

Engineering Intelligent Nanosystems for Enhanced Medical Imaging

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Medical imaging serves to obtain anatomical and physiological data, supporting medical diagnostics as well as providing therapeutic evaluation and guidance. A variety of contrast agents have been developed to enhance the recorded signals and to provide molecular imaging. However, fast clearance from the body or nonspecific biodistribution often limit their efficiency, constituting challenges that need to be overcome. Nanoparticle-based systems are currently emerging as versatile and highly integrated platforms providing improved circulating times, tissue specificity, high loading capacity for signaling moieties, and multimodal imaging features. Furthermore, nanoengineered devices can be tuned for specific applications and the development of responsive behaviors. Responses include in situ modulation of nanoparticle size, increased intratissue mobility through active propulsion of motorized particles, and active modulation of the particle surroundings such as the extracellular matrix for an improved penetration and retention at the desired locations. Once accumulated in the targeted tissue, smart nanoparticle-based contrast agents can provide molecular sensing of biomarkers or characteristics of the tissue microenvironment. In this case, the signal or contrast provided by the nanosystem is responsive to the presence or concentration of an analyte. Herein, recent developments of intelligent nanosystems to improve medical imaging are presented.

1. Introduction

Medical imaging is one of the mainstays of current medical practice, as the patients' anatomy and internal physiology data are of key importance for diagnostics and therapy guidance. This data may aid in detecting a disease at early stages and monitor the effectiveness of its treatment. Most commonly used imaging

modalities are magnetic resonance imaging (MRI), X-ray computed tomography (CT), ultrasound (US), and nuclear imaging, which includes positron emission tomography (PET) and single photon emission computerized tomography (SPECT). The technical characteristics of these modalities are summarized in Table 1. In preclinical stages, optical and photoacoustic imaging also play a pivotal role, although translation to the clinical setting is still not fully accomplished. The selection of the imaging modality depends on the disease, the organ or tissue to be explored and the type of data that is required (Box 1).^[1] MRI is typically used to identify diseases of the central nervous system (e.g., aneurysms and stroke) and cardiovascular diseases (heart size and function, extent of damage after infarct, or structural alterations in major vessels). It is also applied to the detection of tumors or abnormalities in different organs and to the evaluation of joint abnormalities and bone infections. CT finds application in the diagnostics of bone disorders, tumor location, and

image-guided surgery, biopsy or radiation therapy. US imaging, universally known for its application in the monitoring of the evolution of the fetus during pregnancy, is also useful in cardiovascular diseases, image-guided treatment or biopsy of tumors and detection of abdominal abnormalities. Finally, nuclear imaging techniques can be helpful in diagnosing brain disorders (e.g., Alzheimer's disease and dementia),^[6–8] cardiovascular diseases,^[9,10] and different types of cancer.^[11,12]

Current approaches in medical imaging often require the use of contrast agents, which are compounds that improve imaging quality and diagnostic value by increasing the signal in the region of interest (Table 2). In addition, contrast agents for molecular imaging are able to report biochemical dynamics, yielding not only anatomical but also physiological and molecular information.^[2] These contrast agents are required to provide high signal intensities on the imaging modality or modalities of choice.^[14] However, molecular agents may suffer from fast metabolism and/or limited or nonspecific accumulation in the target tissue.^[1] One example of the latter is [¹⁸F]fluoro-2-deoxy-D-glucose, a PET tracer widely used to evaluate glucose metabolism, which shows preferential uptake in glycolytic tumors but also in inflamed and infected areas, eventually leading to false-positive results.^[15] Furthermore, the implementation of multimodal and multifunctional agents still remains an unmet challenge in the field.

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Table 1. Comparison of various imaging modalities.^[1–5]

	MRI	CT	PET	SPECT	US	Optical imaging ^{a)}	Photoacoustic imaging
Type of energy measured	Radio waves	X-rays	Gamma rays	Gamma rays	High-frequency sound waves	Visible or NIR light	High-frequency sound waves
Spatial resolution ^{b)}	10–100 μm	50–200 μm	1–2 mm	1–2 mm	50–500 μm	1–5 mm	50–500 μm
Temporal resolution ^{c)}	Minutes to hours	Minutes	Seconds to minutes	Seconds to minutes	Seconds to minutes	Seconds to minutes	Seconds to minutes
Sensitivity	μM – mM	mM	pM	pM	NWC ^{d)}	nM	pM
Depth	No limit	No limit	No limit	No limit	A few cm	mm to $\text{cm}^{\text{e)}$	A few cm
Information	Anatomical; physiological; molecular	Anatomical; physiological	Physiological; molecular	Physiological; molecular	Anatomical; physiological	Physiological; molecular	Physiological; molecular
Cost	High	Medium–high	High	Medium–high	Low	Low	Low
Stage	Clinical	Clinical	Clinical	Clinical	Clinical	Preclinical	Preclinical

^{a)}Includes bioluminescence imaging and fluorescence imaging; ^{b)}Estimative values are provided. Spatial resolution depends on many factors including size of the species (preclinical and clinical), manufacturer of the equipment, specific application, and so on; ^{c)}Higher temporal resolution can be achieved at the cost of sensitivity and spatial resolution; ^{d)}NWC: not well characterized; ^{e)}Penetration depth of visible light is very limited, whereas NIR light provides a penetration depth of a few centimeters.

Table 2. Contrast agents commonly used for medical imaging.^[1,2,13]

Imaging modality	Material type	Imaging agent ^{a)}
MRI	Gadolinium-based	Gd, Gd^{3+} , Gd_2O_3
	Iron oxides	Fe_3O_4 , $\gamma\text{-Fe}_2\text{O}_3$
	Manganese oxide	MnO , MnO_2 , Mn_3O_4
	Metal alloys or transitional metals	Mn^{2+} , Fe^{3+} , Cu^{2+}
CT	Iodine-based	Iodine or iodine-containing compounds
	Barium-containing	Barium sulphate (gastrointestinal applications)
	Other heavy elements	Au, Bi, Gd, Ce, Tb, Dy, Yb, Lu, Th, Pb, W, Ta or Re
PET	Positron emitter-labeled entities	^{18}F , ^{15}O , ^{13}N , ^{11}C , ^{14}O , ^{44}Sc , ^{64}Cu , ^{62}Cu , ^{64}Cu , ^{72}As , ^{124}I , ^{76}Br , ^{82}Rb , ^{68}Ga , ^{86}Y , ^{89}Zr
SPECT	Gamma emitter-labeled entities	$^{99\text{m}}\text{Tc}$, ^{131}I , ^{123}I , ^{111}In , ^{166}Ho , ^{177}Lu , ^{188}Re
US imaging	Gas phase nano- or microbubbles	Carbon dioxide, oxygen
	Liquid-to-gas compounds	PFCs
Optical imaging	Fluorescent dyes	FITC, Rhodamine, Cyanine-based dyes, Alexa dyes, etc.
	Fluorescent or bioluminescent proteins	GFP, RFP, Luciferases
	Quantum dots	CdSe , CdTe , GaAs , HgTe , InAs , InP , PbSe , PbTe
	Rare-earth upconversion materials	Er^{3+} , Ho^{3+} , Nd^{3+} , Pr^{3+} , Yb^{3+}
Photoacoustic imaging	NIR dyes	Cyanine-based dyes, Rhodamine, Alexa Fluor dyes
	Fluorescent proteins or endogenous chromophores	GDP, RFP, Biliverdin etc.
	Azo chromophores	Methylene blue, Evans blue
	Metal-based	Au, Ag, Pd, Cu
	Carbon-based	Graphene, carbon nanotubes, nanodiamonds
	Polymer-based	Conjugated polymers, porphyrin-related agents

^{a)}These materials can be formulated in nanosize, or incorporated in nanocarriers such as liposomes, dendrimers, nanoemulsions, polymeric NP, mesoporous silica NP, or others.

Engineering imaging agents at the nanoscale may be used to adequately meet these requirements. The use of nanoengineered systems for bioimaging has gained significant attention over the past decade due to the possibility of tuning their size,

composition, surface properties and charge, all of them contributing to modulate the biodistribution and pharmacokinetic profiles. Moreover, such nanosystems can be further engineered to increase signal strength, either by exploiting their inherent

properties as contrasts or by functionalizing and loading large amounts of signaling moieties.^[1,2] In addition, they facilitate simultaneous incorporation of therapeutic and imaging capacities, thus enabling theranostic approaches based on real-time monitoring of drug delivery and evaluation of the therapeutic response.^[16,17] The ability to provide signals for multiple imaging modalities is another example of the benefits of using multifunctional nanosystems, as imaging modalities are often combined in medical practice. For such multimodal particles, imaging with different modalities can be carried out using only one contrast agent, with the consequent regulatory advantages and reduction in cost, time, and patient discomfort.^[2]

Irrespective of the imaging modality, high accumulation of contrast agents at the target site is achieved by both penetration into the tissue of interest and an adequate retention. The use of nanoparticles as imaging agents or contrast agent carriers may yield improved biodistribution profiles compared with small molecules, as the particles may benefit from passive accumulation in tumor tissue or inflammation sites due to enhanced permeability and retention (EPR) effect.^[18,19] The EPR effect has been so far attributed to the presence of large gaps in the endothelium of the blood vessels, which facilitates leakage of macromolecules and particles into the interstitial space.^[19] However, a recent study suggests that the mechanism by which nanoparticles are accumulated

in solid tumors is strongly driven by active transepithelial transport, rather than by passive entry through interendothelial gaps.^[20] To further increase accumulation at the target site, nanoparticles can be functionalized with targeting moieties with affinity to specific epitopes of the tissue, a strategy referred to as active targeting.^[21] Such moieties may include antibodies,^[22–24] aptamers,^[25–27] ligands,^[28–30] or a combination of those,^[31–34] with high affinity for epitopes that are exclusively (or to a larger extent) present in the target tissue. Both active and passive targeting benefit from circulation time, which may be prolonged by optimizing particle size, shape, or charge, and by evading the immune system.^[35–38] Still, only a small part of the injected dose of nanoparticles may actually accumulate in the disease site,^[39] and the distribution within the tissue is usually heterogeneous. Hence, the introduction of intelligent, active components into nanosystems may enhance effectiveness and improve medical imaging. We define such intelligence by responsiveness of the nanosystem to endogenous stimuli or to external impulses. Endogenous stimuli may constitute aberrant properties of the tumor microenvironment such as increased levels of certain enzymes or reactive oxygen species, or a decreased pH.^[40,41] Intelligent particles may respond to these stimuli to adapt their own characteristics or behavior (such as a change in size or the induction of self-actuation) or to modify their environments (by increasing the

Box 1. Medical imaging modalities

In MRI, strong magnetic fields and radiofrequency pulses (5–100 MHz) are used to evaluate the relaxation times of protons in water (^1H), although other nuclei such as fluorine (^{19}F) can be used as well. The longitudinal (T_1) and transversal (T_2) relaxations are most often measured, and hypo- and hyperintense contrasts may be recorded. The relaxation times are dependent on interactions between the water molecules and their surroundings, which vary per tissue compartment as density and compositions differ. In chemical exchange saturation transfer (CEST) MRI, a radio pulse in the resonance frequency of the imaging agent is used to obtain magnetic saturation of protons. Saturated protons from the agent can then be exchanged with the surrounding water, and the MRI signal in water is used as an indirect but sensitive measure for the imaging agent.^[42,43] MRI is one of the most used modalities, as it is safe, offers high spatial resolution and high contrast in soft tissues, as well as no depth limit.^[1] Alternatively, X-ray CT offers high-resolution imaging without depth limits by measuring the attenuation of an electromagnetic beam through a sample or body. The beam source and detector spin around the body so that images are taken at every angle, which can be computed into 2D and 3D images. The technique is very cost-effective and offers fast image acquisition and processing, but the ionizing radiation may cause long-term genetic damage, and soft-matter tissue contrasts are suboptimal.^[1] Distribution of radiotracers (chemical entities labeled with a radionuclide) can be observed using PET or SPECT. In PET, positrons emitted after spontaneous radioactive decay annihilate with an electron, resulting in the formation of two photons travelling in diametrically opposite directions. Two opposing detectors detect these photons (coincidence event), allowing the definition of a line of response (electronic collimation) where the radioactive decay occurred. In SPECT, gamma rays directly emitted by the radionuclides are detected using physical collimators. In both imaging modalities, the detection of millions of photons enables the reconstruction of a 3D image with information about the spatiotemporal distribution of the labeled tracer.^[44,45] SPECT and especially PET show unparalleled sensitivity, but offer low spatial resolution and cannot provide anatomical information. Therefore, they are often combined with CT or MR imaging. SPECT, despite offering lower spatiotemporal resolution than PET and limited quantification options, is significantly cheaper and hence more commonly used in the clinics.^[44,45] US imaging is one of the cheapest imaging modalities available, and is often used in obstetrics, cardiology, surgery guidance, and urology. The technique is safe, portable, and using (targeted) contrast agents capable of molecular quantification. US detects the reflection of high-frequency sound waves by the internal structures of the body, and uses this data to compute 2D and 3D images. US offers high spatiotemporal resolution, but limited imaging depth.^[46,47] In photoacoustic imaging, a laser pulse is fired at the tissue, leading to thermoelastic expansion which generates US waves. The technique can be used separately, or in combination with US, and tomography can be applied.^[48] Optical imaging is based on the detection of light and is often used in combination with fluorophores such as organic dyes or quantum dots for molecular and cellular imaging. The technique is relatively easy and cheap, and many probes (including targeted and/or signal-altering probes) are available and in development. Often, the near-infrared (NIR) window is used, as light in these wavelengths partially overcome tissue absorption. However, while clinical applications are being evaluated, approaches are predominantly used in preclinical stages.^[49]

permeability of the extracellular matrix [ECM] or mucus layers). All these responsive behaviors contribute to increased tissue accumulation, thereby enhancing image contrast. In addition, this responsiveness may be harnessed to obtain (patho)physiological data, by the modulation of the signal or contrast in correlation to the presence of a certain analyte, analogous to molecular contrast agents. Such intelligent nanosystems may therefore report additional information without the need for invasive testing, to improve pretreatment diagnostics or to monitor treatment efficacy. In this Review, we discuss the aforementioned intelligent design strategies and we present representative examples.

2. Size or Shape Reconfiguration upon Endogenous Stimulus

To obtain clear images with specific signals from the tissue relevant for medical diagnostics, the accumulation of the contrast agent at the target tissue is needed. In this regard, the biodistribution of a nanosystem and the circulating time are mainly driven by particle size. Generally, after intravenous (IV) administration particles smaller than 6 nm are filtered quickly by the kidney, which has an effective pore size of 6 nm.^[38,50] Therefore, a size of 10 nm minimum (or 40 kDa) is advised to avoid quick renal filtration.^[19,51] Larger particles (100–200 nm and above) may be captured by the liver, spleen, or mononuclear phagocytic system, whereas microscale particles (2–5 μm) mainly accumulate in the lung capillaries.^[38,50,52,53] Particles taken up in the liver may result in prolonged retention in this organ, or excretion through the biliary system.^[50] First, specific extravasation in the target tissue is mediated by the EPR effect, possibly in combination with active transepithelial transport. Due to the stochastic nature of these passive targeting effects, particle accumulation increases with circulation time.^[37] The EPR effect is dictated by the leakage of particles from defective blood vessels into the interstitial space in tumors. Moreover, the flow in tumor tissue is erratic: blood flow may stop or reverse direction, which may cause excessive extravasation. Subsequently, the particles are retained in the tissue, as lymph drainage is similarly impaired.^[19,54] In contrast to conventional passive targeting of low molecular weight contrast agents, the EPR effect observed with colloidal imaging agents is characterized by long periods of retention in the tumor.^[19] Particle sizes of 100–200 nm are advised for optimal EPR effect, and considering the aforementioned circulatory size considerations, the particles should be larger than 10 nm but smaller than 400 nm preferably.^[18,38,51] While most nanosystems are designed for cancer imaging, the EPR effect has been witnessed in rheumatoid arthritis and in (early stage) inflamed tissue as well.^[55,56] Noteworthy, the EPR effect in cancer also shows limitations. First, the high interstitial pressure often found in tumors and increased contractility of stroma fibroblast-like cells hampers the accumulation of nanosystems in the vicinity of tumor cells.^[54] Second, it has been proven that EPR is an heterogeneous phenomenon, that significantly varies from patient to patient and even within each individual tumor, and results obtained in animal models can be hardly translated to humans.^[17,57] For an in-depth discussion of the EPR effect, we refer to previous reviews.^[18,19,38,39] Recent findings by Chan and coworkers challenged the relevance

of EPR for particle accumulation. The group systematically found that passive extravasation could only explain a fraction of the nanoparticle entry in tumors, whereas active processes accounted for 75–97% of particle accumulation. Nevertheless, size-dependent effects have been observed in this study too.^[20] These results warrant further discussion and investigations about the nature of passive targeting. Next, tissue penetration and retention are size dependent as well. While smaller particles penetrate into tissues with higher efficiency, larger particles are better retained. Therefore, a rational design is needed to ensure optimal circulation time, tissue penetration, and retention.

Aiming to find such a balance, various intelligent, environment-responsive nanosystems capable to change their size in the presence of certain cues have been developed. These cues might be enzymes or compounds such as matrix metalloproteinases (MMPs) or glutathione, often present in increased concentrations in cancer, or the low pH often found in the tumor microenvironment. Both in situ assembly and degradation can be used to enhance nanosystem efficiency, for different purposes. Generally, degradation strategies aim to improve tissue penetration, whereas in situ assembly promotes tissue retention. Both strategies result in enhanced accumulation of the probes in the region of interest, generating strong and long-term signals.

2.1. Decreasing Particle Size for Enhanced Penetration

Optimal penetration in the target tissue may be achieved by reducing the size of the nanosystem after extravasation. For example, Chen et al. proposed albumin–gold nanoparticles (BSA–Au; <10 nm) clustered by MnO_2 into 60 nm complexes (Figure 1A).^[58] In the slightly acidic tumor microenvironment, MnO_2 dissolves and the small BSA–Au particles are released. The system was investigated in mice bearing 4T1 tumors: both the small BSA–Au and the larger BSA–Au– MnO_2 clusters were administered intravenously. Fluorescence imaging indicated significantly better accumulation of the latter, expectedly due to the quick clearance of the small BSA–Au particles from the circulation. Interestingly, although not mentioned in this work, the release of free Mn^{2+} from manganese oxide particles can be further exploited to provide T1 MRI contrast (Section 5).^[61–63] In another example, nanoclusters responsive to matrix metalloproteinase 2 (MMP2) were developed by Sun et al. (Figure 1B).^[59] Thirty nanometer copolymer micelles were assembled into a 200 nm nanoclusters using polyethylene glycol (PEG), dextran and MMP2-cleavable sites as a supportive network. The researchers showed that upon reaching the tumor, the nanoclusters were successfully cleaved by MMP2, and the micelles were released. For imaging purposes, the particles were loaded with a DiO fluorescent dye, which allowed optical particle tracing in mice. The results indicated that the larger nanoclusters accumulated better in the tumor, with the MMP2-responsive clusters outperforming noncleavable control clusters. Free micelles were removed quickly from the circulation, presumably due to renal filtration.

Apart from the high concentration of metalloproteinases, tumor microenvironment is characterized also by slightly acidic pH. In this regard, Yan and coworkers developed an MRI and optical imaging multimodal nanosystem of ultrathin (5.7 nm) Gd_2O_3 nanoscrolls (about 180 nm average diameter) that

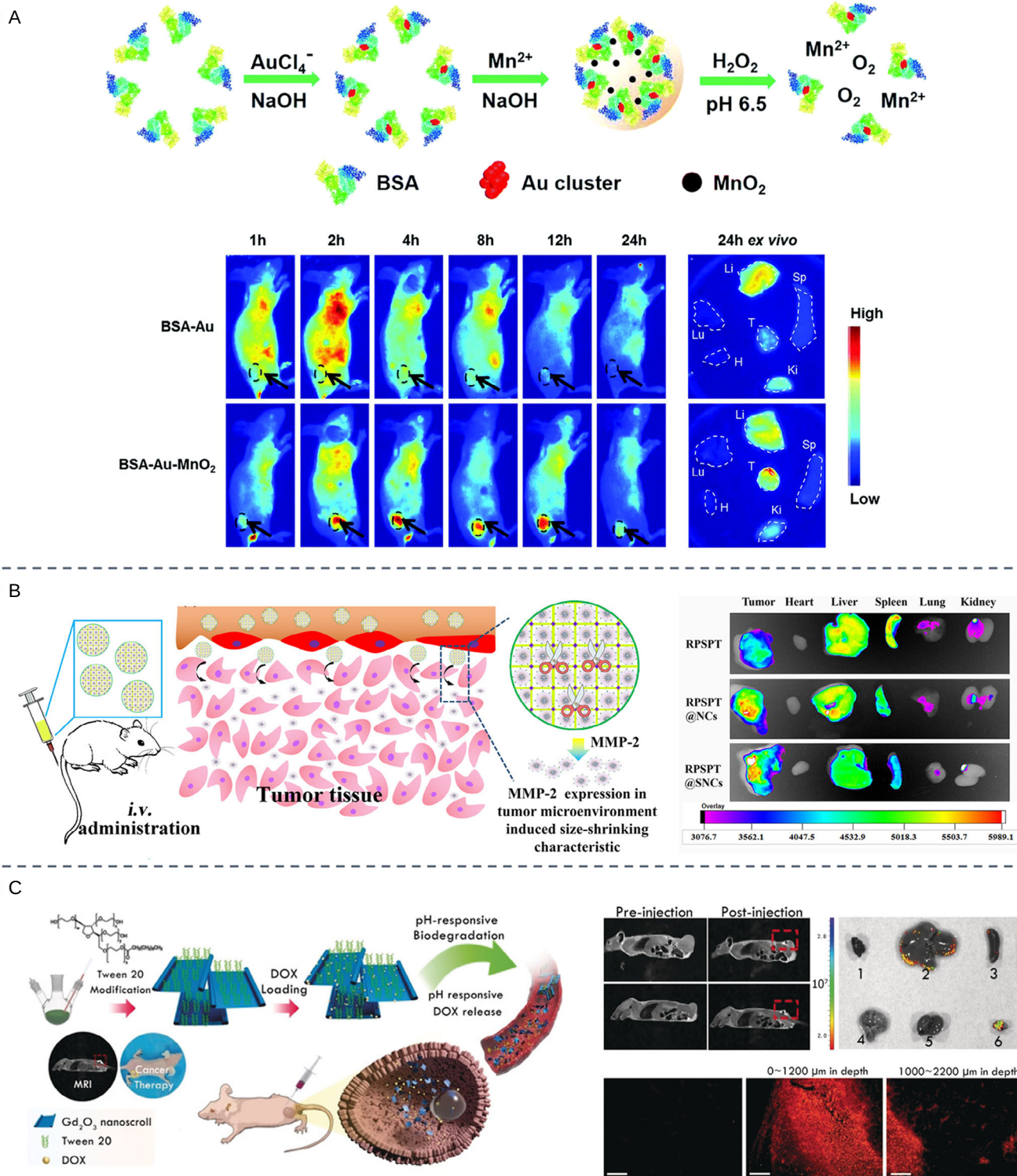


Figure 1. Decreasing particle size allows for increased penetration into the tissue. A) BSA–Au–MnO₂ composite particles degrading into smaller BSA–Au clusters in response to an acidic environment and high H₂O₂ concentrations, improving probe accumulation. Adapted with permission.^[58] Copyright 2017, Royal Society of Chemistry. B) MMP2-responsive nanoclusters dissociate into single copolymer micelles for improved penetration into tumors. Adapted with permission.^[59] Copyright 2019, American Chemical Society. C) pH-induced degradation of ultrathin fluorescent Gd₂O₃ nanoscrolls aim to improved tissue penetration and increased T1 MRI contrast. Adapted with permission.^[60] Copyright 2019, Wiley-VCH.

degraded under acidic conditions (Figure 1C).^[60] MRI T1 relaxation times correlated to nanoscroll concentration, and the T1 weighted signal was increased in tumor-bearing particle-treated mice 30 min postinjection, compared with preinjection values. Moreover, the addition of Eu^{3+} enabled optical imaging to study biodistribution of the nanoscrolls *ex vivo*, indicating strong uptake in the tumor 24 h postinjection. However, while the researchers reported particle degradation and tumor tissue penetration, they did not prove a causal relation between these observations. Interestingly, gadolinium in free form has well-known toxic effects, and is therefore only used in chelated formulations.^[1,14] Here, the investigators claim that degradation-induced Gd^{3+} release induces a therapeutic cytotoxic effect *in situ*.^[60] Finally, Huynh et al. reported the *in situ* conversion of microbubbles (2–8 μm) into nanoparticles (5–500 nm) using low-frequency US.^[64] The perfluoropentane gas microbubbles were contained in a porphyrin-containing lipid shell and provide US, photoacoustic and fluorescent signal. The authors aimed to utilize the micro-to-nano conversion to deliver the particles to the tumor, independent of the EPR effect. Extravasation is suggested to be mediated through burst-induced transient pores in blood vessels, a strategy further discussed in Section 4.

2.2. Increasing Particle Size for Enhanced Retention

In contrast to degradation, particle assembly may be used as well, as an *in situ* increase in particle size may lead to improved retention of the particle in the tissue. Therefore, such probes benefit from both the high penetration capacity of small molecule agents, as from improved retention upon self-assembly. For instance, Hai et al. developed MRI-detectable gadolinium-based particles that were assembled in the presence of γ -glutamyl transpeptidase (GGT) and glutathione (GSH) (Figure 2A).^[65] The cell surface-associated GGT enzyme is involved in the cellular production of cysteine and found to be upregulated in several tumors, including in ovarian, liver, and cervical cancer.^[69] In this case, GGT on the cell membrane first cleaves a Glu-structure in the probe. Subsequently, an intracellular reduction reaction by GSH primes the molecular probe to first dimerize, and then self-assemble into nanoparticles by π - π stacking. The *in situ* assembled nanoparticles of about 50–60 nm exhibit increased MRI T2 contrast of GGT-overexpressing tumors in mice, at high magnetic fields. Compared with conventional gadolinium-based contrast agents, the probe had higher uptake levels, indicating that a lower dose could be applied. However, as the authors noted, current clinical MRI systems are generally limited to 3 T field strengths, while these probes require stronger magnetic fields to provide sufficient T2 contrast. Similar approaches were used to image the enzyme furin,^[70,71] an enzyme biomarker that correlates to tumor progression: Bulte and coworkers used an assembly approach with the cell-penetrating peptide RVRP conjugated to the anticancer agent Olsalazine (Olsa-RVRP: Ac-Arg-Val-Arg-Arg-Cys(StBu)-Lys(Olsalazine)-CBT).^[71] Upon cell entry, Olsa-RVRP undergoes reduction by GSH and cleaving by the enzyme furin. Subsequently, particles self-assemble due to dimerization of the cleaved RVRP-Olsa and π - π stacking interactions. This system was detectable by CEST MRI, which is very sensitive to molecular agents. In this case, the hydroxyl-proton

in Olsa was used to provide contrast. *In vivo* results showed a 6.5-fold increase in CEST signal, with high correlation between imaging signal and therapeutic response. Furthermore, furin-mediated self-assembly has been reported for other modalities as well.^[72,73] Size increase in response to caspase may serve to monitor levels of apoptosis in tumor tissue.^[66,74] An et al. designed a fluorescent peptide probe that self-assembles into fibrous superstructures (5.8 $\pm$ 0.6 nm in diameter) upon enzymatic cleavage by active caspase-3/7, leading to an enhanced accumulation in tumor regions (Figure 2B).^[66] In mice, the uptake and retention of these assembled nanostructures in tumor was significantly improved compared with nonassembling control molecules, with an increased fluorescent signal over at least 120 h. Furthermore, photoacoustic imaging revealed improved tissue penetration compared with typically used liposomes and silica nanoparticles of $\approx$ 100 nm diameter. Xie et al. developed another photoacoustic probe, which is able to self-aggregate into nanoparticles by a ROS-induced hydrophobicity of the macromolecular probe.^[75]

The *in situ* self-assembly strategy has also been applied in the context of nuclear imaging, although only a few examples have been reported in the literature. Lin et al. developed a low molecular weight, fluorine-18 (^{18}F , a positron emitter with a half-life of 109.7 min) labeled activatable probe, capable to dimerize under reducing conditions to form hydrophobic species, which under physiological conditions condense into 120–140 nm nanoparticles due to π - π interactions.^[76] The authors proved that after subcutaneous injection in mice, retention of the labeled tracer in the administration site was higher when coadministered with GSH. High tracer accumulation was also observed in the tumor and in the liver and kidney of nude mice after subcutaneous administration of the labeled tracer alone, consistent with the *in vivo* expression level of GSH, thus demonstrating that formation of the dimer and subsequent *in situ* condensation into nanoparticles enhances concentration of the radioactive signal. These results position the tracer as a PET imaging agent for detection of GSH levels in living subjects. More recently, Lin and coworkers have reported a similar peptide probe for PET imaging of tumor apoptosis (Figure 2C).^[67] The probe was activated by caspase-3 to undergo intermolecular cyclization and the subsequent formation of nanosized ($\approx$ 138 nm) particles. The authors demonstrated increased tumor accumulation of the labeled peptide in mice treated with doxorubicin (a chemotherapeutic agent known to increase expression levels of active caspase-3) when compared with untreated animals, confirming that the activation of the probe and formation of nanoparticles are essential to improve concentration of radioactivity in the target tissue. The same group has recently reported a similar approach for sensing furin activity in tumors.^[77] Other enzyme-mediated self-assembly probes have been reported, including nanosystems responsive to alkaline phosphatase, gelatinase, or myeloperoxidase.^[68,78–80] For example, Chen and coworkers presented an activatable ^{18}F -labeled probe^[68] and a CT-fluorescent probe^[81] for the detection of myeloperoxidase, a proinflammatory enzyme that may function as biomarker for inflammation in patients with cardiovascular, neurological, or rheumatological diseases (Figure 2D). Myeloperoxidase activation allows the probe to bind to plasma proteins (resultant size between 55 and 70 kDa, corresponding to the size of albumin), resulting in improved retention

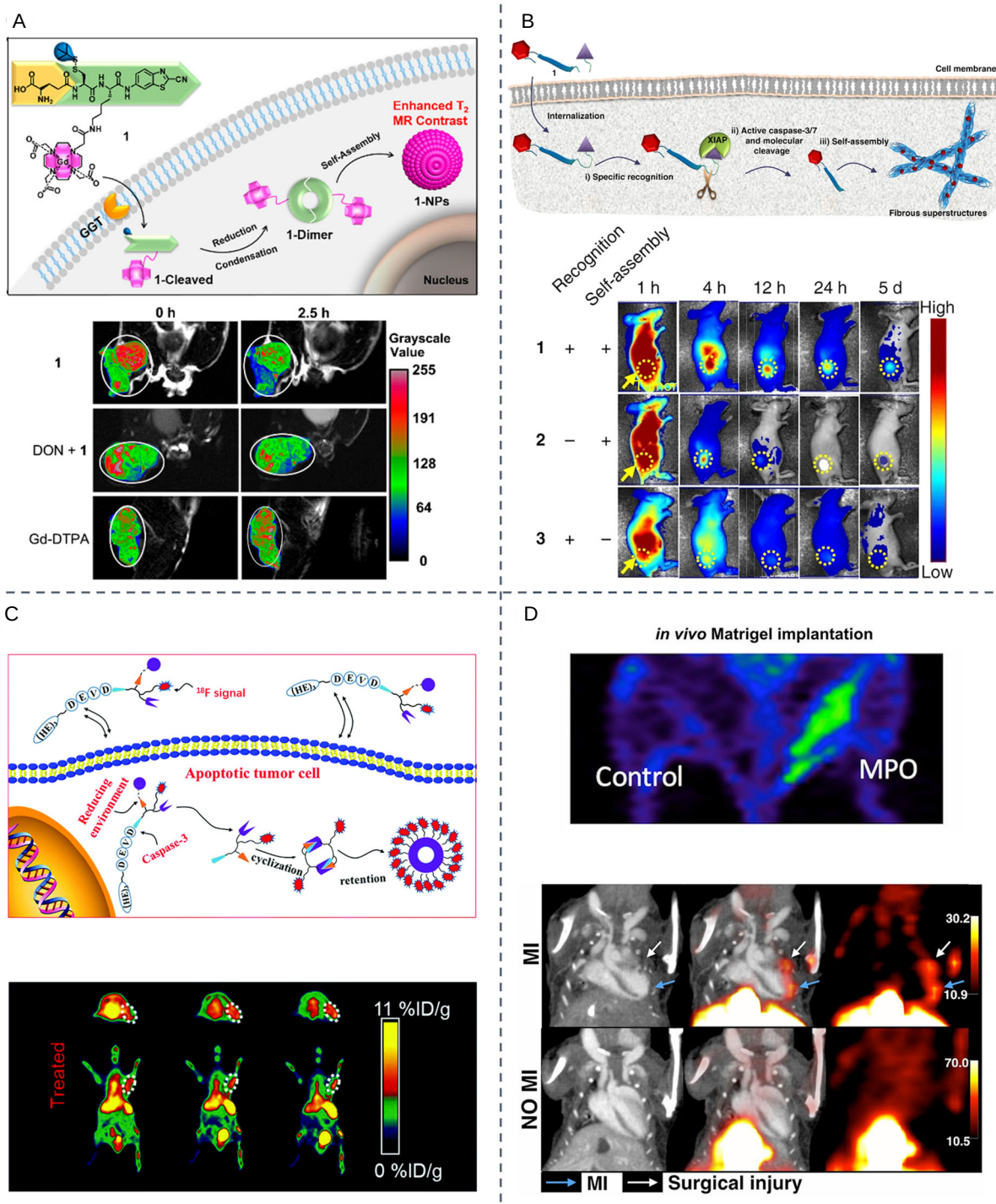


Figure 2. Increased retention in the targeted tissue due to a responsive increase in size. A) Nanoparticles providing T₂ MRI contrast are assembled intracellularly in cells in response to GGT and GSH, and outperform a conventional MRI contrast agent. Adapted with permission.^[65] Copyright 2019, American Chemical Society. B) A fluorescent probe is subject to molecular cleavage by active caspase-3/7 after recognition by X-linked inhibitor of apoptosis and subsequent self-assembly into fibrous nanostructures, which are retained in the tumor tissue. Adapted with permission.^[66] Copyright 2019, Springer Nature. C) A PET probe is reduced by GSH and cleaved by caspase-3 after cellular uptake, condensed into dimers and subsequently aggregated into nanoparticles to be retained in the tumor with constitutive PET signal. Adapted with permission.^[67] Copyright 2020, Royal Society of Chemistry. D) Myeloperoxidase-induced activation of a PET probe was demonstrated in mouse models of myocardial infarction. Upon activation the probe binds to proteins, allowing the probe to remain and thus accumulate at the disease site. Adapted with permission.^[68] Copyright 2019, US National Academy of Sciences.

and accumulation at the site of activation.^[68] In addition to in situ particle assembly, activation-induced aggregation of multiple particles may be used.^[82]

3. Active Motion for Improved Diffusion and Navigation into the Tissue of Interest

Inspired by nature, micro- and nanodevices able to move at the nanoscale (from now on micro- or nanomotors) have emerged as a very powerful tool in nanotechnology for a wide range of applications including biomedicine,^[83–85] sensing,^[86–91] or water remediation.^[86,92–95] Self-propulsion at the nanoscale is not trivial due to the need for overcoming viscous and Brownian forces.^[96] To circumvent this issue, researchers have explored the use of either in situ available chemical fuels^[97–99] or external power sources.^[100,101] Here, we will focus on those aimed to be used in nanomedicine, expected to present better performance than passive counterparts. Currently, significant efforts are being conducted to explore their actuation in complex biological fluids and the development of medical imaging techniques as effective tools to track their activity in vivo and validate their potential as drug delivery carriers and medical imaging tools.^[102] Usage of motorized nanorobots swarming through the body to autonomously sense, signal, and treat, is more realistic than ever. Still, a significant research effort is required to bring self-propulsive nanoagents to daily medical practice, as most of nanomotor publications just cover in vitro work.

3.1. Chemically Powered

One of the advantages of chemical propulsion is the use of endogenous fuels to trigger the activation of micro- and nanomotors in situ.^[97] Particularly, enzymes are natural catalysts that convert bioavailable substrates into products,^[103] making them excellent candidates for the fabrication of biocompatible motor/fuel complexes.^[98] So far, different enzymes, including glucose oxidase,^[104–106] catalase,^[92,105,107–110] acetylcholinesterase,^[111] and urease,^[112,113] or the bienzyme glucose oxidase and catalase cascade reactions,^[114–117] have been successfully used as catalytic engines. Recently, their potential application for improved cell and tissue uptake has been explored. For instance, Wilson and coworkers reported the development of motile polymeric asymmetric capsules, named stomatocytes, where catalase was encapsulated in the inner cavity. Upon the decomposition of hydrogen peroxide, often present in tumor microenvironments, the reaction products were released outside the cavity leading to self-propulsion, which resulted in an improved diffusion across a vascular endothelial model in vitro (Figure 3A).^[108] The versatility of enzyme-propelled systems allows to tailor their function according to the desired application. In this sense, motivated by the high urea concentration in the bladder, Sánchez and coworkers developed urease-powered silica-based nanomotors to enhance anticancer drug delivery^[112] and to improve the targeting and penetration of 3D bladder cancer spheroids (Figure 3B).^[113] In their work, the authors also reported that the decomposition of urea into carbon dioxide and ammonia leads to self-propulsion in both simulated and real urine. Moreover, these nanomotors not only demonstrated a

higher spheroid penetration but also a higher targeting and therapeutic capabilities of the anti-FGFR3 antibody, which specifically targets bladder cancer cells and induces cell death. The penetration of cancer spheroids by nanomotors was also achieved by Städler and coworkers, who developed a polystyrene-based motor, coated with both collagenase and manganese ferrite nanoparticles for the combination of enzymatic and heat-induced ECM disruption (Figure 3C).^[118] A higher penetration into the spheroid was found for motile particles, compared with their passive counterparts. Within the body, nanosystems may be subject to harsh conditions, such as the highly acidic environment of the stomach, demanding for specific designs. In this regard, Wang and coworkers developed magnesium-based micromotors that are able to self-propel in gastric media.^[120] Recently, the same group developed magnesium-based micromotors for the oral delivery of vaccines, where self-propulsion led to an enhanced retention and uptake efficiency at the small intestine (Figure 3D).^[119]

An additional feature of chemically powered systems is the possibility to guide them toward target locations using endogenous chemical gradients produced by the organism. For this purpose, Schmidt and coworkers developed carbonate-based Janus micromotors that are able to move in slightly acidic local microenvironments generated by HeLa cancer cells in vitro (Figure 4A).^[121] Similarly, Wilson and coworkers reported the movement of catalase-powered PLGA microspheres toward hydrogen peroxide gradients generated by activated macrophages in an in vitro culture model of inflammation (Figure 4B).^[122] Chemotaxis was also used by Battaglia and workers to facilitate the crossing of the blood–brain barrier in vivo. In their study, asymmetric polymersomes with encapsulated glucose oxidase and catalase demonstrated a chemotactic movement toward increasing glucose concentrations. In vivo, a higher accumulation in the brain was observed compared with passive particles, which was attributed to the naturally occurring glucose transport toward the brain tissues in high concentrations, compared with the surrounding tissues (Figure 4C).^[123]

3.2. Physically Powered

The propulsion of micro- and nanomotors can also be provided by external energy sources, including light,^[124–126] electric fields,^[127–129] US,^[91,130–136] or magnetic fields.^[137–142] One of the main advantages of externally actuated devices is the possibility to be propelled or steered in a noninvasive fashion, which could serve to guide imaging agents toward the intended region of interest with a high precision. Furthermore, the techniques and equipment used for acoustically and magnetically propelled devices are very similar to those used in medical imaging, such as US or MRI, respectively. In this regard, a few examples have focused on the use of US fields to increase cell penetration. Esteban-Fernández De Ávila et al. presented acoustically propelled biocompatible gold nanowires able to penetrate HEK6 and MCF-7 cells and efficiently deliver siRNA, where a 13-fold increase in gene silencing was observed when using motile nanomotors compared with static ones.^[133] In addition, optical imaging revealed that nanowires were moving intracellularly (Figure 5A). Similar approaches have been used for intracellular

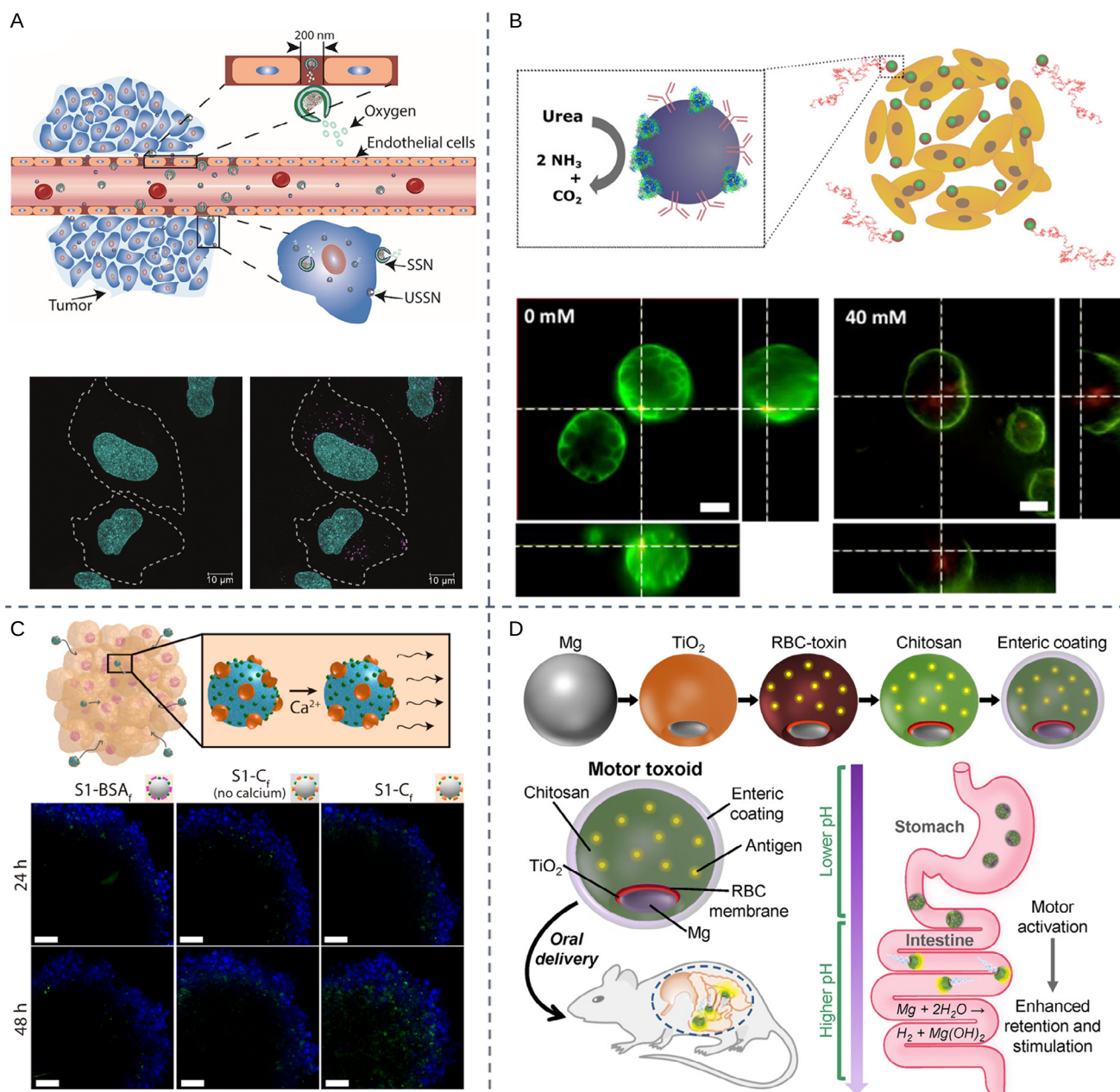


Figure 3. Enhanced cell and tissue penetration by chemically propelled micro- and nanodevices. A) Polymeric stomatocytes with encapsulated catalase are self-propelled by bubble generation, which allows for an enhanced intracellular accumulation in an endothelial vascular model in vitro. Adapted with permission.^[108] Copyright 2019, American Chemical Society. B) Urease-powered silica-based nanomotors show improved 3D bladder cancer spheroid penetration and cell death, which is due to the combination of self-propulsion and the incorporation of the FGFR3-targeting therapeutic antibody. Adapted with permission.^[113] Copyright 2019, American Chemical Society. C) Enhanced penetration of tumor spheroids by collagenase/manganese ferrite on polystyrene nanomotors. Adapted with permission.^[118] Copyright 2019, American Chemical Society. D) Oral administration of antigens using magnesium-based micromotors, providing increased retention and uptake efficiency in the small intestine. Adapted with permission.^[119] Copyright 2019, American Chemical Society.

real-time sensing of miRNA-21,^[144] mRNA,^[131] oncoproteins,^[145] and the delivery of functional Cas9/sgRNA.^[134] Despite these examples, the use of magnetic fields as a power source for biomedical applications is much more established, as their unique controllability turns magnetically propelled devices into very promising miniaturized robotic tools for

minimally invasive microsurgery, therapeutic, and diagnostic.^[101] Self-propulsion with high precision control over the trajectory can be achieved using rotating magnetic fields. Figure 5B shows a recent example where magnetic helical microstructures were coated with a biocompatible zinc-based metal–organic framework, responsive to pH. The authors demonstrated that

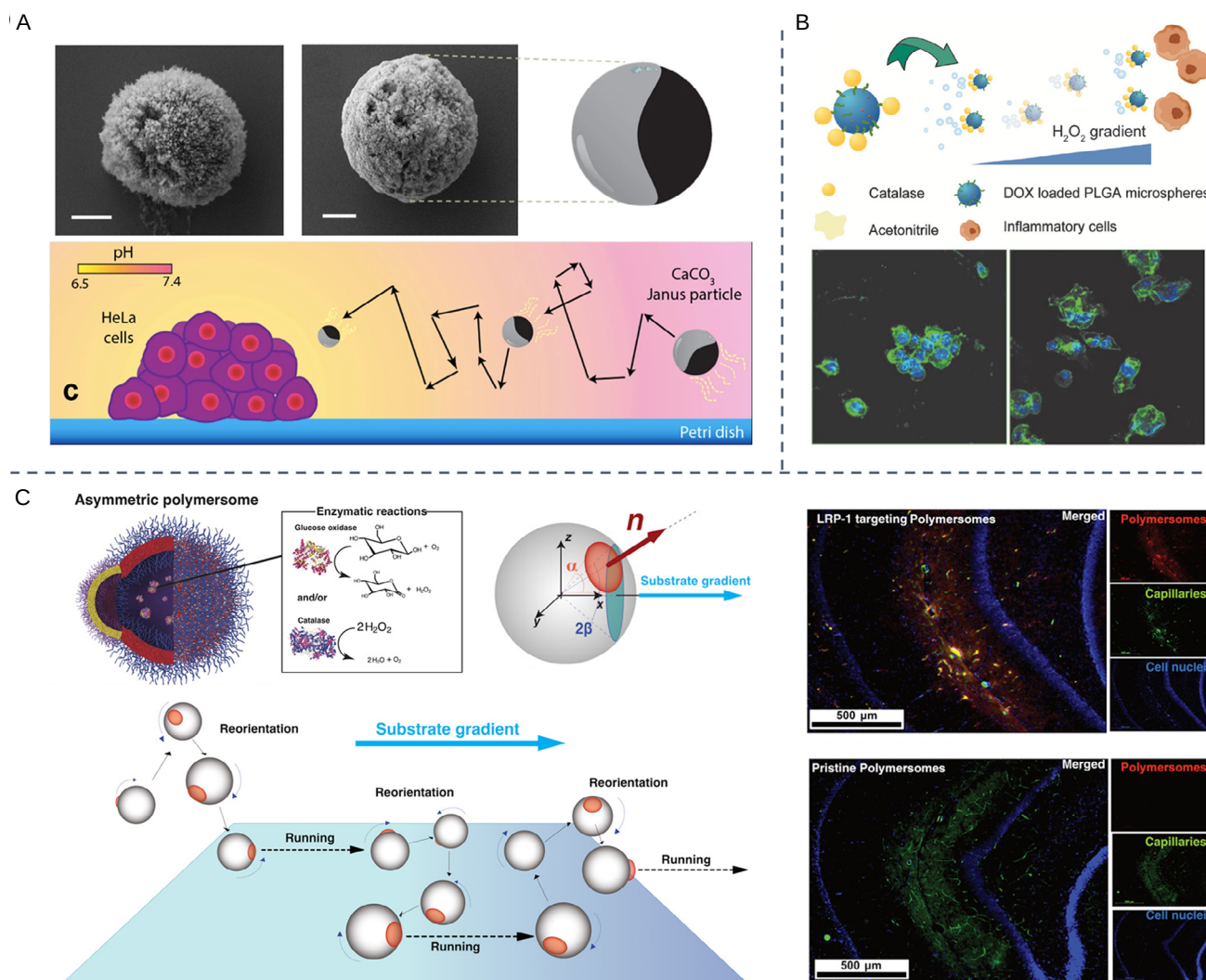


Figure 4. Chemotactic motion of micro- and nanomotors using endogenous gradients. A) Carbonated Janus micromotors are able to move in slightly acidic conditions generated by HeLa cells in vitro. Adapted with permission.^[121] Copyright 2016, Springer Nature. B) PLGA microspheres move toward hydrogen peroxide gradients generated by activated macrophages. Adapted with permission.^[122] Copyright 2020, Wiley-VCH. C) Chemotactic polymer-some vesicles were designed to cross the blood–brain barrier by motion toward glucose gradients. Adapted with permission.^[123] Copyright 2017, AAAS.

these microrobots were able to follow predefined trajectory paths and target single cells in 2D cell culture models. In the vicinity of the cells, acidic pH triggered the release of fluorescent rhodamine-B as a model drug, demonstrating a highly functional integration into a single device.^[143] Although the majority of studies have focused on in vitro models and Newtonian fluids, for an efficient translation into real clinical applications several aspects need to be addressed, such as the motion capabilities in highly viscous media and across complex biological matrices that may act as a barrier. Several strategies have been developed in this direction, such as the design of screw-like micromotors for the penetration and swimming across complex viscoelastic fluids based on hyaluronan^[146] and the vitreous of the eye^[147] in vitro. Remarkably, Fischer group achieved for the first time the design of nanopropellers that could be actively and controllably propelled across the vitreous humor upon intraocular injection in a porcine eye and reach the retina (Figure 5C).^[140] The

actuation of such nanopropellers was monitored using standard optical coherence tomography, used in clinical applications, thus opening the door for future real clinical applications. Moreover, the motion control of microrobotic swarms in vivo was achieved by Servant et al., who developed bacteria-like microrobotic flagella.^[148]

3.3. In Vivo Monitoring and Guidance of Micro- and Nanomotors Actuation using Medical Imaging Technologies

The rapid and exciting advances on the use of micro- and nanomotors as powerful tools in biomedicine is demanding the use of medical imaging techniques to monitor and control their performance. In turn, the advanced capabilities of micro- and nanorobots might improve the precision and targeting of specific regions of interest for medical imaging

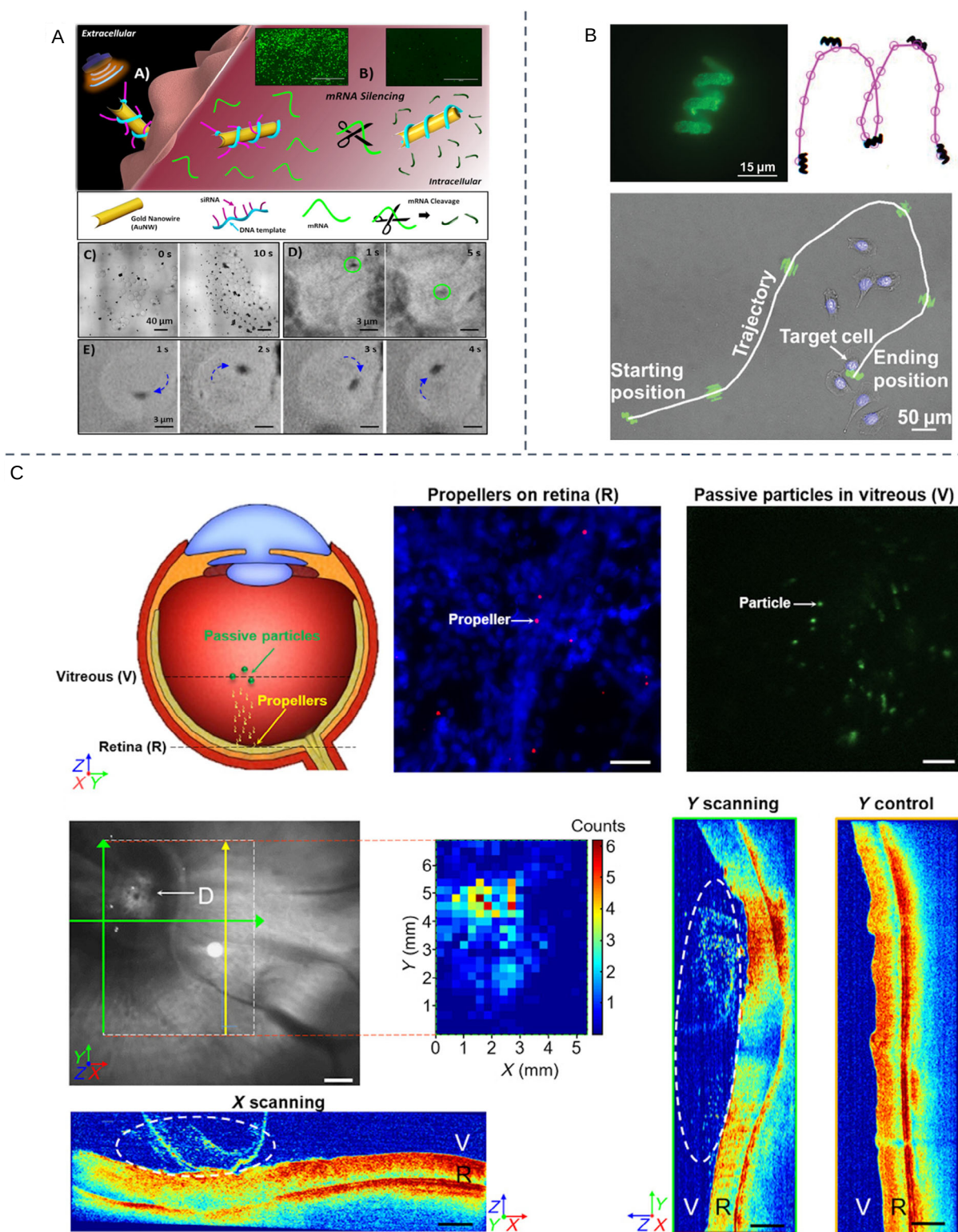


Figure 5. External actuation of motors provides guidance and enhances cell and tissue penetration. A) Biocompatible acoustically propelled nanomotors move intracellularly and deliver siRNA for gene silencing. Adapted with permission.^[133] Copyright 2016, American Chemical Society. B) Magnetic helical microstructures coated with zinc-based metal–organic frameworks are capable of pH-responsive delivery of Rhodamine-B as model drug. Adapted with permission.^[143] Copyright 2019, Wiley-VCH. C) Nanopropellers in the vitreous humor of the eye are actively propelled to reach the retina, monitored by optical coherence tomography. Adapted with permission.^[140] Copyright 2018, AAAS.

techniques. A multidisciplinary approach is thus required, where synergistic approaches combining active propulsion and medical imaging guidance could revolutionize current theranostic approaches. Although the field is still at an early stage, several milestones have been achieved, such as the use of medical imaging techniques for the tracking and controlled actuation of micro- and nanomotors. The bubble generation by catalytically and US propelled micro- and nanomotors can be used for US imaging. For instance, Magdanz and coworkers were able to control the trajectories of self-propelled microjets, which used hydrogen peroxide as fuel, where the generation of oxygen bubbles could be imaged by B-mode US imaging, which provided feedback for the guidance of their trajectories using magnetic fields.^[149] In a similar approach, Olson et al. presented a new form of US molecular imaging, which was tested in vivo.^[150] The system was based on the visualization of bubbles generated by micromotors, which resulted from the decomposition of hydrogen peroxide released by neutrophils, making this a diagnostic tool for inflammation. The in vitro tracking of catalytic micromotors using nuclear imaging techniques has also been reported, as Vilela et al. developed radiolabeled tubular microjets powered by hydrogen peroxide.^[151] The authors were able to monitor the movement inside phantoms using PET-CT (Figure 6A). Alternatively, SPECT was used by Iacovacci et al. for the tracking of thermoresponsive soft microrobots in vitro and using ex vivo rodent models, through the inclusion of radioactive labeling.^[152] The authors reported not only the visualization of the micromotor but also their configuration, where folding and unfolding in response of a temperature change could be observed (Figure 6B). The use of MRI for the control and guidance of micromotors in vivo also holds a great potential for imaging guided therapy. In this regard, Yan et al. pioneered magnetic microrobots based on the *Spirulina* microalgae coated with magnetite (Fe_3O_4).^[153] The authors demonstrated that these magnetic microrobots could not only be tracked but also propelled and steered inside the stomach in rats using rotating magnetic fields (Figure 6C). More recently, Xie et al. demonstrated magnetic actuation with photoacoustic tracking of magnetized *Spirulina* and off/on fluorescent detection of a bacterial infection in mice.^[155] Photoacoustic computed tomography (PACT) has been used for the in vivo monitoring of a microrobotic system by Wu et al.^[154] The authors designed a gastrointestinal tract-targeted system consisting of gold-magnesium micromotors, which were encapsulated into a hydrogel to protect them from the harsh conditions of the stomach. Upon oral administration, their distribution through the bowels was monitored by PACT. Once they reached the intended area in the intestine, NIR irradiation was used to activate the release of micromotors from the capsules, triggering their motion capabilities and subsequently their penetration and in the intestinal wall (Figure 6D).^[154] Active nanoparticles hold a great promise as future medical nanotools due to their enhanced mobility across biological environments, which could improve the targeting and accumulation of nanocarriers at the target locations, where they could be used for drug delivery and imaging purposes. However, despite these exciting outcomes, there is still a gap in the clinical translation of active particles for medical imaging purposes. In this regard, the implementation of imaging modalities to visualize and track active nanoparticles is in its infancy. Recent proof-of-concept

studies have focused on the use of medical imaging to track of these devices, which is a key milestone in the field. However, motion control and guidance of active nanoparticles in vivo is not trivial due to the heterogeneity and complex dynamics found in the organism, such as blood flow rates, body movement, lung expansion, highly viscous media, and so on. Moreover, deeper insights into the interactions of motile particles with the biological barriers are needed. In addition, motile particles might display collective behavior. Understanding how swarms of active nanoparticles will behave in vivo is of paramount relevance, where multidisciplinary approaches that combine medical imaging technologies to track active nanoparticle swarms in vivo, as well as biodistribution studies will significantly contribute to the advancement in the field. Moreover, exhaustive studies on their biocompatibility, biodistribution, and biodegradability will be necessary to implement these devices in a safe manner.

4. Modification of the Surrounding Environment

Before reaching the intended destination, a nanoparticle must cross many biological barriers.^[38] These barriers may include avoidance of the immune system, escape from the blood vessel into the tissue, as well as traversing the ECM to deeply penetrate tumors or other tissues. In tumor tissue, ECM composition is often altered, where increased levels of collagen are present within the ECM and surrounding tumor vasculature, constituting a limitation for the extravasation and penetration of particles deep into the tissue.^[156,157] Moreover, epithelial surfaces such as the gastrointestinal tract or the airways are covered by a layer of protective mucus. Application of intelligent colloids through these entry routes therefore requires penetration of this layer. In this section, the recent advances in the engineering of nanoparticles that modify their surrounding microenvironment to facilitate their accumulation at the target locations are discussed.

4.1. Editing the ECM

In some tumors, the collagen networks in the ECM may be especially dense, which hinders the diffusion of therapeutic or diagnostic agents (Figure 7A).^[156,158] Indeed, researchers found that tumors with dense collagen networks exhibited higher interstitial resistance to macromolecule penetration compared with tumors with loose collagen networks. This problem is especially relevant for large-sized agents such as colloidal systems.^[156] Others found a negative correlation between collagen content surrounding vessels and extravasation of particles, but not small molecules.^[157] Hence, even though the concept of higher accumulation of particles compared with molecular agents through mechanisms, such as the EPR or through active transepithelial transport, has been widely accepted, not all aberrant properties of the tumor microenvironment support these effects. To further increase the accessibility of nanoparticles to the tissue, (partial) degradation of the ECM has been perceived as a possible solution. Often, these approaches are aimed at achieving a reduction or breakdown of key ECM components such as collagen or hyaluronan. For example, to reduce ECM density, Diop-Frimpong et al. attempted to reduce the production of collagen by tumor stroma fibroblast

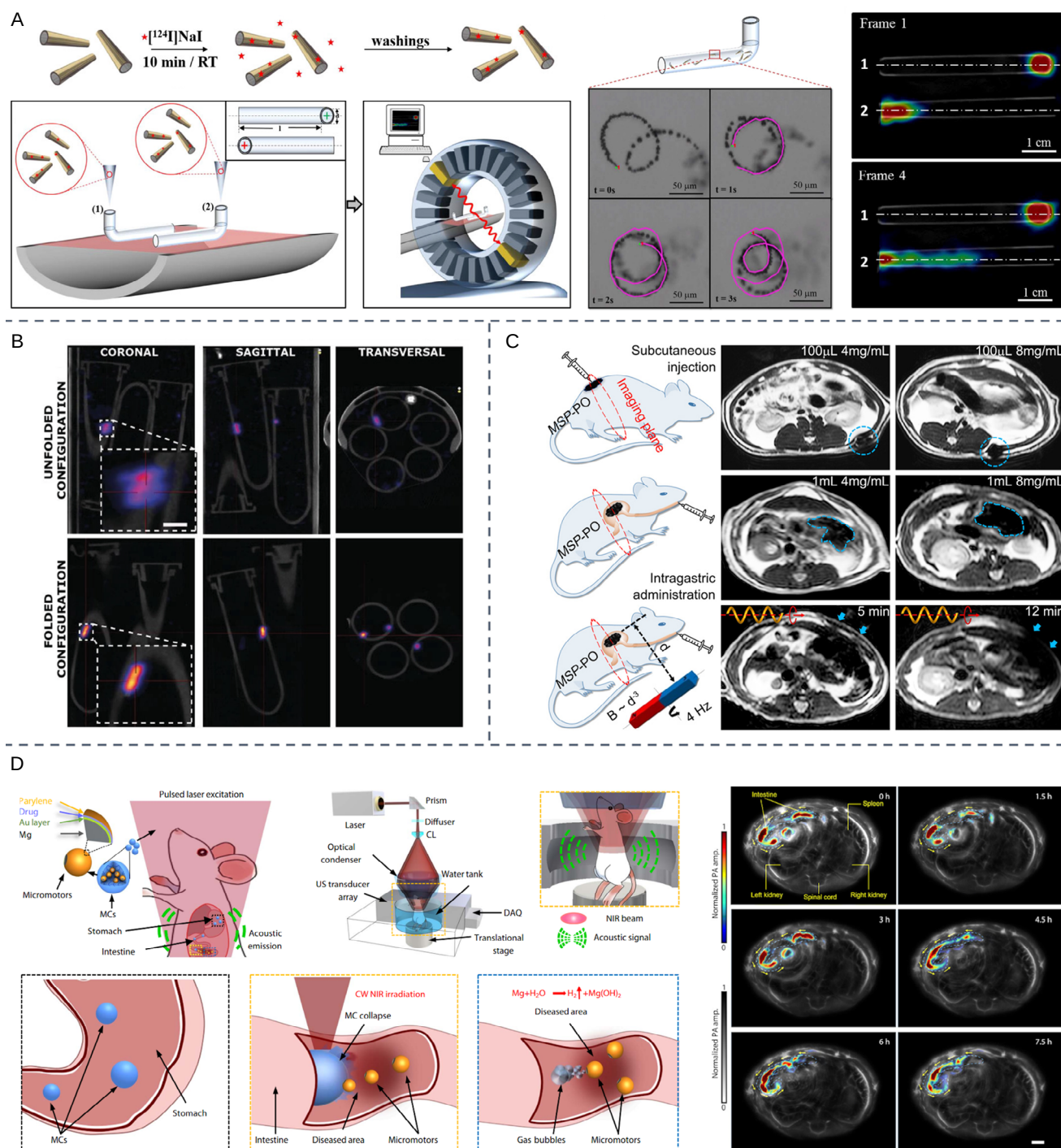


Figure 6. Tracking and control of micro- and nanomotors using medical imaging techniques. A) Radiolabeled tubular microjets are powered by hydrogen peroxide, and movement in tissue phantoms was monitored using PET-CT. Adapted with permission.^[151] Copyright 2018, American Chemical Society. B) SPECT-monitoring of radiolabeled soft thermoresponsive microrobots. Adapted with permission.^[152] Copyright 2019, Wiley-VCH. C) MRI imaging of biohybrid magnetite microrobotic swarms in vivo. Adapted with permission.^[153] Copyright 2017, AAAS. D) PACT was used to monitor micromotors through the gastrointestinal tract. Upon NIR stimulation, motion is triggered, which leads to increased penetration into the intestinal wall. Adapted with permission.^[154] Copyright 2019, AAAS.

cells using treatment with angiotensin-II receptor antagonist losartan, which resulted in an improved accumulation and tumor penetration of colloidal agents.^[161] Another option is the

treatment of the ECM with proteolytic enzymes, such as collagenase or hyaluronidase. These enzymes affect the breakdown of collagen and hyaluronan networks, respectively, both major

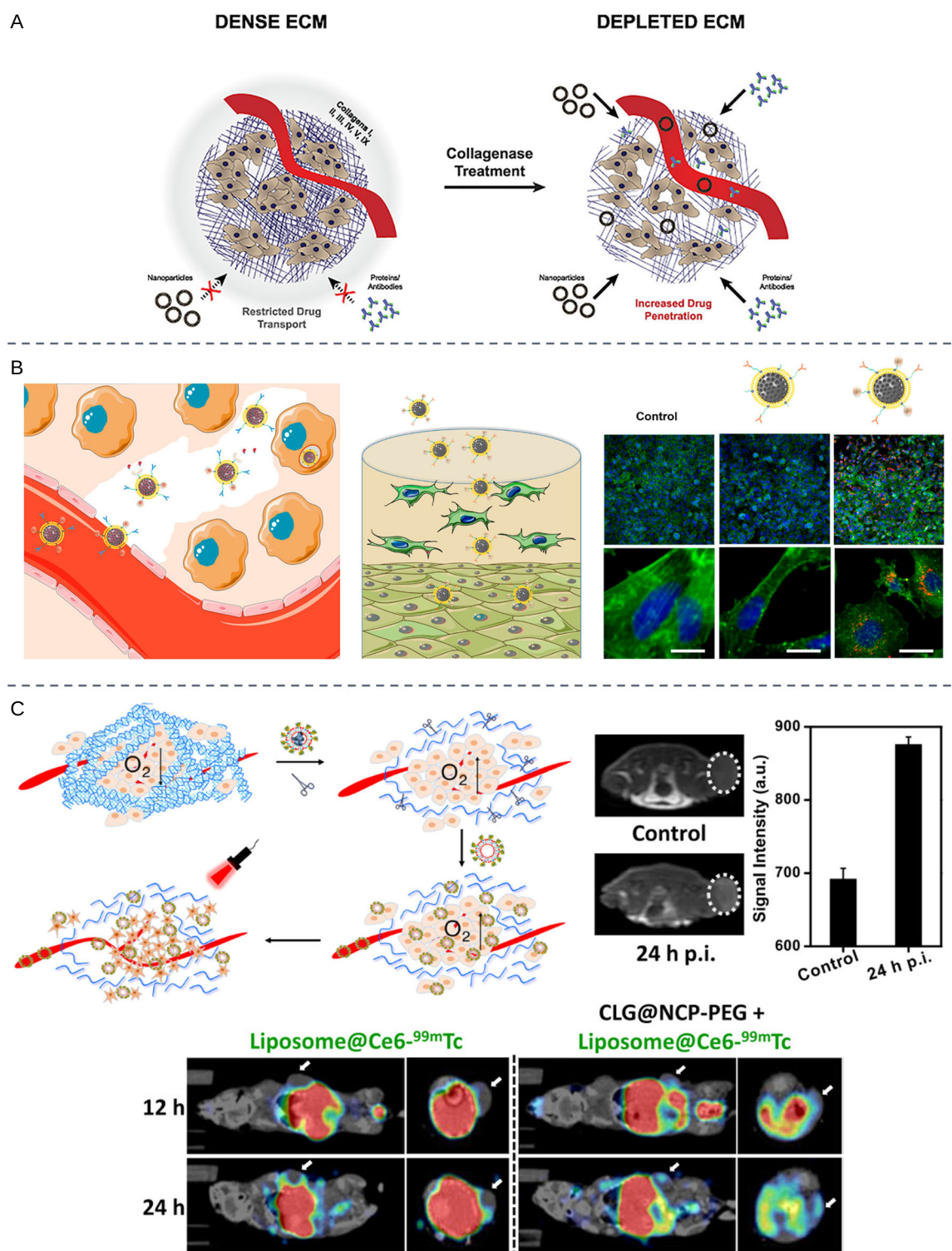


Figure 7. Destruction of the ECM improves imaging agent access. A) Penetration of therapeutic or imaging agents to the tumor microenvironment may be enhanced by destruction of the ECM. Reproduced with permission.^[158] Copyright 2018, ACS Publications. B) To induce proteolytic effects, collagenase is released from polymeric nanocapsules assembled onto the surface of protocell nanosystems, in acidic environments. Adapted with permission.^[159] Copyright 2018, American Chemical Society. C) Low pH levels lead to the release of collagenase and manganese ions from polymeric particles, increasing ECM access to a secondary wave of SPECT-detectable particles, and proving MRI contrast. Adapted with permission.^[160] Copyright 2018, American Chemical Society.

components of the ECM. For instance, Hassid et al. showed that pretreatment with collagenase could increase the concentration and improve the distribution of a conventional gadolinium MRI contrast agent within tumor tissue in mice.^[162] While both intravenous and intratumoral injection of these enzymes has been reported, the latter may result in longer lasting efficacy.^[158,163] Moreover, to increase delivery precision, such enzymes can be codelivered together with the imaging agent itself.^[118,147,159,160,164,165] For example, an early in vivo study was conducted by Murty et al., who presented a modest increase in the accumulation of collagenase-coated gold nanoparticles in murine tumor xenografts compared with bare gold nanoparticles.^[165] More recently, Villegas et al. designed a protocell system carrying collagenase-containing polymeric nanocapsules (Figure 7B).^[159] In acidic conditions such as the tumor microenvironment, the capsules dissolve to release the collagenase and induce a local proteolytic effect. In a 3D in vitro setup, the authors demonstrated the improved penetration of the fluorescently labeled collagenase-carrying protocells through collagen gel, compared with noncollagenase controls. Moreover, Liu et al. developed a nanosystem containing collagenase and manganese (Figure 7C).^[160] Both could be released from the polymer particles in response to an acidic tumor microenvironment. The free manganese ions would increase MRI contrast, whereas the release of collagenase induced enhanced tumor perfusion, reduced local hypoxia, and improved penetration of a second wave of IV-administered SPECT-detectable liposomes.^[160] A similar approach was used by Nelson and coworkers, where they used different surface coatings, including the enzymes hyaluronidase and collagenase to improve swimming capabilities of microrobots in the eye vitreous.^[147] In this study, the presence of both hyaluronidase and collagenase resulted in the reduction of the viscosity of the vitreous, which lead to more than 40% increased mobility when compared with nonenzymatic hydrophilic coatings such as PEG or TiO₂.^[147] Nevertheless, the role of the ECM and vascular permeability in tumor progression and metastasis, as well as in drug and nanoparticle penetration, is still subject to extensive debate. Over-treatment with ECM-deconstructing agents might lead to tumor collapse, resulting in a reduction of particle diffusion, and increased chance of metastasis or unintended degradation of collagen in healthy tissue.^[158] Indeed, other nanosystems seek an opposite strategy, a good illustration of this being gadolinium metallofullerenol nanoparticles developed to inhibit ECM degradation by MMPs, to prevent cancer metastases.^[166] And despite the antifibrotic effects of angiotensin-II antagonists, agonists to this receptor have been used to enhance the effect of EPR-based selective accumulation of particles in tumors.^[19,54] In addition, using proteases to degrade the ECM results in the release of cleaved ECM fragments, that may lead to macrophage activation and to the induction of an immune response.^[167] Perhaps, these issues could be resolved using a highly targeted approach (e.g., using active targeting moieties), or the administration of treatments with concomitant local immune response modulation and by optimizing the applied therapeutic dose. Altogether, such effects should be evaluated in multidisciplinary studies, combining pharmacokinetics and pharmacodynamics, inflammatory response monitoring, and measurements of therapeutic effects on tumor growth and metastases.

4.2. Liquefying Mucus Layers

In specific tissues, such as the lungs and the gastrointestinal and urogenital tracts, reaching the epithelial surfaces and underlying tissue involves the interaction of contrast agents with the protective layer of mucus. This layer prevents pathogens to reach these tissues and also limits nanosystems' penetration capacity. Nanoparticles have shown facilitated diffusion across mucous microenvironments due to inherent properties^[168,169] or surface-modifications such as PEG, chitosan, or bile salts, although many efforts have not yet progressed to in vivo research.^[170–174] For instance, Hanes and coworkers loaded PEG-coated liposomes with a CEST MRI contrast agent and studied the distribution and retention of the nanosystem in cervicovaginal mucus, demonstrating its potential for mucosal theranostics or imaging.^[174] For instance, Taipaleenmäki et al. developed mucopenetrating polymer–lipid particles in mucoadhesive alginate carriers, and studied the delivery and retention of the particles in the gastrointestinal tracts in rats (Figure 8A).^[169] The polymer–lipid particles were retained in mucus layers after oral administration, although no added benefit of alginate encapsulation was found in vivo. Interestingly, several biological pathogens have developed sophisticated methods to actively manipulate their environments to gain access to their targets. This may be through the use of metalloproteinases (*Vibrio cholerae*), serine proteases (*Trichuris muris nematodes*), or urease enzymes (*Helicobacter pylori*) to dissolve mucus linings.^[176–178] The latter increases its flagella-induced motility in the stomach wall to reach the epithelial layer by liquifying the mucus lining. The secreted urease converts urea into ammonia, raising the local pH, affecting the rheological parameters of the surrounding mucus.^[177] Inspired by these naturally occurring examples, Fischer and coworkers developed urease-coated micropropellers, consisting of SiO₂ helices with a thin magnetic section (Figure 8B).^[175] Rotating magnetic fields may induce propulsive motion, while reduction of the viscosity of the surrounding mucus by urease activity increases the speed. These properties improved access to the endothelial layers covered by mucus. Similarly, the reaction of magnesium with water in the microrobotic system reported by Wu et al., and previously described in this Review, causes elevated pH levels, which improves mucus penetration.^[154] In addition, the authors claim that the free magnesium cations result in collapse of the mucus layer. The magnesium nanoparticles had higher diffusion and retention compared with passive silica particles used as control.^[154] Finally, Bernkop-Schnürch and coworkers presented several mucolytic coatings for drug carriers, including particles decorated with mucin-cleaving enzymes such as trypsin, papain, or bromelain.^[179–181] Their results show increased mucus penetration and subsequent particle retention in the intestinal mucosa in rats.^[181]

4.3. Mechanical Deconstruction of the Tumor Microenvironment

Alternatively to these enzymatic or chemical approaches, the structural integrity of the target tissue and its vasculature can be weakened mechanically, and the generation of microbubbles

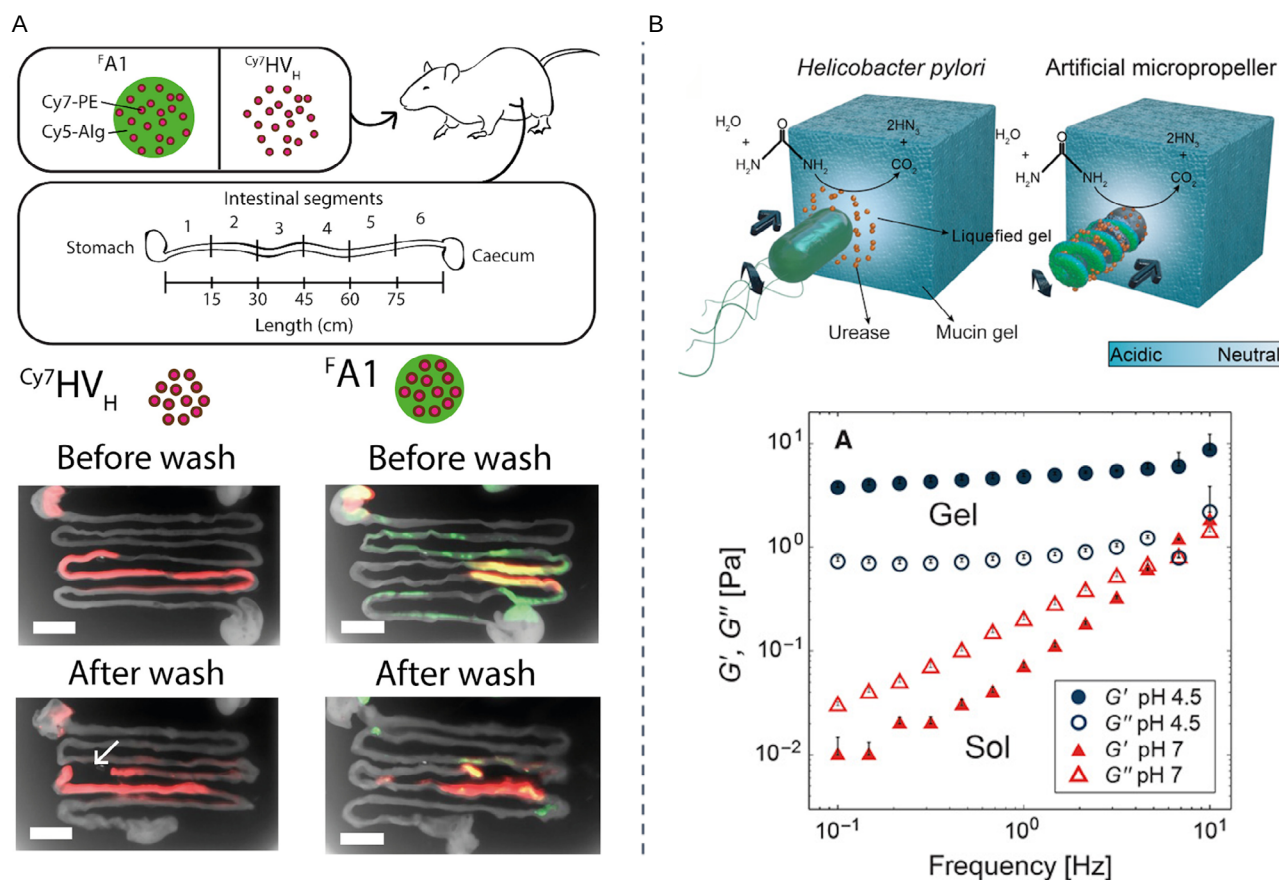


Figure 8. Increased penetration in mucus layers. A) Delivery of mucopenetrating polymer–lipid nanoparticles (red signal) by mucoadhesive alginate carriers (green signal) to the gastrointestinal tract wall was evaluated in rats. By washing the intestines ex vivo, the carriers were removed but the nanoparticles were retained in the intestinal wall. Adapted with permission.^[169] Copyright 2020, Elsevier. B) *H. pylori* bacteria produce urease to increase the local pH, resulting in reduced viscosity of the mucus. Similarly, magnetic helical micropropellers are functionalized with urease to convert water and urea in the stomach to ammonia and CO₂, creating a basic environment, facilitating movement through the mucus. Reproduced with permission.^[175] Copyright 2015, AAAS.

is a prime method to induce this mechanical stress.^[182–184] Microbubbles can be created by US pulses causing acoustic droplet vaporization of liquid perfluorocarbons (PFCs). Noteworthy, there is already ample experience in the literature with this type of compounds, as PFCs have been used to provide contrast for US imaging and for MRI for years.^[46,185,186] Zhang et al. produced perfluoropentane nanodroplets from which microbubbles were generated upon the application of US pulses (Figure 9A).^[182] After the induced phase transformation of perfluoropentane, US contrast was enhanced and increasingly strong photoacoustic signals could be detected over time. Moreover, optical imaging could be used to observe the biodistribution, which indicated accumulation in the tumor, as well as in the liver and the lungs. In addition, the researchers claim that the acoustic pressure gradient acts as a driving force on the nanodroplets and bubbles forcing them into the tissue, further improving penetration. Analysis of histological sections observing the distribution of particles relative to tumor vasculature indicated increased tissue penetration of PFC droplets compared with nonbubble-generating liposomes.^[182] Moreover, Ho et al. investigated acoustic droplet vaporization

of micro- and nanodroplets PFC by intravital microscopy in tumor-bearing mice (Figure 9B).^[184] In this study, tissue penetration of a second wave of fluorescent liposomes was compared with the conventional passive EPR effect. The results indicate significantly improved particle access after micro- or nanodroplet vaporization, the latter being the most efficient. Similarly, bubble-mediated propulsion of nano- and micromotors, as discussed in the previous section, may be advantageous in tissue deconstruction as well.^[187–189] Kagan et al. demonstrated PFC-loaded microbullets capable of tissue penetration, as well as inducing tissue expansion and splicing under acoustic droplet vaporization (Figure 9C).^[187] The incorporation of photothermal agents in PFC droplets allows for optical droplet vaporization by laser light, which could be advantageous for photoacoustic imaging.^[190,191] Collagen disruption and ECM remodeling can be induced using external forces as well, by applying pulsed high intensity-focused US. As a result, tumor targeting and penetration of fluorescent chitosan nanoparticles was improved in mice (Figure 9D).^[163] Alternatively, a photothermal effect, which is found in many nanosystems, may induce endothelial damage further increasing access of the particles

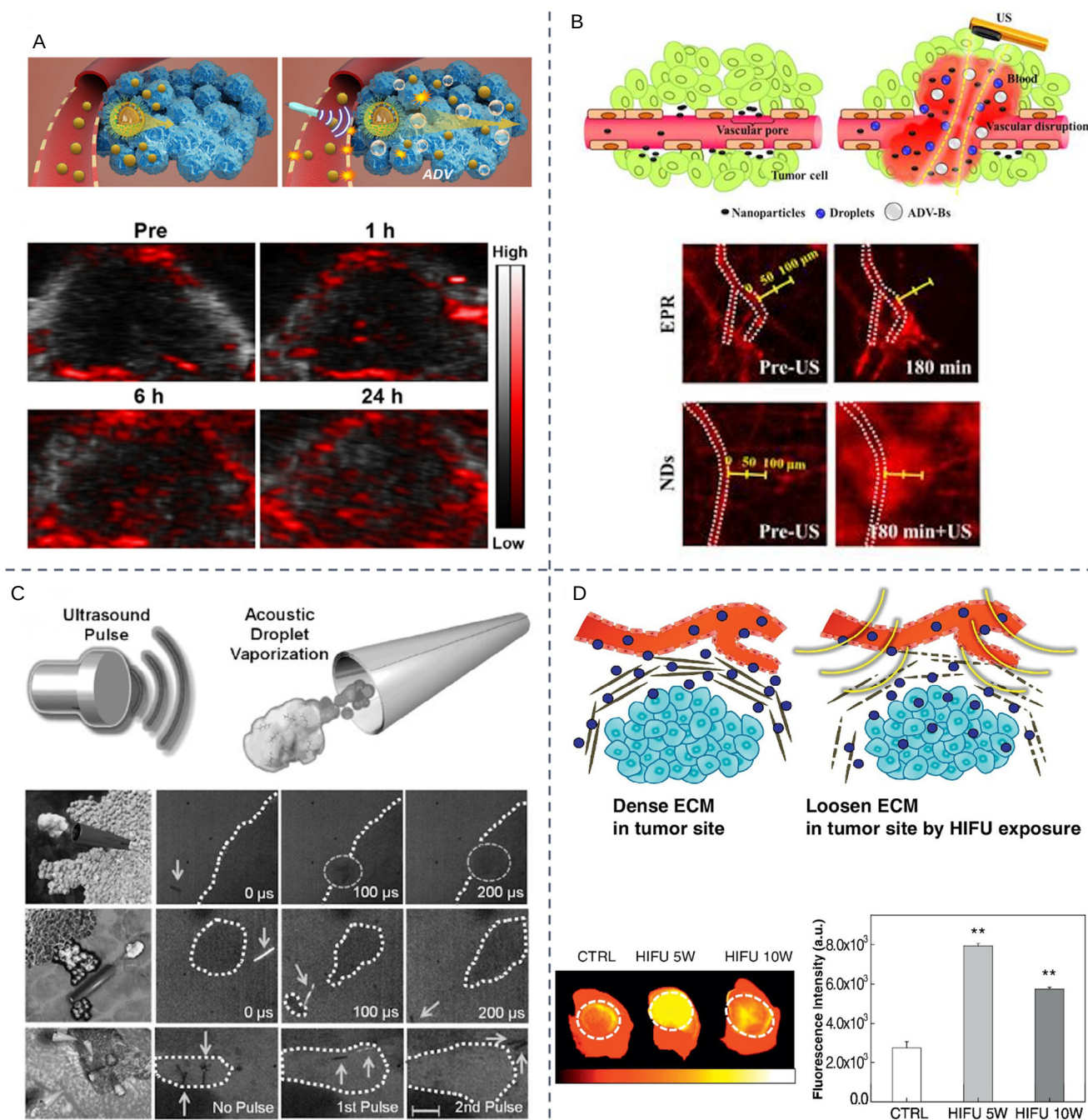


Figure 9. Mechanical deconstruction of tissue to promote particle penetration. A) Acoustic droplet vaporization of perfluoropentane nanodroplets by USs stimulation increases tissue penetration by inducing vessel disruption and tissue damage. Adapted with permission.^[182] Copyright 2019, ACS Publications. B) The induction of tissue erosion through bubbles generated by acoustic droplet vaporization of PFC nanodroplets allows increased tissue penetration of a second wave of liposomes loaded with a fluorescent dye, compared with passive EPR accumulation. Adapted with permission.^[184] Copyright 2016, Ivyspring. C) PFC-loaded microbullets are capable of inducing tissue swelling and splicing, and exhibit tissue penetration as a result of US-induced acoustic droplet vaporization. Reproduced with permission.^[187] Copyright 2012, Wiley-VCH. D) High intensity-focused US is used to loosen the ECM structure, enabling nanoparticle access to tumors. Adapted with permission.^[163] Copyright 2017, Elsevier.

to the tumors.^[192] This therapy may be targeted specifically to vessels, for example, by functionalization with an arginine–glycine–aspartic acid (RGD) peptide targeting $\alpha_v\beta_3$ -integrins in angiogenic tissue.^[18] However, as mentioned earlier, methods

to disrupt the tumor stroma integrity may increase the chance of metastasis. Therefore, investigators should find a balance between promoting particle penetration and preserving sufficient tumoral tissue integrity.

5. Sensing of Physiological Analytes by Nanosystems

Diseased tissue generally exhibits an altered microenvironment, and aberrant levels of signaling molecules or metabolites may be used as biomarkers to track disease progression or therapeutic effect. Molecular imaging to report this physiological information may conventionally be applied by actively targeting the imaging agent toward molecular markers related to the process under scrutiny, such as targeting to glucose transporters, tumor antigens or biomarkers of angiogenesis, or apoptosis.^[31,193–196] Furthermore, the signal of a contrast agent can be actively modulated in response to certain conditions. In this manner, the presence of a certain analyte or cell type can be detected, without the need to perform invasive tests. Moreover, responsive contrast agents for fluorescent, photoacoustic, or MRI may provide ratiometric measurements. In this case, two different signals are being measured, one of which is modulated in response to the presence of the analyte. The ratio between the two signals is then calculated and used as the final readout. This approach offers self-calibration, mitigating the effect of signal interference due to tissue scattering or heterogeneous probe accumulation. Thus, intelligent nanosystems may be used as *in vivo* sensors to obtain physiological information and improve medical diagnostics or therapy monitoring.

5.1. Detecting pH, Hydrogen Peroxide and Oxygen Levels

The tumor microenvironment is often characterized by a slightly acidic pH, increased levels of hydrogen peroxide and hypoxia. In other diseases similar changes occur, such as an increased production of hydrogen peroxide in inflamed tissue. Current nanosystems can modulate their properties in response to these conditions, resulting in enhanced imaging contrast in the affected areas. This physiological information may aid in detecting diseased tissue. For instance, MRI contrast may be modulated by increasing the interaction between the contrast agent and protons in the surrounding water in response to low pH.^[197–199] For instance, Li et al. presented assemblies of extremely small iron oxide nanoparticles (7 nm hydrodynamic diameter) crosslinked into larger assemblies (about 80 nm).^[197] In an acidic environment, the assemblies disassemble into single nanoparticles greatly enhancing water accessibility, which is further enhanced by the hydrophilic surface of the particles (**Figure 10A**). The shortened T1 relaxation time of protons in the surrounding water results in a brighter T1 MRI signal. A pH-responsive gadolinium-based MRI nanosystem based on the same principle of water interaction was presented by Liu and coworkers.^[198] Therefore, while these systems are able to reconfigure their size, the main imaging enhancing effect is not due to improved tissue penetration, as discussed in Section 2, but to an increase in contrast signal as a response to pH. The release of Mn²⁺ from manganese-containing particles in acidic and/or reducing tumor microenvironment is also a popular method to enhance MRI contrast.^[61–63,204] However, there are concerns that chronic exposure to free magnesium ions may result in toxic effects.^[205] Tissue pH levels may be determined by CEST MRI. Based on the pH-sensitive proton chemical exchange

rate between the imaging agent and water, both molecular and nanoformulated agents may be used for this purpose.^[42,206,207] Moreover, observing pH using optical^[89,208–210] or photoacoustic imaging^[200,211–213] is enabled by a variety of responsive probes. For instance, Jo et al. presented a ratiometric probe based on an optical pH indicator encapsulated in polyacrylamide nanoparticles and demonstrated its use in tumor-bearing mice (**Figure 10B**).^[200] The probe allowed for quantitative imaging of pH without interference from biological tissue, as well as for evaluation of tumor hemodynamic properties. The ratiometric nanosystem consisted of polyacrylamide nanoparticles encapsulating an optical pH indicator, and could be excited at 565 and 600 nm, with the emission signal of the latter being pH dependent. Furthermore, Patiño et al. made use of pH-sensitive ratiometric DNA-nanowires developed in Ricci's lab^[214] and assembled them onto enzyme-powered micromotors.^[89] The researchers claim that in future works, motorized nanowires could be used to report intracellular or intratissue pH. As introduced in previous sections, microbubbles can be used to provide US contrast due to their scattering effect on US waves. However, microbubbles are short lived and unable to extravasate vessels due to their size. As a result, circulation times are short, and the contrast is limited to the vascular space.^[46] Nanoformulated bubble generators capable of penetrating tissue can address these issues, and US contrast may be generated in response to pH.^[215] For instance, ketallized maltodextrin nanoparticles were reported to undergo acidic degradation, causing a hydrolytic conversion of carbonate to CO₂ microbubbles. In a mouse liver failure model, the particles were shown to improve US contrast due to acidic conditions in the inflamed tissue.^[215]

In addition to measuring pH levels, nanosystems responsive to hydrogen peroxide have been developed, including systems for optical sensing,^[216–219] MRI,^[220,221] echogenic,^[201,220,222] or photoacoustic contrast.^[202,223] For example, an optical ratiometric hydrogen peroxide sensor was developed based on upconversion nanoparticles.^[216] The particle core upon excitation emits fluorescent light at two wavelengths (540 and 660 nm). In the presence of H₂O₂, its absorption spectrum changes and the 540 nm signal is quenched. Therefore, the ratio between the signals could be used to detect H₂O₂ *in vivo* in mice. Moreover, particles releasing CO₂ or O₂ microbubbles in response to hydrogen peroxide have been reported for US imaging.^[150,201,224,225] Jung et al. prepared nanoparticles to detect peripheral artery disease: ischemic injury was induced in mice hindlimbs to model this disease, and US imaging was conducted 7 days after insult (**Figure 10C**).^[201] Enhanced contrast due to increased H₂O₂ levels was observed at the injection site of the particle system, compared with a non-ischemic control surgery, a healthy leg with control particles, and an ischemic tissue without control particles. The same group reported a thrombosis-targeted theranostic nanosystem providing contrast for both US and photoacoustic imaging through H₂O₂-mediated signal amplification.^[225] Alternatively, Prussian Blue nanoparticles or catalase-loaded nanoparticles catalyze the breakdown of hydrogen peroxide into oxygen molecules, providing O₂ bubbles amplifying US and/or photoacoustic signals in response to H₂O₂ in mice.^[220,222] Moreover, because of the paramagnetic properties of oxygen, T1 MRI contrast of the affected tissue may be enhanced in the presence of such a nanosystem.^[220] Liu and coworkers demonstrated liposomal nanoprobe

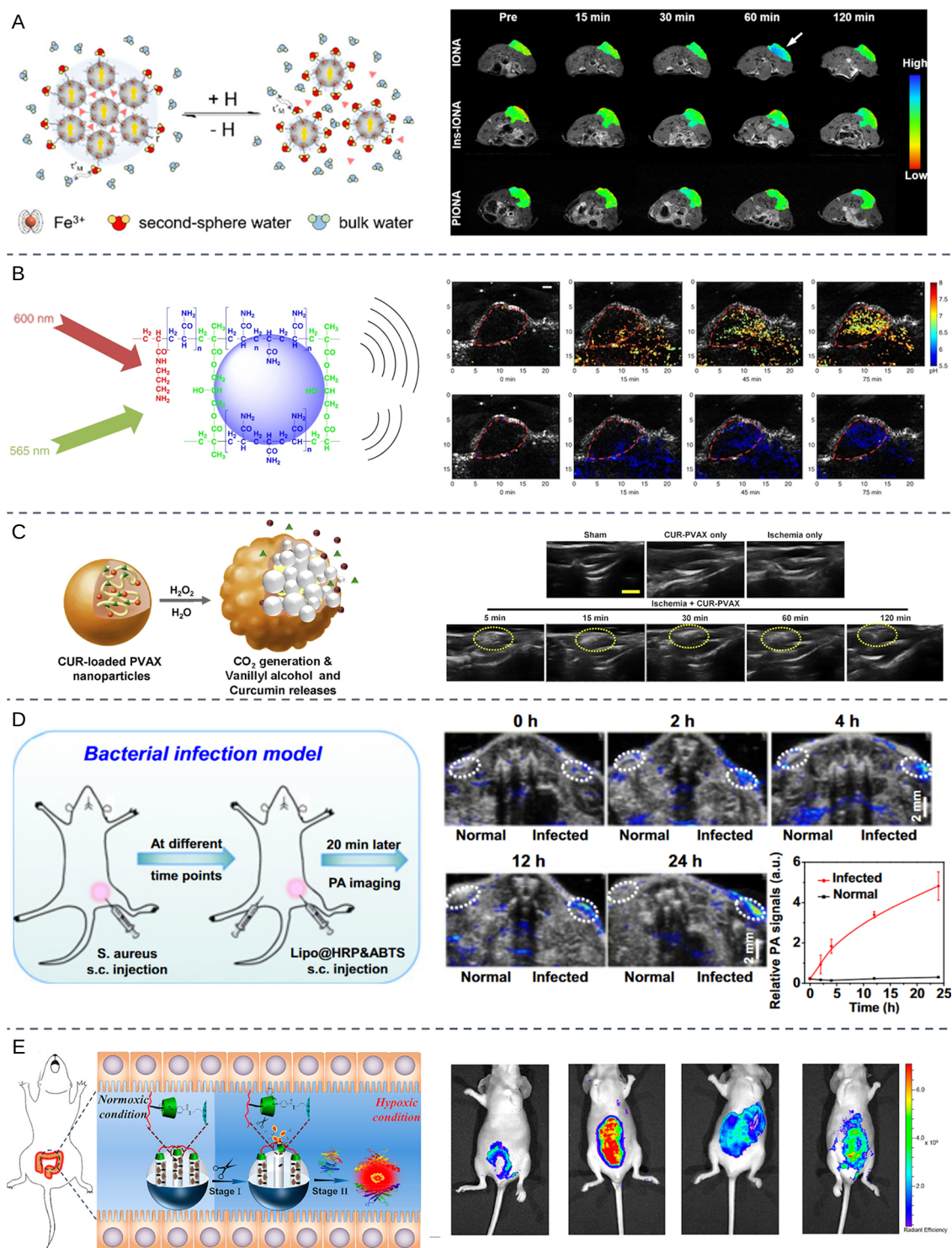


Figure 10. In vivo detection of pH, hydrogen peroxide, and oxygen. A) Dissociation of iron oxide nanoparticle assemblies under low pH increases the accessibility of protons in water to the iron oxide, enhancing T1 MRI signal. Adapted with permission.^[197] Copyright 2019, American Chemical Society. B) Ratiometric photoacoustic imaging of pH using polyacrylamide nanoparticles containing an optical pH indicator. The pH in the center of the tumor was shown to be lower compared with the outer regions. Adapted with permission.^[200] Copyright 2017, Springer Nature. C) Antioxidant vanillyl alcohol-incorporated copolyoxalate (PVAX) particles react with H_2O_2 to generate CO_2 microbubbles, providing contrast for US imaging to detect peripheral artery disease. Adapted with permission.^[201] Copyright 2018, Elsevier. D) A substrate is oxidized by horseradish peroxidase in liposomes in the presence of H_2O_2 , increasing NIR absorbance. Adapted with permission.^[202] Copyright 2017, US National Academy of Sciences. E) Fluorescent hypoxia probes were demonstrated in colitis. β -CDP gatekeepers are attached to the particle surface, but released in hypoxic conditions, leading to the release of the dye and quencher with subsequent increase in fluorescent signal. Adapted with permission.^[203] Copyright 2020, American Chemical Society.

for photoacoustic imaging of inflammation and cancer (Figure 10D).^[202] The liposomes contained horseradish peroxidase and a substrate, which is oxidized by the peroxidase in the presence of hydrogen peroxide. This oxidation increased the NIR absorbance of the substrate, enabling photoacoustic imaging. The liposomes were used to detect the increased levels of H_2O_2 in bacterial infections, tumors, and metastatic lymph nodes in mice.

Tumors often show low levels of oxygen, due to impaired vasculature and blood supply. This hypoxia worsens patient prognosis, as it increases the risk for metastases and resistance to therapy. Low tissue oxygen levels play a role in other conditions as well, including cardiac ischemia and wound healing. Therefore, imaging hypoxia is important for medical prognostics and therapy monitoring, and responsive imaging nanosystems have been developed for MRI,^[226–228] optical imaging,^[203,229–231] and photoacoustic imaging.^[232] Moreover, as tumor oxygen levels affect the efficiency of photodynamic therapy, oxygen mapping may be used to guide the procedure.^[230] Most MRI is based on 1H proton relaxation in water molecules and molecular oxygen may function as paramagnetic T1-shortening contrast agent in MRI.^[228] However, due to the high water-to-oxygen ratio, T1 change by oxygen is limited. In contrast, the solubility of oxygen is six times higher in lipids than in water, turning MRI into a suitable tool for sensing oxygen levels. To monitor oxygen deprivation in tumors, Nel et al. developed lipid nanocapsules to increase lipids levels in the tissue, enabling O_2 mapping in tumors by MRI. Indeed, oxygenation changes after a carbogen challenge could be detected in mice.^[228] Furthermore, hypoxia is one of the characteristics of inflammatory bowel disease (IBD). Zhou et al. developed a mesoporous silica nanoparticles loaded with an squarylium fluorescent dye and a BHQ2 quencher to image this characteristic (Figure 10E).^[203] The surface of the nanosystem was decorated with β -CDP gatekeepers, linked by hypoxia-sensitive azobenzoic acid groups. In a hypoxic environment (<20% oxygen), the squarylium dye is released from the particles, activating fluorescence. The authors demonstrated that signal strength increased with decreasing oxygen levels, and interactions of the dye with cytoplasmic protein further increased signal strength. Moreover, the use of the probe was demonstrated in mice models of colitis. Interestingly, Martel and coworkers used magnetoaerotactic live bacteria which migrate to hypoxic tissue. Such an approach could benefit medical imaging of hypoxic regions in tumors in future developments.^[233]

5.2. Sensing Other Biological Markers

While many efforts are dedicated to sensing pH and hydrogen peroxide, many more biomedically relevant analytes may be measured, such as levels of ions,^[63,234–237] reactive oxygen or nitrogen species,^[238–246] enzymes,^[81,247–254] glutathione,^[255–260] and other biomarkers.^[261–263] An example is the ratiometric hypochlorite sensor developed by Zou et al. for the detection of arthritis (Figure 11A).^[264] These upconversion nanoparticles can be excited at 980 or 808 nm, and the absorbance of the latter wavelength is mediated by a fluorescent dye on the particles. As the absorbance of this dye decreases upon reaction with ClO^- , the ratio between signals after 980 or 808 nm excitation serves

as readout for the hypochlorite concentration. The authors found a clear difference between the healthy and diseased leg, with a concentration of $10 \mu M ClO^-$ in the latter. Interestingly, hypochlorite in solution is in equilibrium with hypochlorous acid, which can be detected by yet other ratiometric biosensors.^[265,267] For instance, the probe developed by Wang et al. was used to detect lymphatic inflammation in mice, and the excitation spectrum in the second NIR window allowed for high spatial resolution (Figure 11B).^[265] The probe can be excited at two wavelengths, one of which is mediated through a hypochlorous acid-degradable dye. However, the degradation of the dye is irreversible, which is important for in vivo sensing.

A photoacoustic ratiometric probe detecting two analytes simultaneously was introduced by Ai et al. for measuring reactive nitrogen species (RNS) and reactive oxygen species (ROS), which show altered levels in pathophysiology (Figure 11C).^[245] Lanthanide-doped upconversion nanoparticles were functionalized with ROS-responsive and RNS-responsive fluorophores, and the specific emission maxima were measured using multi-spectral photoacoustic tomography in mice. An increase in ROS resulted in a signal increase at 640 nm, and an increase in RNS resulted in a decrease in signal at 780 nm. Treatment of mice with ROS-inducing lipopolysaccharide (LPS) or RNS-inducing acetaminophenol could be observed at different wavelengths. Degradable MnO_2 nanosheets were presented for the detection of intracellular Zn^{2+} , a biomarker in breast and prostate cancer.^[63] The sheets carry DNA oligomers and fluorescent silver nanoparticles. Upon endocytosis, the nanosheets degrade, the oligonucleotides are released, and three-way DNA junctions are formed due to Zn^{2+} -dependent hybridization, leading to a high fluorescent signal. Moreover, as each arm in the three-way junction contains one AgNP, the self-assembly in the presence of zinc ions amplifies the signal. In addition, the free Mg^{2+} enhances MRI signal. Similarly, a similar system was presented for measuring ascorbic acid vitamin levels based on free magnesium ions.^[268] However, as MRI-based sensing of other analytes has been proposed based on MgO_2 degradation, including common conditions such as acidic pH, the development of sensing particles based on this premise should carefully consider that the signal may not be entirely specific, thus limiting its application in medical practice. Moreover, the sensitivity of MRI to pathological changes and to contrast agents is relatively low. To increase the MRI sensitivity, the detection of ^{19}F instead of 1H nuclei can be used.^[266] ^{19}F is present in much smaller quantities endogenously, and thus offers a much higher signal-to-noise ratio. As an example for this approach, Phospholipase A2 (PLA2)-responsive ^{19}F -Gd nanoprobe were administered in vivo as phospholipid-encapsulated particles by Guo et al. (Figure 11D).^[266] In the presence of elevated levels of PLA2, which may be found in certain tumors, cardiovascular disease or inflammation, the nanoprobe disintegrated, resulting in an increased mobility of the ^{19}F -Gd probes and consequent increased T2 MRI signal.

5.3. Detecting Gene Expression

In addition to biomarkers, valuable diagnostic data can be retrieved by reading out genomic transcription levels for genes

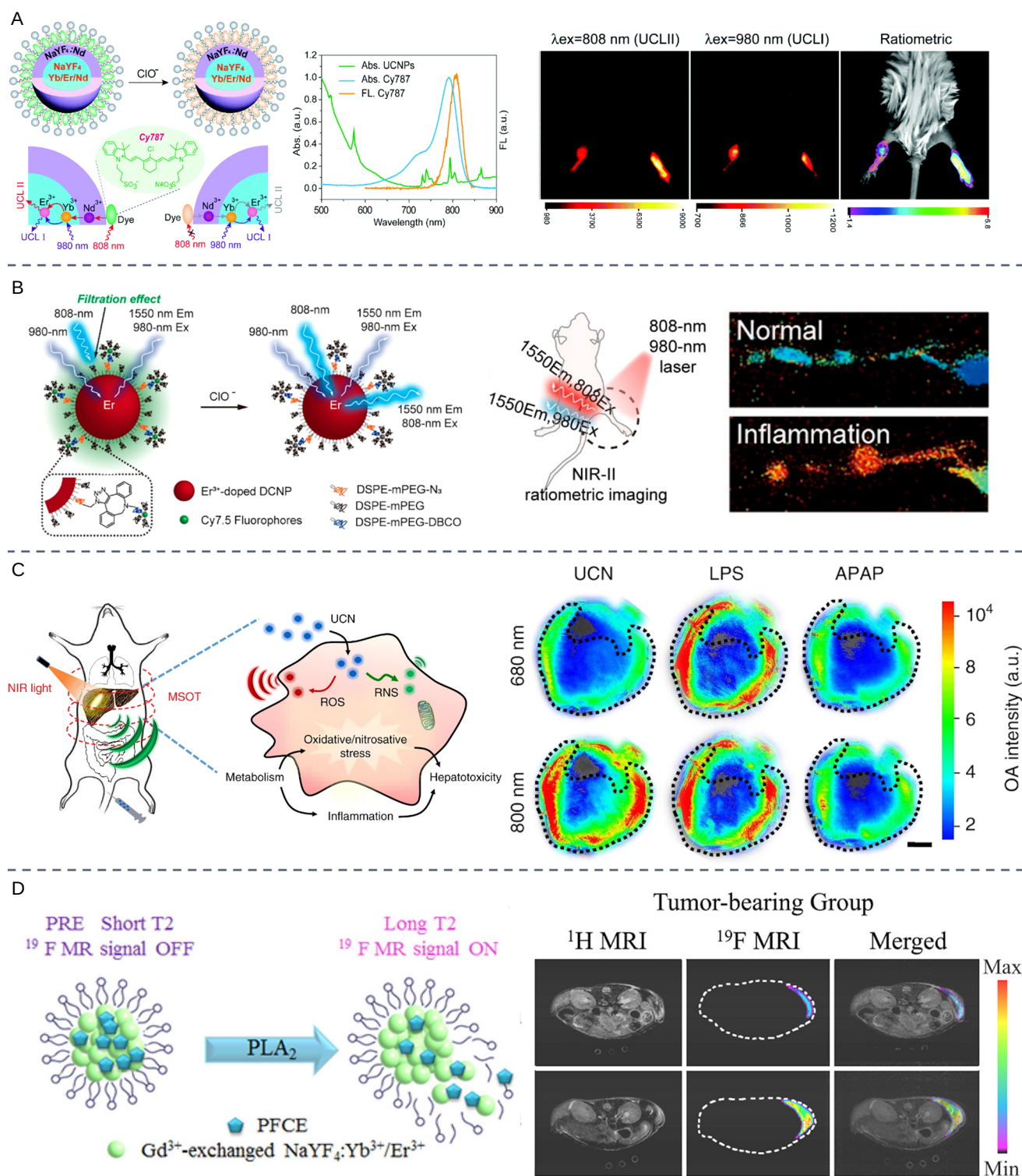


Figure 11. Sensing biological markers in vivo. A) Ratiometric probing of hypochlorite in arthritis using a Cy787 dye-sensitized nanosystem, indicating increased levels of hypochlorite in the left leg. Adapted with permission.^[264] Copyright 2019, Royal Society of Chemistry. B) Ratiometric fluorescent probes developed to image hypochlorous acid in vivo. LPS-induced lymphatic inflammation in mice was imaged using the nanoprobe. Adapted with permission.^[265] Copyright 2019, American Chemical Society. C) ROS and RNS were observed with a dual responsive upconverting nanoparticles. The probed allowed for detection of LPS-induced ROS at 680 nm, and for the detection of acetaminophen (APAP)-induced RNS at 800 nm. Adapted with permission.^[245] Copyright 2019, Springer Nature. D) Phospholipase A₂-responsive nanoprobe disintegrate to release ¹⁹F-Gd contrast agent, with a subsequent increased T2 contrast and switching on of the ¹⁹F MRI signal. Adapted with permission.^[266] Copyright 2019, American Chemical Society.

of interest. Complementary strands to RNA sequences can be assembled onto nanosystems, to create sensors specific for these sequences. It has been shown that tethering DNA sensors to a membrane scaffold, such as a liposomes or other types of particles, results in more robust and responsive systems.^[269] Various systems were developed for such purposes.^[270–273] An example of such a DNA-based sensor was reported by Gao et al., who investigated the use of mesoporous silica particles for the detection of human mutT homologue (MTH1) expression in cancer with AuNPs immobilized on the particle surface by recognition sequence single-strand DNA.^[271] Upon binding a complementary MTH1 mRNA strand, the AuNPs are released, which interrupted quenching of fluorophores on the silica particles. Ren et al. developed an upconversion nanosystem for fluorescent imaging of miRNA 122 expression, a marker regulating fatty acid and cholesterol metabolism in the liver.^[272] Moreover, Yang et al. demonstrated a fluorescent photoacoustic probe for the detection of TK1 mRNA, a marker of tumor growth (Figure 12A).^[273] In the presence of the target mRNA, the recognition strands with Cy5.5 fluorophores are released from the surface, resulting in increased fluorescence and reduced photoacoustic signal. The presence of TK1 in mice bearing MCF-7 or HepG2 tumors could be confirmed based on the changes in the photoacoustic signal.^[273]

5.4. Tracking Cells

In addition to the tracking of molecular changes, responsive nanosystems may be used to track specific cell types. For example, the tumor microenvironment is enriched with macrophages,

which are associated with transition of tumors toward malignancy.^[275] Furthermore, the variable distribution of these macrophages contributes to tumor heterogeneity. To detect these macrophages, an optoacoustic reporter has been developed based on a bacterial system, using phototropic bacteria.^[276] These bacteria absorb light at lower wavelengths compared with endogenous compounds such as hemoglobin and lipids. The 800 and 860 nm two-peak spectrum and the spatiotemporal changes of the spectral shape are dependent on phagocytosis by macrophages, which can be detected using multispectral optical tomography. The authors report that the use of the probe led to better understanding of macrophage heterogeneity within the tumor. Other investigators produced “nanopomegranates” for the detection of Kupffer cell macrophages (KCs) and the characterization of their structural organization in hepatic lobules, aiming to evaluate liver function (Figure 12B).^[274] The 400 nm nanopomegranates consist of 4–5 nm self-quenched fluorescent seeds, and disintegrate upon phagocytosis by KCs. While intact, the particles function as contrast agents for KC uptake, imaged by photoacoustic microscopy. Upon degradation by KCs, the particles lose photoacoustic signal, but as the seeds are no longer quenched, a fluorescent signal can be measured. Therefore, these particles can not only be used to locate KCs in the hepatic lobular structure, but also to evaluate KC degradative function. Moreover, Kim et al. demonstrated the release of manganese ions after uptake in KCs, aiming to characterize hepatic tumors using MRI.^[277] Next, Dhada et al. used a photoacoustic nanoprobe to track mesenchymal stem cells in vivo.^[278] The gold nanorods were coated with a ROS-sensitive infrared dye, which serves to report cell death. Therefore, the system is able to report viability

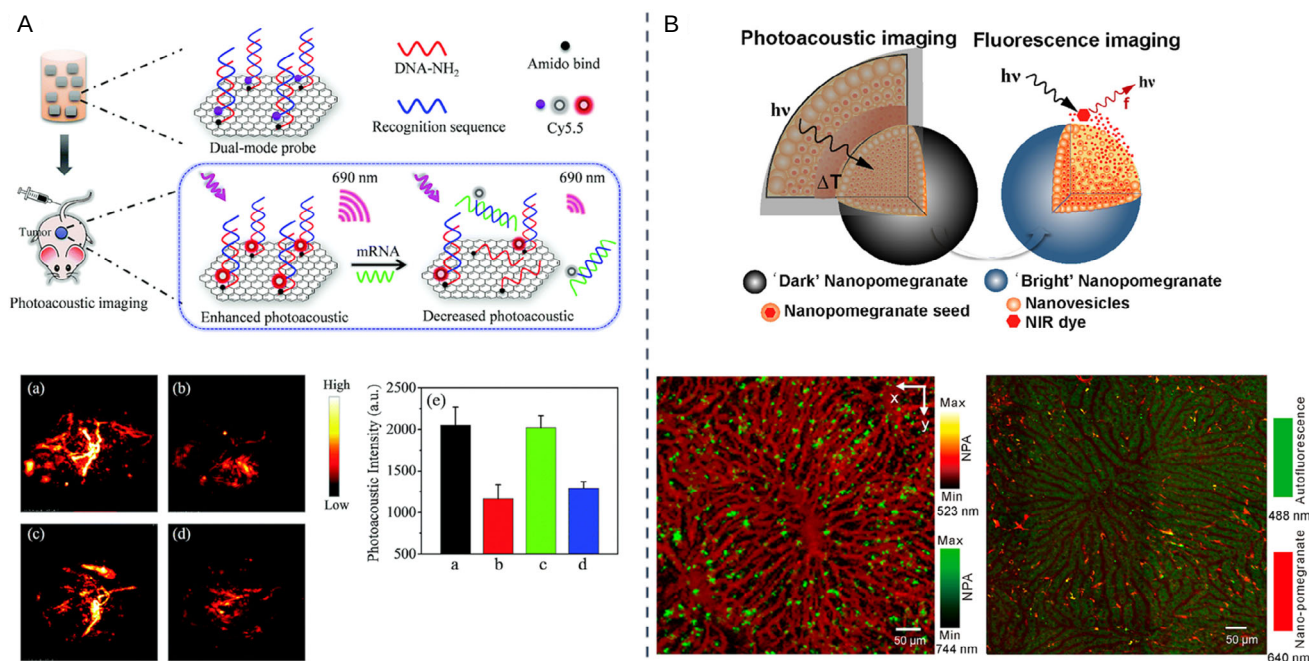


Figure 12. In vivo visualization of gene expression and cell activity. A) Fluorescent and photoacoustic imaging were used to detect TK1 mRNA, a tumor biomarker. Recognition of the mRNA results in the release of the recognition strand, resulting in increased fluorescent and decreased photoacoustic signal. Adapted with permission.^[273] Copyright 2018, Royal Society of Chemistry. B) Self-quenching nanopomegranates provide photoacoustic contrast and fluorescent signals for the characterization of Kupffer cell distribution in hepatic lobules. Upon phagocytosis, fluorescent signal increases and photoacoustic signal is reduced. Adapted with permission.^[274] Copyright 2019, American Chemical Society.

of the stem cells after transplantation. In this study, the nanoprobes were loaded into the stem cells, which were then transplanted into the lower limbs of mice, and stem cell viability was recorded over 10 days. Finally, the intracellular levels of Ca^{2+} increase in response to T-cell activation. Chen et al. demonstrated a dendrimer probe with entrapped gold nanoparticles to monitor the distribution and activation of transplanted T-cells in mice using CT and fluorescent imaging, aiming to monitor the effects of immune therapy.^[279] CT contrast was provided by the gold, whereas the levels of free calcium ions could be monitored by a responsive fluorescent dye.

In this section, no systems were presented exploiting PET or SPECT imaging. These nuclear imaging techniques rely on the detection of gamma rays emitted as a result of the decay process that occurs when gamma or positron emitters undergo disintegration. The radioactive decay is a process that depends exclusively on the physical properties of the radionuclide, and is not affected by the chemical structure of the labeled molecule, the environment (pH, chemical composition, and physiochemical properties) or the temperature. Because of this, the signal detected in PET or SPECT depends exclusively on the concentration of the radionuclide in a particular location. In this context, the application of nuclear imaging techniques to the identification/quantification of analytes or investigation of physiological/biological processes cannot be used, unless a selective accumulation of the labeled entity or a labeled byproduct, that is, a metabolite or a condensation product, is accumulated as a result of the occurrence of the process or the presence of the analyte to be investigated. Examples showing the development of smart, small-molecule-based radiolabeled systems capable of undergoing in situ condensation (and consequent enhanced retention) in certain locations due to the presence of reducing agents or enzymes, have been mentioned in Section 2.^[68,76,77,280] Noteworthy, the accumulation of the "intact" administered radiolabeled entity contributes to background signal that can be incorrectly interpreted as the presence of the metabolite, or the occurrence of the phenomenon under investigation. Hence, careful validation of the approach is paramount to obtain truly reliable qualitative or quantitative data.

6. Conclusions

Using nanosystems offers significant benefits in creating multifunctional and intelligent imaging agents, including a great flexibility in design and the opportunity to serve multiple imaging modalities. In summary, intelligent features, such as the change of size as a response to physiological environments, active navigation across tissues, and modification of the extracellular environment, may significantly enhance tissue penetration and retention of contrast agents. Furthermore, signal strength and quantitative analysis can be performed using nanosystems with sensing capabilities, in which the signal or contrast is correlated to the presence or concentration of specific analytes or cells. Nonetheless, some challenges are yet to be overcome. While nanotechnology offers the benefit of designing multifunctional systems, overly complex devices might not necessarily meet clinical needs. In addition, researchers are often professionals in one scientific field, while multimodal and multifunctional probes

require expertise in multiple areas, including nanotechnology, imaging techniques, and medical biology. Therefore, in early stages of nanosystem design, the eventual medical application as well as the specific imaging needs for the intended clinical use case should be discussed between (chemical) engineering and medical experts. In addition, it might be difficult to extrapolate the results from preclinical to clinical environments, due to differences such as a smaller blood pool volume and faster clearance in laboratory animals.^[1] Many proposed nanosystems, especially those with dynamic signaling functionality, rely on forms of optical imaging, which are very useful in (small) animals. Generally speaking, this modality is rarely used in clinical practice, and the development of adequate optical imaging systems for humans is a future perspective. Therefore, in developing imaging nanosystems, it might be appropriate to pay attention to other modalities, such as MRI or PET. Moreover, while a good retention of a probe in the body increases the time window in which imaging can be performed, there is a trade-off with safety. Specifically, long-term retention and the incapacity of the body to degrade certain types of nanomaterials may lead to toxic effects. Specifically, the removal of particles taken up by the mononuclear phagocytic system is dependent on intracellular degradation, meaning that particles that cannot be broken down will remain in the body.^[50] In contrast, other nanomaterials may yield toxic effects upon degradation, such as free gadolinium released from Gd-containing particles. In such cases, toxic imaging agents may need protective encapsulation in the nanosystem to prevent harm. Therefore, to achieve clinical translation of nanomaterials, understanding the biocompatibility and long-term effects of nanosystems is crucial in the evaluation of safety. Not only does this demand for the development of stable, but eventually biodegradable probes, these characteristics should be adequately evaluated in every in vivo study. Moreover, most research efforts are dedicated to cancer. This is understandable, considering the world-wide burden of the disease and the passive accumulation achieved by EPR effect. However, other diseases might benefit from smart imaging systems, if research efforts are dedicated to design intelligent nanosystems for these purposes. Altogether, while the development of intelligent nanosystems for advanced imaging is challenging, the rewards are profound. Continuous improvements made to these systems promise a great future in medical imaging.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

in vivo sensing, intelligent nanosystems, medical imaging, molecular imaging, nanomotors, smart contrast agents

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