-HMRG targeting cathepsin was prepared (Fig. 8A). Enzymatic cleavage produced an intense fluorescent signal with the probe showing a 200 fold increase in fluorescence upon cleavage. This small turn-on probe turned out to be able to rapidly image tumour nodules in a mouse model with a high signal to background ratio. In addition, local spraying of the probe allowed the visualization of the tumour margins by fluorescence endoscopy in vivo proving its potential in clinical applications.
10.4. Caspases

In an effort to generate an optical probe selective to caspases which are signature enzymes of apoptosis, Bogyo and co-workers exploited the same strategy for cathepsins that makes use of irreversible inhibitors. AB50-Cy5 had an electrophilic fragment (an acyloxymethyl ketone) containing the peptidic sequence Glu-Pro-Asp labelled with Cy5. Although cross-reactivity with legumain was observed, the probe enabled in vivo imaging of the kinetics of apoptosis in different mouse models by labelling caspase 3 and 7.

Shi et al. generated a fluorescent "light-up" probe by exploiting the aggregation-induced emission (AIE) of tetraphenylethene (TPE). Thus, they conjugated the peptidic sequence DEVD (general sequence susceptible to caspase cleavage) to the dye creating a water-soluble and poorly fluorescent adduct Ac-DEVD-PyTPE (Fig. 8B). Upon caspase cleavage a strong fluorescence was observed because of the aggregation of the hydrophobic TPE residues, however there must always be concerns about probes that induce aggregation with respect to human imaging.

A further strategy for a turn-on optical probe to image apoptosis was reported by Ye et al. In this case the probe endowed with a NIR
dye had a peptidic substrate cleaved by caspase 3 and 7 linked to a disulphide moiety that under the reducing tumour microenvironment and caspase cleavage activity underwent an intramolecular condensation to form a cyclic compound in situ. The latter self-assembled into fluorescent aggregates allowing real-time imaging of tumour cell death in vivo. It is important to remark that the bio-orthogonal reaction that leads to the formation of the assembled nanoparticles occurred intracellularly, although the level of fluorophore self-quenching in aggregates might be a concern. That is important for their retention in apoptotic cells, improved specificity and signal-to-noise in cancer imaging. Studies in HeLa tumour-bearing mice with doxorubicin treatment showed that the probe was able to non-invasively image apoptosis providing information about tumour response over the course of chemotherapy.

10.5. Matrix metalloproteinases (MMPs)

MMPs are one of the most widely studied enzyme families due to their relationship with cancer and inflammation. MMPs are a group of zinc containing endopeptidases known to degrade ECM proteins such as collagens, gelatins, fibronectin, and laminin. MMP activity is involved in many disease-related processes including cancer progression, invasion and metastasis, rheumatoid arthritis, pulmonary diseases and cardiovascular diseases. Since the first description of ACPPs targeting MMPs, much progress has been made using these probes to visualize tumour margins during surgery as proved recently by Orosco et al. for detection of residual microscopic cancer foci (1.2 mm in size) or to detect metastases with increased sensitivity and specificity. MMPs have also been imaged by the hydrophobic membrane anchoring of a FRET probe (synthesized using two fluorophores) in cells derived from a murine bronchoalveolar lavage, although specificity to MMP-12 needs to be evaluated.

Probe design in this area has evolved in different ways. For example the probe described by Li for the detection of MMP-2 and caspase-3 consisted of a central fluorophore (FAM) attached to two peptidic substrates for each enzyme both ending with a quencher unit (Dabcyl) with sequential detection (rather than simultaneous), and relying on a single fluorophore. Other recent advances include the targeting of two biomarkers producing probes with dual functionality, such as an activity-based probe that has both detection (FRET) and affinity (binding) components to improve the accumulation in the target site. One example of this strategy is the probe that combines a cleavable MMP peptide and cRGD peptide targeting MMP-2 and integrin αvβ3 simultaneously.

Commercially available probes MMPSense™ (PerkinElmer) containing the sequence -PLGVR- have been extensively used in animal studies. Recent examples include MMPSense680™ for...
intravital microscopy in a model of breast cancer to provide insight into cellular composition and real-time dynamics of the stromal microenvironment. Also Chi et al. recently reported the use of MMPSense750TM to facilitate the detection and removal of breast cancer tumours. The selectivity of these probes to specific MMPs, or for example to common proteases such as plasmin is a question. Combined probes for diagnostic and photodynamic (PDT) treatment is an emergent field. For instance an MMP-2 imaging and PDT probe (Fig. 9) which after tail vein injection accumulates at MMP-2-overexpressing tumour sites. This consisted of an ACPP with the sequence R9GPLGLAGE8 and a protoporphyrin which under irradiation (singlet oxygen generation) showed in vivo inhibition of tumour growth.

Currently there is an ongoing phase-1 clinical trial (NCT02604862) with a peptide-based MMP probe that images intrapulmonary MMP activity in patients.

10.6. Human neutrophil elastase (HNE)

HNE is a serine protease secreted by neutrophils under physiological conditions but when uncontrolled is also involved in the

Fig. 8. A) The enzymatic cleavage of Z-Phe-Arg-HMRG "decages" the modified rhodamine dye to produce an intense fluorescent signal. B) The probe Ac-DEVD-PyTPE. C) Cleavage by caspase induces an intramolecular cyclization of C-SNAP that self-assembles into aggregates.

Fig. 9. Imaging and PDT with an ACPP probe for MMP-2. Once the ACPP-PoIX conjugates get into the tumour tissue, activation by extracellular MMP-2 leads to internalization. Reprinted with permission from Li S-Y, Cheng H, Qiu W-X, et al. ACS Appl Mater Interfaces 2015, 7, 28319–28329. Copyright 2015 American Chemical Society.
pathogenesis of acute and chronic inflammatory diseases, for these reasons monitoring its activity is therapeutically important. Gehrig et al.\textsuperscript{10} described two FRET peptides based on methoxycoumarin/-coumarin343 or coumarin343/TAMA and cleavable sequences QPMAV\textbackslash{}VQSVPX (X = Q or EE) respectively for the soluble enzyme or for insertion in the plasma membrane, and applied them in a mouse model with acute neutrophil lung inflammation. Avlonitis et al.\textsuperscript{12} developed internally quenched multi-branched (three or six) scaffolds containing the peptide APEEIMXRQ (X = D or R).

After successfully completing a phase 0/1 study,\textsuperscript{10} a multi-branched probe is currently undergoing 2 studies in the setting of Adult Respiratory Distress Syndrome in the Intensive Care Unit (NCT02804854). The study involves the delivery of microdoses of a Neutrophil Activatable Probe by intra-pulmonary instillation to patients in ICU with pulmonary infiltrates and imaging through probe-based confocal laser endomicroscopy (pCLE) to test the utility and safety of this probe.

10.7. Legumain

Legumain is a lysosomal cysteine protease associated with a number of inflammatory diseases and cancer, upregulated in the majority of solid tumours. A recent approach to image legumain activity in vivo has been described using a substrate that contains the peptide EPD, Cy5/QSY21 as a FRET pair and an acyloxymethyl ketone (see Section 10.4. above).\textsuperscript{11} This probe was used to image legumain activity in two murine models of cancer, with advantages over the previous strategy based on an epoxide opening reaction.\textsuperscript{11}

10.8. Thrombin

Thrombin is a key enzyme in blood coagulation and plays an important role in atherosclerotic disease development. Peptide-based probes have been described in the last years for live imaging in plaques using a thrombin sensing ACP.\textsuperscript{12,13} The ACP labelled with Cy5 and improved ratiometric versions labelled with Cy5/Cy7 provided good results in mice after systemic injection. The ratiometric version could detect thrombin activity more quickly and turned out to be more reliable and selective due to minor changes in the peptide sequence. Although further studies are needed, in clinical applications, such as deep vein thrombosis and stroke, there are quite broad clinical applications, especially as intravascular endoscopic catheters may in the future enable optical determination of vulnerable atherosclerotic plaques. Thrombin activity is also a major contributor to fibroproliferation and optimized thrombin substrates with high selectivity and resistance to plasmin degradation have been also reported for rapid sensing in lung tissue.\textsuperscript{11}

11. Conclusions and perspectives

The ultimate goal of clinical imaging approaches are to improve the diagnosis, prognosis and treatment of patients and to provide a greater understanding of human biology in both health and disease. Whilst more established imaging modalities such as PET, MRI, CT and ultrasound have entered routine clinical use, primarily as structural imaging platforms, the versatility of optical molecular imaging (OMI) has the potential to have a significant and major impact on approaches of molecular imaging using peptidic imaging probes. OMI is now at the leading front of advances in augmenting image-guided surgery to guide real-time clinical and also in the future robotic decision making.

The ability to develop targeted and molecularly specific fluorescent peptidic probes has driven the progress in optical imaging in the last two decades along with low cost and safety. The advances in fluorescence imaging technology should now be focused on overcoming some of its current limitations, such as its narrow tissue penetration and tuning specificity – as too many probes are activated by by-stander enzymes. In developing OMI as a platform for observing molecular events in humans in situ, studies need to show specific and full knock-down with pharmaceutical inhibitors.

The development of novel optical probes is a key element in the evolution of OMI, and combined with the discovery of novel biomarkers has the potential to increase clinical applicability in a broad number of diseases. Regarding peptide-based probes, their synthesis relies on standard chemistry methods, where building blocks can be easily modified for special requirements, and conjugation with dyes is usually straightforward. In addition, peptides are smaller, have less immunogenicity, and have faster rates for in vivo localization than antibodies. All this taken together suggests that peptides will remain as important features in many imaging probes in the future. As they become more sophisticated in design with novel turn-on approaches and targeted strategies, further applications will follow with ensuing clinical benefit.

Despite the promising results reported with peptide-based probes in animal models, it remains difficult to predict how fast dual imaging/PDT probes will enter clinical practice and there needs to be much less reliance on poor murine models and much more on diseased human tissues. The implementation of these theranostic agents still faces several obstacles such as co-development of devices and the real in vivo environment is exceptionally complex, requiring strict control over energy delivery and evidence of low toxicity. However, PDT has great potential to transform current pathways of diagnosis and therapy as advances in instrumentation progress to theranostic approaches and interventional image-guided therapy.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2017.09.039.

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