Nanomed. J. 9(2): 107-130, Spring 2022

REVIEW PAPER

Impact of nanovectors in multimodal medical imaging

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ABSTRACT

Medical imaging is currently revolutionizing the diagnosis and treatment of a variety of diseases. Several imaging modalities have been developed based on advances in science and engineering. The impact of these imaging tools has been further improved with the advent of various modern chemistries, leading to the development of contrast agents that serve further to localize the detection of diseased tissues. Several researchers are recently involved in engineering contrast agents that can generate contrast differences between tissues in multiple imaging modalities, enabling cross-referenced determination of anomalies. To establish these multimodal imaging agents, nanovectors have gained significance due to their key physicochemical properties. The major focus of this review is on the engineering strategies of nanovectors for multimodal medical imaging. The review conceives the basic principles, major parameters, and limitations of imaging modalities, namely, magnetic resonance imaging (MRI), computed tomography (CT), and fluorescence imaging at the beginning. Drawbacks of traditional contrast agents and the demand for new contrast agents are established. The importance of multimodal imaging and the need for a single contrast agent for these imaging applications are elaborated. Finally, the advantages, limitations, and design considerations of nanovectors based on magnetic and metallic nanoparticles with surface modifications to reduce toxicity and enable targeted delivery as multimodal imaging agents are also emphasized.

Keywords: *CT, Medical imaging, MRI, Nanovectors, Nanomedicines*

How to cite this article
Gowtham P, Haribabu V, Daniel Prabhu A, Pallavi P, Girigoswami K, Girigoswami A. Impact of nanovectors in multimodal medical imaging. Nanomed J. 2022; 9(2): 107-130. DOI: 10.22038/NMJ.2022.63459.1664

INTRODUCTION

Diagnosis of diseases, especially cancers, has always been challenging the healthcare industry for decades. A step forward, molecular imaging has proved to be a boon to physicians in diagnosing cancer, infections, organ damages, and many more. Due to the noninvasive characteristics with a higher ability to visualize cellular changes and functions, real-time analysis of processes in diseased tissue is conceivable for molecular imaging [1, 2]. With these potentials to better diagnose disease conditions, molecular imaging can promote life expectancy with improved treatment plans.

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Note. This manuscript was submitted on December 3, 2021; approved on Febuary 21, 2022

Molecular imaging refers to the quantitative, non-invasive, and repetitive imaging of targeted biomolecules leading to a sequential follow-up of biological processes in a living subject. Specific biological mechanisms can be relevant in disease conditions that can be imaged. With the advantage of monitoring the biological changes that occur from time to time, molecular imaging offers the ability to diagnose a variety of diseases and disorders at an early stage [3, 4]. The demand for attending in details in diagnosis has been at extremes ever since the introduction of X-ray-based radiological imaging. Imaging anatomical conditions and functional anomalies have always been a challenge for decades until the emergence of molecular imaging modalities.

These extreme demands lead to the innovation of several modalities, and they can be categorized

into two groups based on the intelligence they have to offer. Magnetic resonance imaging (MRI) and computed tomography (CT) can be grouped as they are highly efficient in delivering anatomical evidence. Furthermore, optical imaging, positron emission tomography (PET), single-photon emission computed tomography (SPECT), and functional MRI (fMRI) can be grouped for their ability to determine functional or molecular anomalies [5]. On the other hand, ultrasound has proved its importance by generating real-time images with its sophisticated probe design and is considered as the first-line imaging tool for determining pregnancy and growth of the fetus at every trimester. Each imaging modality is unique in its principles, capabilities, and limitations for enabling diagnosis. To establish the major focus of the present review, the following sections are confined to magnetic resonance imaging, computed tomography, and fluorescence imaging modalities.

Physics of magnetic resonance imaging

The ability to generate tissue contrast differences is remarkable with the versatility offered by MRI. Being a noninvasive imaging technique without the use of ionizing radiation like x-rays or γ-rays, MRI has proved to be an efficient tool in the diagnosis of several disease including cancers [6]. The use of non-ionizing radiation in MRI allows the imaging and diagnosis of foetal anomalies as well to correlate with findings that are inferior to ultrasonography, a well-known first-line imaging tool in pregnancy [7]. MRI relies mainly on high magnetic fields and radiofrequencies (RFs). It is also dependent on the relaxation times of protons in various mobile molecules such as water, proteins, and lipids that are present in various organs. These dependencies offer superior spatial resolution in soft tissue anatomical images with better endogenous contrast differences that ensures MRI to be highly useful in diagnosis [8].

MRI typically accredits on the applied magnetic fields of higher strength in which the sample (a patient in the clinical setting) is placed (Fig. 1). Following the Faraday's law of electromagnetic induction, hydrogen nuclei acquire a magnetic moment (magnetism) due to the presence of net charge and precession (motion) leading to either parallel (Spin-up nuclei) or antiparallel (spin-down nuclei) alignment to the direction of the external magnetic field. This alignment depends mainly

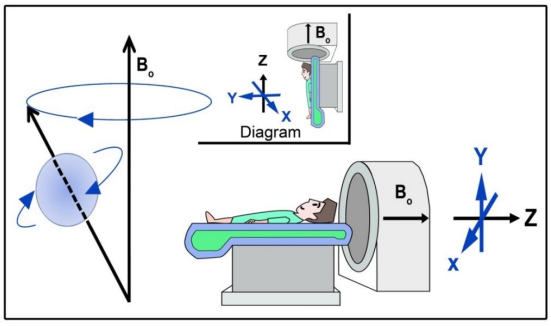

Fig 1. Representation of the basic principle of magnetic resonance imaging

on the quantum theory where hydrogen nuclei exhibit either low or high energy quantity. A radiofrequency (RF) pulse is applied to the aligned nuclear spins for resonance absorption, thereby moving the hydrogen nucleus spin away from equilibrium. After this disturbed alignment, the RF pulse is turned off, thereby resulting the hydrogen spin to align back into equilibrium. During this realignment to achieve equilibrium, transfer of energy occurs, and this excess energy is received by receivers, which is further computed to form visualizable MR images [9].

Water molecules are a valid source for MR Imaging since highly sensitive hydrogen nuclei possessing water molecules form most of the human body [5]. Hydrogen atoms are of concern in MRI since their protons exhibit a higher gyromagnetic ratio of 42.58 MHz/T along with the ability to generate nonzero magnetic moments. Gyromagnetic ratio is a constant that exhibits the relation between the angular momentum and magnetic moment of nuclei in deliberation for MR imaging. Larmor equation gives this relationships as $\omega_0 = B_0\gamma$, where ω_0 is the frequency of precession, B_0 is the applied magnetic field strength, γ is the Gyromagnetic ratio of the precessing nuclei. Every nuclei that are MR active spins on its own axis with a wobbling motion called as precession. Gyromagnetic ratio is explained in relation to this precession which is specific for MR active nuclei at a given magnetic field [10]. Although several alternatives including carbon-13 (C^{13}) and fluorine-19 (F^{19}) are being researched for improving MR image resolution, hydrogen (H^1) and oxygen-17 (O^{17}) are proposed to be highly effective. This contemplation is due to their abundance in the living subject along with the ingrained knowledge of precessional frequencies of various magnetic fields for decades in diagnosing diseases and functional anomalies [11].

The phenomenon of resonance, where the precessional frequency of the hydrogen nuclei in the external magnetic field is matched by the RF frequency that is applied, is a major part of MRI. As explained previously, the precessing hydrogen nuclear spins absorb the matching RF frequency and gains energy to resonate. This excess energy allows the nuclear magnetic vector (NMV) to deviate from the alignment with the applied magnetic field. Based on the energy quanta received by the nuclear spins, the NMVS tends to move at an angle out of alignment with the magnetic field. This angular change in NMV depends on the parameter called flip angle, which is dedicated to the amplitude and duration of the RF pulse application. When the RF supply is deprecated over the above change to NMV, the nuclear spin to realign with the magnetic field to achieve equilibrium. To move back to equilibrium, the nuclear spins liberate the excess energy absorbed from the resonant RF frequencies and this process is termed as relaxation. Relaxations are from two independent processes, namely, T1 recovery or longitudinal relaxation (T1 relaxation) and T2 decay or transverse relaxation (T2 relaxation). These relaxation properties of hydrogen nuclei depend on the tissues in which they are present and the macromolecular interactions in their microenvironment. Based on the plane in which this relaxation occurs, the NMV reduces or increases leading to a free induction decay signal that is further processed to form visualizable MR images [12, 13].

T1 relaxation

T1 relaxation or longitudinal relaxation devotes to the time required for the magnetic moment to retire back to equilibrium magnetization along z-axis. During the T1 relaxation process, the absorbed energy gained from RF energy is dissipated to the neighbouring molecules in the lattice. This allows the nuclear spin to relax and gain the net magnetization along the longitudinal plane (z-axis) resulting in Boltzmann equilibrium. Since this longitudinal changes to the net magnetization depends on the energy transfer between the spin and the surrounding lattice, this T1 relaxation is also called as spin-lattice relaxation [14]. As mentioned earlier, the biological environment in various tissues determines the T1 relaxation properties of hydrogen nuclei of concern. The hydrogen nuclei present in water molecules exhibit a T1 relaxation that is longer compared to those present in lipids. This indicates that the transfer of energy to the surrounding environment is efficient in lipids [14, 15]. Therefore, the T1 relaxation time for water is longer than that for fat and the growth of net magnetization is slower in water compared to fat.

T2 relaxation

T2 relaxation, also termed as T2 decay, occurs along the transverse plane (xy plane) away from the plane in which the magnetic field is applied. In the case of T2 relaxation, the loss of coherence of the net magnetization to return to equilibrium after the resonating RF field is turned off. The magnetic interactions of the nuclear spins of hydrogen molecules without dissipation of energy to the surrounding lattice leads to a more rapid dephasing. Additionally, the magnetic field inhomogeneities causes dephasing leading to the decay of net magnetization along the transverse plane [15]. T2 relaxation is also called as spin-spin relaxation since the gained energy is transferred among spins that are directly interacting with the surrounding, thereby enabling the loss of coherence in the transverse plane. It is important to note that spin-spin relaxation has an impact on the spin-lattice relaxation since the growth of the magnetic vector in the longitudinal plane is impossible without the decay along the transverse plane [16]. T2 relaxation occurs faster due to the direct involvement of interacting spins in a domain leading to the transfer of energy at a faster rate. The coherence in transverse magnetization is lost and reduces in a short time to direct interaction of spins. This is evident from the extended time of hydrogen nuclei present in the water molecules compared to fat. The dissipation of energy in water is slow as the hydrogen proton density is higher compared to fat, which is indicative that water has longer T2 relaxation time than fat [15, 17].

MRI pulse sequence

Pulse sequences in MRI play a major role in determining the quality of MR images and the type of weighting in which the images are represented. Pulse sequences are generally classified into two types vis-à-vis i) spin echo sequences and ii) gradient echo sequences. The basic mechanism of pulse sequences has been explained previously in section 2. Briefly, an excitation pulse is applied and turned off to allow the excited nuclear spin

to return to equilibrium [5]. As the magnetization dephases, a refocusing pulse is applied to rephase this magnetization, which is later followed by the collection of echoes and converted into a visualizable MR image. In the case of spin echo sequences, the refocusing is achieved by applying a RF pulse of 180° flip angle while that in gradient echo sequence is replaced by a gradient. Additionally, the exciting pulse in a gradient echo sequence is variable, while in the spin echo it is fixed at a flip angle of 90°. Other parameters are also varied based on the type of imaging that is intended [17].

Spin echo sequences are considered the gold standard in MR imaging as they possess the ability to determine the anatomy as well as pathology. Conventional spin echo, turbo or fast spin echo inversion recovery sequences are some of the examples of spin echo pulse sequences [17]. On the other hand, gradient echo sequences allow for reduced scan time and breath hold image acquisition of abdomen. Few examples of gradient echo sequences include coherent gradient echo, incoherent gradient echo, steady state free precession, balanced gradient echo, and echoplanar imaging sequences. These pulse sequences are evolving with the interest of reducing the scan time with improved image quality of the region being investigated [18].

Concept of contrast in MRI

The appearance of tissues varies in the image depending on many factors and this difference is termed as 'contrast'. The determination of how a tissue appears comes from the signal density that is collected. The tissue appears dark when the signal intensity is low and shows a bright spot when the signal intensity is high. The major reasons for this contrast difference are - inherent energy of the tissues, molecular package and the match between molecular tumbling rates and Larmor frequency of hydrogen.

MR imaging depends mainly on T1 contrast, T2 contrast, and proton density contrast [17]. To understand the mechanisms involved in the above types, considering the tissues including fat, cerebrospinal fluid (CSF), and muscle would be appropriate. In the case of T1 contrast, the net magnetization along the longitudinal plane that grows to 63% is taken into consideration. Fat molecules require a relaxation time that is shorter compared to CSF which has more water in its composition. This allows the net magnetization of fat to grow faster in the longitudinal plane, whereas that of CSF takes longer. Hence, fats show up as bright regions in the image and CSF appears dark. This difference creates a contrast and is called as T1-weighted MR imaging. On the contrary, T2 contrast depends on the 63% decay of net magnetization along the transverse plane. As explained earlier, T2 decay of water molecules is slower than that compared to fat. Hence net magnetization of CSF presents a longer time giving out high signal intensities, however fat shows diminished signal [17, 19]. Thus, a contrast difference is generated and since this difference is based on the transverse decay, this imaging is typed as T2-weighted MR imaging.

A third type of MR imaging, termed as proton density (PD) imaging, generates contrast with the support of the total number of hydrogen protons present in the unit volume of a tissue. The number of protons that gets aligned with the applied magnetic field determines the net magnetization that is flipped to the transverse plane in that microenvironment which in turn expresses the signal intensity. When the proton density in a tissue is high, the image appears bright due to the large transverse component but appears dark if the proton density is low [17, 19]. It is important to remember that each contrast mechanism is unique and is influenced by several parameters explained previously, that affect the signal-to--to-noise ratio and contrast-to--to-noise ratio, which are key factors to achieve better image quality in MR imaging.

Limitations of MRI

Although MRI has become the heart of diagnosing diseases, especially cancer, limitations do persist with this massive modality. As mentioned earlier, several factors influence the SNR and CNR of the acquired images in MRI. These factors are usually modulated to achieve better contrast difference between normal and diseased tissues [5]. The use of T2-weighted MR imaging is a way to interpret pathologies since water plays a major role in these disease conditions in the form of edema. One major step taken toward improving contrast differences in MR images is to use contrast agents that can alter the relaxation properties to a greater extent, leading to changes in the availability of net magnetization in the longitudinal plane or transverse plane depending on the type of weighting engaged [17].

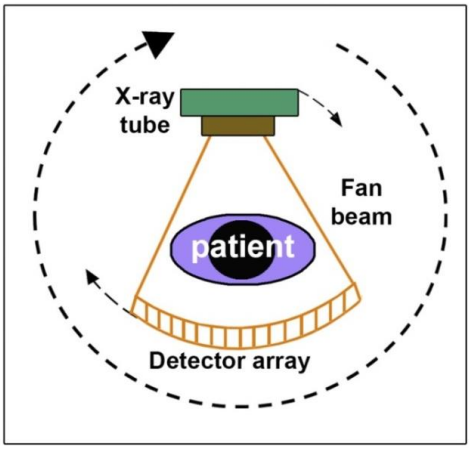

Fig 2. Diagram shows the process to generate CT images

Computed Tomography imaging

In the 1960s and 1970s, Godfrey Hounsfield and Allan Mcleod Cormack developed the Computed Tomography (CT) imaging technique with x-rays as source. A CT scanner depends mainly on the x-ray source and the detector arrays that are made to rotate around the patient (Fig. 2). The basic principle of CT imaging is that the internal structure of an object (patient in a clinical setting) can be reconstructed from multiple projections of that object [20]. This multiple projection is achieved by transmitting x-rays from the x-ray source through the object where most of the x-ray photons are absorbed and part of these photons pass through and reach the detectors that record the x-ray flux. In the most common configuration of CT scanners, the x-ray source and the detector are made to rotate in synchronization around the patient (object) that allows the collection of a 360° dataset of absorbed and transmitted x-ray photons at all known angles. This dataset supports the reconstruction of a section or a slice of the area of interest. Finally, the CT images are reconstructed from the obtained datasets using reconstruction algorithms [21]. Currently, the filtered back projection-based reconstruction method is predominantly engaged, while iteration model-based methods are also being utilized.

Initial CT scanners were able to image only a single thin section of the patient due to the use of single row detectors and a pencil beam of x-rays with constant motion of the patient that required long scanning times to generate volume image sets. The reconstruction of such volume imaging datasets took hours, which paved the way for innovations in CT scanner design. The 'step and shoot' fashion of scanning was replaced with the use of helical or spiral fashion of x-ray source and detector motions with constant patient movements in modern CT scanners [22]. This change may lead to a tremendous reduction in scan time along with the reconstruction time that came down to 1 min. Modern CT scanners have up to 320 detector rows and a 64 detector row scanner can acquire 14 cm of volume data equalizing heart of a normal patient in less than 5 sec. Reconstruction is completed in around 1 min leading to faster and reliable images for diagnosis.

Grey scale images are widely displayed in CT imaging and are constructed based on the linear attenuation coefficients of each pixel in the imaging matrix. These linear attenuation coefficients of the pixels are given a specific value called the CT number. CT numbers are calculated based on the following equation.

$$CT\ Number = \frac{K(\mu_p - \mu_w)}{\mu_w}$$

Where K = magnification constant, μ_p = pixel linear attenuation coefficient, μ_w = water linear attenuation coefficient

More precisely, a bone with a higher attenuation coefficient offers larger CT numbers, and water with a low attenuation coefficient has a low CT number. This difference in CT number stands the key in generating grey scales for each pixel of the image that is reconstructed. CT numbers with a magnification constant of 1000 are called Hounsfield Units (HU). Since this magnification constant is maintained at 1000 in modern CT scanners, CT number and HU stand the same for these scanners [23]. A variety of 3D rendering techniques are used to display anomalies of high attenuating structures such as bone with false colors. Although limitations prevail, CT scanners have become a tool for first-line diagnosis of pathologies pertaining to gastroenterology, musculoskeletal, cardiovascular, and nervous systems.

Factors influencing the quality of CT image

Image quality in CT imaging is not accurately defined since most of the factors are interrelated and proportional to inherent properties. However, it is necessary to understand those factors to improve image quality by controlling them from the operator's end [24, 25]. The following are the three basic factors that control the image quality in CT.

(i) ***Quantum mottle*** **–** Quantum mottle or noise is the result of statistical fluctuations that occur from the inhomogeneous entities that are imaged. The variation in the number of x-ray photons detected by the detector contributes to this quantum mottle. This noise is a major issue in refining the CT image quality but can be controlled by increasing the patient x-ray dose with a compromise on side effects caused due to radiation.

(ii) ***Resolution*** **–** Resolution in CT depends on the ability of the scanner to determine objects that are close to each other in a slice (spatial resolution) and display them as separate entities (contrast resolution). Attempts are to be made with at most care since the noise level images may increase, decreasing the contrast resolution when the patient dose is increased to achieve better spatial resolution. Another way to improve the resolution is by improving the image matrix (pixels and voxels), leading to a higher resolution. It is also important to note that partial volume averaging may reduce since the materials' attenuating coefficients falling within one volume element (or in a pixel) would be further divided in the matrix is increased on the image display.

(iii) ***Patient exposure*** **–** Patient dose or exposure to CT has always been a challenge. This is because improvements in spatial as well as contrast resolution can be achieved with increasing patient dose. As mentioned earlier, increasing patient dose can increase the contrast resolution as there will be an increase in x-ray photons available to improve counting statistics. To improve spatial resolution, smaller pixel and voxel sizes are necessary, leaving the patient dose unchanged. This leads to a decrease in dose per volume but will lead to an increase in noise. Hence, optimal patient exposure is required to achieve image quality that would enable better detection limits in the final image.

Limitations of CT imaging

Over the modern developments in imaging, x-ray CT imaging has become an integral tool in modern medicine. As elaborated above, CT scanners determine tissue anomalies due to the absorption effect of various tissues in humans. The spatial resolution in CT imaging has improved widely due to the development and application of multislice and multi-layered CT scanners [26]. Rendering techniques are highly useful in reconstructing bone deformities since bones have higher X-ray attenuation compared to the surrounding tissue. This is made possible with better reconstruction that can reduce scan time to a greater extent. However, soft tissue and other tissue that has minimal X-ray attenuation suffer anatomical localization in CT imaging [27]. A good example to illustrate this limitation is the visualization of blood vessels, pulmonary organs, and the heart. Since these structures are filled with blood, more of this being water, the transmitted x-rays are not absorbed, thereby making it difficult to detect them as different entities in the CT image. Similarly, diagnosing a cancerous tissue in the liver or any organ for that matter is challenging, leading to false interpretations [28]. To overcome the limitations caused by differences in x-ray attenuation of various tissues being imaged under CT, contrast agents are being used.

Optical imaging

Recent developments in techniques with the ability to rapidly investigate molecular mechanisms at the cellular and subcellular levels optical imaging modalities have picked up pace in molecular imaging. Optical imaging tools are inexpensive and sensitive in generating real-time images with improved resolution that has gained plausible interest from researchers. Fluorophores play a major role in fluorescence microscopy and imaging with their ability to absorb energy when excited with light at a specific wavelength and re-emit at another specific wavelength (Fig. 3). The wavelength and the amount of the emitted energy depend on the nature of the fluorophore and its chemical environment [29]. Although several principles have been interpreted over the years,

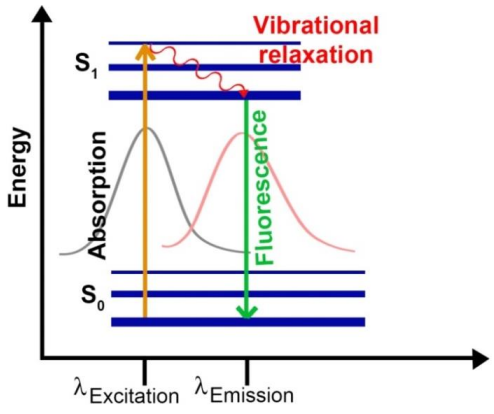

Fig 3. Jablonski energy diagram to represent fluorescence mechanism

the fluorescence emission can be well explained with the Jablonski diagram that illustrates the possible mechanisms by which fluorescence or phosphorescence is achieved.

Electrons in a molecule possess spins that are prevalent in various electronic states. These electronic states can be widely divided as singlet states and triplet states where these electrons are spin paired or unpaired, respectively [30]. The fluorophores generally reside at one of the multiple vibrational levels of an excited single state when they are suitably excited with a specific wavelength of light. Depending on the interaction of the electrons involved in the transition with the electric field of the excitation light, the occupation of singlet states by the molecules is determined. Following this excitation, internal conversion occurs where the molecules in higher singlet states (S_n) relax to higher vibrational levels of the first excited singlet state (S_1), within 10^{-11}–10^{-14} *s*. With the loss of energy through collisions, molecules in these higher vibrational levels of S_1 return to the lower vibrational levels. This molecule can further lose energy from the lowest vibrational level of the first excited singlet state via non-radiating internal conversion and further vibrational relaxation [31].

On the contrary, the molecule might again be excited to a higher singlet state due to the absorption of a second photon. This singlet-singlet absorption for a molecule in S_1 to move to S_n with subsequent ionization leads to a possible photobleaching pathway [30]. The efficiency of this process depends on the transition strength of the absorption wavelength of the higher excited singlet states resonating with the excitation light. Fluorescence might also be spontaneous based on the molecular structure leading to depopulation in the higher excited state. According to Franck-Condon principle, the vertical transition to a higher excited vibrational level is followed by the vibrational relaxation to reach thermal equilibrium based on Boltzmann distribution [32]. Thus, the vibrational relaxation and internal conversion leading to heating of the solvent offers the determination of the fluorescence quantum yield of fluorophore molecules through measurements of solvent temperature in relation to other parameters. Molecules of loose arrangement offer low fluorescence intensity due to several rotations and vibrations that may occur upon excitation [29].

The intersystem crossing can also reverse the spin of an excited electron that leaves the molecule in the first excited triplet state (T_1). Although the triplet state is of low electronic energy than the excited singlet state, the intersystem crossing in most organic dyes is inefficient as a spin-forbidden process. If the vibrational levels of the two states overlap, then the probability of intersystem crossing increases. Essentially, depending on the nature of the fluorophore and transition probabilities, intersystem crossing may occur and is generally unpredictable. However, the intersystem crossing rate is known to be higher when heavy atoms are present [33].

Like the singlet state, a molecule with electrons excited to a higher triplet state may absorb a second exciting photon that prevents the radiative degeneration of energy. This again leads to photobleaching pathways and is more common in triplet states since the lifetime of triplet state falls at 100 ms compared to singlet states that last longer. Loss of fluorescent properties due to irreversible changes in the absorption and emission capabilities of a fluorophore is termed as photobleaching. Therefore, different strategies are being designed to escape this photobleaching pathway by increasing the photostability of fluorophores, and enhancing the number of photons that can be emitted to achieve better fluorescence [29].

Several fluorescent dyes are being used to generate highly sensitive fluorescent images. A list of near-infra-red (NIR) fluorescent dyes (tags) and green fluorescent proteins are there that are widely used in fluorescent imaging applications [30]. The efficiency of absorption in triplet states from a lower vibrational level to a higher vibrational level depends mainly on the following factors – (i) the intersystem crossing rate, k_{isc}, that is, the probability of finding the molecules in the triplet state, (ii) the extinction coefficient εT (ν) at the excitation wavelength, and (iii) the triplet-state lifetime τT.

Triplet quenchers such as cyclooctatetraene (COT) or molecular oxygen are added to depopulate the triplet state leading to reduced triplet-triplet absorption. The dye will be transferred into a singlet state due to the addition of a quencher that has a low-lying triplet state acting as an efficient acceptor for triplet-triplet energy transfer. The technique becomes more difficult since higher concentrations of quenchers are needed to achieve better quenching effects on the triplet state transitions. It is necessary to

understand that the fluorescence emission of a dye molecule depends on spontaneous processes where the fluorophore's interactions with the environment through excited singlet states takes place [29].

Fluorescence imaging

Optical imaging (OI) techniques have undergone a variety of improvements from microscopic cellular imaging to visualizing centimeters in animal models, thereby enabling the tracking of the pharmacological activities of a drug being injected. On the other hand, OI probe light sources with high efficiency and optical detectors of higher sensitivity have led to further improvements [34]. OI has been made possible at the NIR regions (700 nm – 1 mm) and visible regions (400-700 nm) of light through fluorescence emissions from biological tissues [35, 36]. In principle, OI depends on three major characteristics, namely, photon absorption, photon scattering, and instrumentation.

The absorption characteristics of a molecule determine the fluorescence properties of that molecule. The theory behind photon absorption and scattering has been covered in the previous section, and hence OI instrumentation is of focus in this section. Optical imagers are generally designed to illuminate various excitation wavelengths of light, usually with a xenon lamp as the source. Additionally, charge-coupled devices (CCD_s) are placed to detect the fluorescence emission wavelength. The setup is coved in a tight light box to prevent interference of light in the measurements of fluorescence and excitation being applied. Mice can be suitably anesthetized and placed in the light box, and fixed to a FOV. The distance between the excitation source and the detector can also be adjusted in modern-day optical imagers [37]. When fluorophores are injected, these dyes interact with the biological environment and generate fluorescence emission. The emitted fluorescence wavelengths are detected, and images with pseudocolors depending on the fluorescence intensity are displayed for analysis on an overlapped animal picture to localize the region of interest. More recently, the exploitation of Cerenkov luminescence and radioluminescence are being employed to achieve better detection of luminescent properties of chromophores [38].

Limitations of fluorescence imaging

When a biological tissue contains natural fluorescent properties where absorption /emission occurs on illumination with light, the phenomenon is called auto-fluorescence. This phenomenon is, unfortunately, the reason for the reduced sensitivity of fluorophores employed in a variety of OI applications. Additionally, the photostability of the traditionally used fluorophores due to quenching poses to be an issue since their ability to differentiate tissues in living organisms is diminished. As explained in the previous sections, photobleaching occurs due to 1) photo-oxidation leading to the formation of singlet oxygen through sensitization of ground-state triplet molecules by the triplet states of fluorophores and 2) photo-ionization that leads to the formation of reactive radical ions by excitation of higher excited states. Photobleaching burdens the emission of fluorescence from fluorescent dyes. Hence, to overcome these issues faced by traditional fluorophores and to improve the fluorescence window of wavelengths, nanoparticles are being researched to be used as fluorescing agents [39].

Role of contrast agents

Contrast agents are those that can overcome the issues of poor resolution posed by imaging modalities, thereby improving the detection of diseases leading to a better prognosis. Several contrast agents are utilized conventionally for this purpose, and their mechanisms differ based on the modality in which they are applied. For the topic taken under this review, the mechanisms of contrast generation by agents marketed traditionally for MRI and CT imaging are explained along with a focus on their drawbacks. Any agents that can generate a contrast difference between various tissues, more specifically, between normal and diseased tissues, are termed as contrast agents or imaging agents. By manipulating the source characteristics or the biological environment of patients, these agents create the image contrast in various modalities. Contrast agents used traditionally for MR imaging applications act by altering the T1 or T2 relaxation properties normally required by the water molecules in the tissues' microenvironment to generate a bright or a dark signal [40, 41]. This, in turn, leads to a contrast variation between normal and anomalous tissues. On the other hand, contrast agents used for CT imaging applications usually are injected to attenuate x-rays. The attenuation is achieved by the accumulation of these agents in the diseased

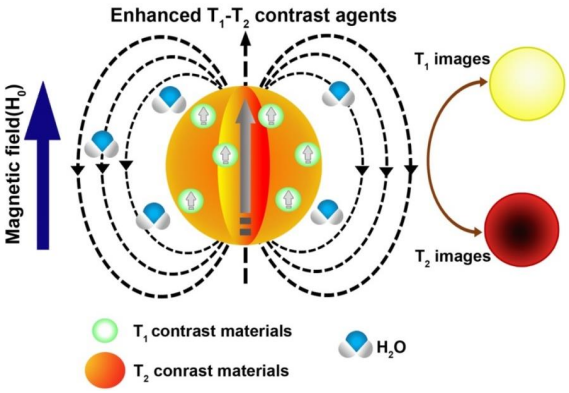

Fig 4. Mechanism of T1 and T2 weighted contrast in magnetic resonance imaging

tissues, thereby enhancing these tissues with regions of high signal density compared to normal ones. CT contrast agents are widely used for cancer diagnosis and angiography studies to determine vascular deformations [28, 42]. The major needs for a contrast agent fall in any one of the following requirements – (i) enhance differentiation among tissues by being sensitive, (ii) offer tissue-specific biochemical information, and (iii) enable functional evaluation of organs/tissues.

Conventional contrast in MRI

It is important to understand that intrinsically, water molecules act as natural contrast agents in MRI. As discussed earlier, the image contrast in MRI depends mainly on the T1 and T2 relaxation properties of the tissue microenvironment (Fig. 4). More specifically, diseased tissues are generally flooded with water in the form of fluid accumulations called edema [43]. However, in certain diseased conditions, the use of these water molecules in generating contrast becomes absurd due to interference from a variety of parameters that may be intrinsic or extrinsic. In these situations, the use of contrast agents comes into play and enhances the detection limits in MR imaging [5]. Contrast agents used in MRI are of two broad types, namely, T1 or positive contrast agents and T2 or negative contrast agents.

Gadolinium in complexed form with chelators is more commonly used to enhance T1 contrast. The interaction of molecules in the biological environment with these gadolinium-based contrast agents undergoes a reduction of their T1 and T2 relaxation properties that offers a difference in contrast. Gadolinium behaves as a paramagnetic material at body temperature and offers seven unpaired electrons. Gadolinium complexed with chelators such as Gd-DTPA or Gd-DOTA offers valence electrons with an open coordinate that allows the interaction of water molecules [14, 44]. In general, water takes a long time to relax and regain its longitudinal magnetization.

It is important to remember that T1 contrast depends on the availability of net magnetization in the longitudinal plane or T1 direction. The interaction of gadolinium complexes leads to time-reduced relaxation, thereby leading to faster growth of net magnetization along the longitudinal direction. This, in turn, causes an enhancement in the signal intensity generating bright areas in the image offering positive contrast [45]. Table 1 shows the list of gadolinium-based agents approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) for clinical use over the years.

On the contrary, T2 contrast has been generated using iron oxide-based contrast agents for years. As described previously, T2 contrast depends on the net magnetization along the transverse plane

Table 1. Overview of gadolinium-based contrast agents

Generic name	Trade name	Structure	Risk class	Elimination route
Gadoterate meglumine	Dotarem	Macrocyclic	Low	Renal
Gadobutrol	Gadavist/ Gadovist	Macrocyclic	Low	Renal
Gadoteridol	Prohance	Macrocyclic	Low	Renal
Gadobenate dimeglumine	Multihance	Linear	Medium	Renal, Hepatobiliary
Gadoxetate disodium	Eovist/ Primovist	Linear	Medium	Renal, Hepatobiliary
Gadopentate dimeglumine	Magnevist	Linear	High	Renal
Gadodiamide	Omniscan	Linear	High	Renal
Gadoversetamide	Optimark	Linear	High	Renal

Foot Notes: Data collected from www.fda.gov and www.ema.europa.eu/en

or T2 direction. The time to relax or decay in the transverse plane is longer for water molecules that contribute to higher signals in T2 weighted MR imaging. When iron oxide interacts with these water molecules, a reduction in decay time occurs, altering the transverse magnetization to a massive drop. This allows for a decreased signal intensity that in turn makes the regions of interest appear dark in the T2 weighted MR images. This contrast difference is termed as negative contrast since the tissues of interest are shown as dark areas compared to the background signal [15, 16].

Iron oxide is being used for T2 contrasting purposes in MRI due to its superparamagnetic behavior. Agents including ferumoxides (Feridex IV, Berlex Laboratories), Ferucarbotran (Resovist, Bayer Healthcare), Ferumoxtran-10 (AMI-227 or Code-7227, Combidex, AMAG Pharma; Sinerem, Guerbet), and NC100150 (Clariscan, Nycomed and VSOP C184, Ferropharm) have been designed and clinically tested, Resovist stands as the only clinically used superparamagnetic iron oxide based T2 contrast agent in several countries [46].

Conventional contrast in CT imaging

As per our discussion in the section, indicating the factors that influence the CT image contrast, k-edge, and atomic density of the substances in the regions of interest play a vital role. With this understanding, contrast agents for X-ray CT imaging have been identified for clinical use based on their k-edge and atomic number. Iodine and barium are the most widely used X-ray contrast agents since they can efficiently attenuate X-rays that are transmitted towards them, thereby creating a contrast difference between tissues based on their accumulation sites. It can also be inferred that by tuning the parameters based on the k-edge absorption possessed by these agents, tissue-specific or organ-specific contrast CT imaging can be established [28]. Although sodium iodide and lithium iodide were the first water-soluble CT contrast agents to be established, these agents were soon withdrawn due to associated adverse effects. Therefore, iodine in covalently bound form came for the rescue to be used as CT contrast agents [47]. Two broad categories of iodinated contrast agents are used for clinical diagnosis. These include - ionic contrast agents and nonionic contrast agents.

Iodinated contrast agents in their ionic forms are usually of high osmolality and viscosity, with most of them possessing negatively charged species. Non-ionic forms of these agents hold a comparative low osmolarity with low molecular weights linked to aromatic chains to reduce toxicity arising from ionic counterparts [48]. Small-molecule iodinated contrast agent-based imaging has been optimized over the past decades and is still being researched to enable improved pharmacological properties. Several of such iodinated contrast agents are approved for clinical use, and a few of them are iopromide (Ultravist, Bayer Healthcare), iodixanol (Visipaque, GE Healthcare), iohexol (Omnipaque, GE Healthcare), iopamidol (Isovue, Bracco Imaging) and iothalamate (Cysto-Conray II, Mallinckrodt Imaging). These agents are marketed all around the globe and are manufactured on a large scale for clinical contrast-enhanced CT imaging [28].

Drawbacks and issues with clinical contrast agents

Contrast agents marketed for clinical use in MRI and CT imaging are widely being acknowledged for their excellent ability to establish contrast differences between tissues that are normal and diseased. Although these agents are applied in everyday practice, a variety of drawbacks and issues persist in using them. Nephrotic systemic fibrosis (NSF) leading to total nephropathy that may cause permanent renal failure is a well-known contrast-induced adverse effect, common to both gadolinium and iodinated contrast agents employed in MRI and CT imaging applications, respectively.

Gadolinium poses serious fatal threats when getting released into the biological environment in their complex chelated forms. In more recent studies with gadolinium-based MR contrast agents, neurological effects leading to chronic neurodegeneration due to deposition of gadolinium in the brain parenchyma have been reported [49]. On the other hand, poor specificity and improper biodistribution are major concerns arising from the use of iron oxide-based contrast agents in MRI. Renal clearance of these iron oxide-based agents poses to be a drawback along with their accumulation at nonspecific sites, which leads to an increase in dose requirements to achieve contrast differences [50].

Iodinated contrast agents used for CT imaging applications are no exception to posting limitations. Iodinated ionic contrast agents potentially lead to

renal toxicity associated with the high intrinsic osmolality. Furthermore, physiological problems, including vasodilation, bradycardia, and pulmonary hypertension are common adverse effects from these agents. With respect to image contrast, ionic agents offer a low radiodensity compared to non-ionic contrast agents [51]. Although non-ionic iodinated contrast agents were brought into light with the idea of circumventing the issues of ionic agents, constant efforts are continuing to reduce renal retention due to high osmolality, increasing water solubility, and reducing viscosity, thereby reducing the associated physiological changes including hypersensitivity and cardiac events [52, 53]. Higher rates of extravasation into capillaries and rapid renal clearance are further issues to be addressed for these agents.

Hybrid diagnostic imaging and it's clinical needs

Hybrid imaging applications are gaining interest among researchers depending on the innovations that are probable in connecting the resulting images acquired from different modalities. One established example is the use of PET-CT, which has now become the standard of imaging cancer, pre-, and post-treatment with chemotherapy and radiotherapy. Hybrid imaging modalities prove to be the current need to extend the interpretation of diagnostic imaging to the next level. The choice of imaging modalities for interpreting different biological conditions depends on their ability to generate intended outputs [54]. For example, MRI is an excellent noninvasive technique that possesses the ability to generate anatomical and functional disabilities of a tissue. PET, with its excellent quality to quantify physiological changes, will not be able to elaborate the localization undergoing the said changes. To circumvent these difficulties among imaging modalities, hybrid imaging techniques are being researched, and few are already in clinical use [55].

Available hybrid imaging modalities

Individual imaging modalities have their own advantages and limitations in providing interpretations towards diagnosis. The impact of PET-CT and PET-MRI combinations has revolutionized these interpretations, more specifically in the diagnosis and follow-up of various cancers. More recently, several combinations for *in vivo* diagnostics are being employed and naming a few, FI-CT, FI-MRI, and FI-MRI-CT hybrids are gaining attention [56, 57]. The excellence in contrast, spatial and temporal resolutions offered by MRI, CT imaging, and fluorescence imaging allows improved detection of anatomical, pathological, and physiological changes [58]. More recently, combined T1 and T2 weighted imaging has received emphasis due to several reasons, including false positives noted on using a single mode. This combination with MRI is usually termed as dual contrast MRI or twin T1-T2 contrast MRI. It proves to be difficult to interpret certain tissues such as bone and bleeding blood vessels in T1 MR imaging as both these tissues generate bright signals. On the other hand, air bubbles and image artifacts are misleading when imaged with T2 weighted MRI with iron oxide-based contrast agents [59]. These difficulties in identifying MRI lead researchers to vest their interest in contrast agents for dual T1-T2 MRI [60]. Hence, the need for hybrid imaging modalities proved to have potential in leveraging the limitations posed by single modalities with the superimposition of images from these modalities in detecting disease conditions.

Limitations of hybrid imaging

Even though hybrid imaging applications can evade several individual limitations of imaging modalities, limitations do exist with these combined modalities. As an example, if we consider PET-CT, the combination requires the use of iodinated contrast agents and 18-Fluro-deoxy-glucose (18-FDG) to generate contrast differences in CT imaging and PET, respectively. These agents, when infused for imaging in PET-CT, show adverse effects that are specific to their own interaction in the biological system [61]. Another good example is the use of gadolinium complexes and iron oxide-based contrast agents for T1 and T2 contrast images, respectively, in MRI. Gadolinium suffers from rapid clearance via the kidneys while iron oxide faces misinterpretation due to improper biodistribution [62]. Furthermore, fluorescent tags used historically in FI are affected by hindrances caused by the additional use of contrast agents for imaging in different modalities since these fluorophores are highly sensitive and undergo photobleaching at a rapid rate compromising resolution. Photofading effects noted with cyanine dyes with increasing size and absorption wavelength as Cy7 > Cy5 > Cy3 stands evident for issues with FI tags [63]. To leverage

these limitations, a multimodal imaging agent that can suffice the need for multiple contrast agents to be used for various imaging modalities is highly desired.

Steering towards possibilities – the advent of Nanotechnology

Nanotechnology is an interdisciplinary field that combines chemistry, physics, biology, and engineering with its ability to establish a variety of applications. Nanotechnology has proved its advent as an alternative for historically used materials in bulk and microforms for a variety of applications in our day-to-day life [64]. Various materials in their nanoscale (typically from 1-100 nm) offer physical, chemical, mechanical, magnetic, and optical properties that can be tuned steering towards their possible applications in different fields. These fields that are benefited from nanotechnology include but are not limited to water treatment, food and crop management, data storage devices, paints, and many more [65]. The advantageous nature of nanotechnology has led to tremendous applications in biology, healthcare, and medicine [65, 66].

Nanomedicine

The application of nanotechnology to medicine and healthcare with advantageous opportunities are extended by nanomaterials of various nature [67]. The European Science Foundation, in 2004, defined the term nanomedicine as "the science and technology of diagnosing, treating, and preventing disease and traumatic injury, of relieving pain, and of preserving and improving human health, using molecular tools and molecular knowledge of the human body" [68]. This definition may appear confusing since researchers have been working with various biomaterials such as nucleic acids and proteins that are also in the nanoscale, in lieu of molecular medicinal applications. Several experts in the field narrowed this definition as the application of nanomaterials' unique properties for medicine. Several products based on nanomedicine have already arrived in the market for clinical use, and their applications in medicine are immense. *In-vitro* diagnostics, therapeutics, diagnostic imaging agents, and medical devices are a few known entries in nanomedicine. Over 200 products based on nanomedicine have reached in the market, replacing the conventional pharmaceutics due to the remarkable properties of nanomaterials that establish better translation into the clinics [69, 70]. Most of these nanomedicines utilize the unique physicochemical properties offered by the nanovectors, which are usually a combination of various nanomaterials and biomacromolecules that are engineered to achieve the required biological applications.

To achieve improved contrast enhancement and additional therapeutic efficiency as theragnostic agents, nanomaterials of various categories are chosen. "Nanovectors" is a broad term used to indicate these nanomaterials that include magnetic nanoparticles, metallic nanoparticles, core-shell nanostructures, nanocomposites, hybrid nanomaterials, dendrimers, polymeric nanomaterials, and nanocarriers that are capable to act as multifunctional nano-formulations [71]. Usually, nanovectors can constitute one or many of the nanoscale materials that can improve the diagnosis and therapy of diseased conditions. The components of a nanovector can be useful for a specific application such as being a nanocarrier for the delivery of anticancer agents or can act individually to establish specific functions in the biological micro-environment [72].

Advantages offered by nanomaterials

Scientists from interdisciplinary fields are fascinated by the physicochemical properties of nanomaterials that make them the choice for application as imaging agents in one or many of the diagnostic imaging modalities. A variety of *in vivo* imaging modalities have been seen to have exploited these nanomaterials' properties to engineer nanovectors in this past and newer venues are being exploited further [73]. The following properties are considered the vital requirements for a nanomaterial to be capitalized, enabling better diagnostic applications. *Stability* – More necessarily, a nanomaterial should be stable in the biological environment with the ability to generate optimal signals for imaging. They should also be characteristically strong enough to escape the immune response that occurs by the attack of macrophages. *Polydispersity* – The surface charge, shape, size, and solvent decide the polydispersity of nanomaterials to be applied for contrast imaging. The broad range of polydispersity determines the aggregation of nanoparticles, which in turn supports the identification of signaling abilities in the imaging modalities of

such nanomaterials. Polydispersity may have both positive or negative effects on the signal generation in dynamic biological situations, and hence, it is a necessary parameter to be controlled during the nanomaterials' preparatory stages [74]. *Biocompatibility* - Nanomaterials for imaging applications are more carefully chosen rather than therapeutics due to the reason that these agents are possibly injected into healthy as well as diseased subjects (patients). In healthy subjects, these imaging agents are not expected to cause an adverse effect leading to serious issues [75]. It is important to remember the limitations noted with the conventionally used contrast agents discussed previously. These nanomaterials are to be cleared over time without causing chronic damage to the system. *Circulation time frames* - Conventional contrast agents suffer issues with time frames with regard to circulation in living subjects. For the assessment of blood vessel anomalies, blood pool agents are required to be in circulation for extended time frames enabling dynamic acquisition of signals without missing out on bolus injection (more common in CT contrast imaging) [76]. It is imperative that a nanomaterial chosen to succeed the traditional imaging agents should maintain improved circulation times, allowing a better window to acquire designated contrast differences among tissues. *Biodistribution* – Accumulation of nanomaterials takes place through extravasation into the capillaries and leaky vasculature through enhanced permeability and retention (EPR) effects in cancerous tissues. This distribution is essential to imitate the contrasting effects offered by marketed contrast agents and is achieved by modulating the shape and size of nanomaterials of choice [77]. *Targeted delivery* – Delivery of nanomaterials to the targeted site is yet another concern, although extravasation through EPR effects occurs naturally upon injection into living subjects. A major issue is that this extravasation and accumulation happens not only in the target tumor site but also in the normal tissue, leading to a question on the distribution and reduction in contrast differences [77]. Hence, targeted delivery is preferred for such nanomaterials to be used as contrast agents through the conjugation of surface moieties or ligands following the biological mechanisms and pathways which are to be discussed later in this review.

Engineering of nanovectors

The use of nanovectors has seen a diverse application in the field of nano-theranostics. More specifically, these nanovectors can be classified into three generations where their applications have superseded their individual capacities at each generation [78]. The first generation nanovectors were designed to utilize the intrinsic EPR effects where they can be accumulated at the tumor site and extravasated into the interstitial space following passive delivery mechanism. The use of metal nanoparticles with polymeric surface coatings has been approved for imaging applications while paclitaxel loaded albumin nanocarriers have been approved for therapeutic applications in breast cancer treatment [79].

Nanovectors that are a step higher in delivering the payload to specific target sites such as tumors fall under the second generation. These nanovectors are functionalized with a small ligand or a targeting moiety that triggers the receptors to actively internalize them through receptor mediation. A well-known case for these second-generation nanovectors includes the antibody-mediated delivery of nanovectors that selectively bind to the antigen that needs to be handled by the nanovector. A collection of targeting agents other than antibodies, including aptamers, small ligands, proteins, small peptide chains, and many more, are being idealized to target specific biomarkers that allow active delivery to the target site [80].

A highly diverse conglomerate of nanoforms included in a nanovector forms the third category nanovectors. Fig. 5 shows a few examples of nanoscale materials that can be useful in designing multifunctional nanovectors. These nanovectors have multiple focus points in their design where they establish multifunctional behaviors. Multifunctional activities may ascertain the use of these nanovectors for imaging in single or multimodalities, drug delivery, and anticancer activity in an actively targeted fashion. These third

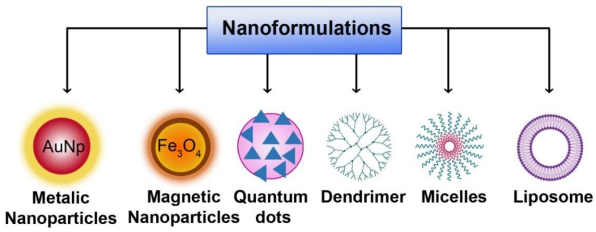

Fig 5. Nanostructures used for engineering nanovectors or nanoformulations

generation nanovectors usually constitute what is known as multifunctional nanoparticles. With multiple functional components in place, these nanovectors are designed to break through the natural biological barriers such as the blood-brain barrier (BBB) [81, 82].

Designing a nanovector for imaging applications requires consensus to achieve high signal generation, improved loading efficiency, better pharmacokinetics, and targeted delivery capacity, clearance from the system without causing toxicity to the normal tissue. A step forward nanovectors with stimuli-responsive activity such as pH and temperature can lead these nanovectors to be the future research perspective in replacing the existing contrast agents and fluorescent tags are being employed currently for modern imaging applications [5].

Micelles

A major scientific focus in the past decade is the development of smart materials that alter their structure and properties based on the environment or on demand. The ubiquitous nature of supramolecular assemblies has been proved to be an inspiration for such developments due to their functionalities and responsiveness [83]. To improve the properties and processes required for various biomedical applications, such as transportation of molecules near an oil/water interface change, wettability, or physicochemical properties, these smart materials are organized. Due to the co-existence of both hydrophilic and hydrophobic entities, surfactant molecules have the ability to self-assemble into various structures [84]. Most importantly, micellar structures, including spherical, disc-shaped, worm-like, or vesicular structures, are widely used for various applications. The multitude of micellar structures that are formed depends on the critical packing parameter, the critical micellar concentration (CMC) that enables this prediction to a certain extent. These micelles are useful as nanocarrier systems for various drugs and prodrugs which could be efficiently delivered to the site of interest.

Amphiphilic molecules exposed to a hydrophobic solvent form an oppositely oriented micelle with hydrophobic parts on the outside and a hydrophilic parts on the inside, called reverse micelles. Although micelles are considered an efficient nanocarrier for drugs, they are not suited for hydrophilic drugs [85]. To overcome this, reverse micelles with hydrophilic ends in the inside of the structure support the loading of hydrophilic drugs. This enables the delivery of these hydrophilic drugs without being lost in the bloodstream [86]. One good example is the delivery of nutrients required for comatose patients as oily injections. Micelles and reverse micelles are also studied to analyze their efficacy for protein delivery, enabling them to be utilized as valuable nanocarriers [87].

Liposomes

Liposomes have proved their ability to act as an efficient nanovector in the past decades. These are concentric vesicles of phospholipids with aqueous volume within and inside the lipid bilayers that can be tuned to carry payloads for diagnosis and therapy. The use of phospholipids in liposomal preparations is a major advantage since it replicates the cell membrane components [88]. This, in turn, enables these liposomal formulations to be eliminated in a safe and biocompatible fashion without causing severe adverse reactions in the biological environment. The surface charge, particle size, and phospholipid composition determine their physical properties, which in turn allows for improved circulation time by escaping the reticuloendothelial system (RES) [89]. A variety of liposomal formulations have been marketed and in clinical trials, and these include Doxil® , LipoDox®, ThermoDox®, Ambisome®, Daunoxome®, and many more. These liposomal formulations are used in treating a variety of cancers [90]. On the diagnostic front, several studies have been performed to determine the applications of these liposomes in molecular imaging. In these formulations, liposomes act as nanocarriers for a variety of nanomaterials that can enhance contrast differences in the imaging of tissues [91]. Few examples of such studies include gadolinium loaded liposome for MRI [92], Iodinated liposome for CT imaging, Technetium-99m loaded liposomes for SPECT/CT imaging [93], and ^{111}In loaded liposome formulation for scintigraphy applications [94]. Being an efficient nanocarrier, liposomes can be targeted to the required site by functionalizing targeting moieties that could attract these formulations to the specific biomolecules in the target site.

Polymeric Micelles

Polymeric micelles are a class of nanostructures that act as a carrier for a variety of chemotherapeutic drugs and imaging agents.

These polymeric micelles are constructed either with the use of synthetic polymers such as polyethylene glycol (PEG) and polyvinyl alcohol (PVA) or natural polymers such as polyamino acids and polysaccharides [95]. Micellar nanocarriers are highly adaptable and processable that could lead to efficient control over the distribution and elimination of the payload in the biological environment. The selection of block copolymers for the preparation of these polymeric micelles depends mainly on the ability to overcome the immune responses while being non-toxic to the system [96]. At the same time, elimination via kidneys is also of importance since their accumulation is unintended. The design of these micelles supports biodegradability wherein they are broken down into monomer units for the purposes of delivering the payload and eliminated from the system avoiding long-term toxicity [97].

Polymeric micelles can be designed to respond to specific stimuli that enable control over functions, including the release of the payload based on the microenvironment. For example, pH cleavage of these micelles loaded with chemotherapeutic drugs can be made possible by modifying the multiple components of such micelles that lead to the release of drugs in the tumor microenvironment due to the presence of unique pH conditions compared to normal tissues [98]. This stimuli-responsive micellar construct can establish high selectivity in the delivery of the drugs to specific targets [99]. On the other hand, enzyme activity in normal and diseased tissues can also be exploited in determining the actions of micelles by conjugating a moiety that is selectively degraded by overexpressed enzyme activity. However, the bulky structure of the nanoformulation based on micelles' multiple compartments may hinder the enzymes' activity, which may require further investigation as the release kinetics are reduced for the drug in the micelles [100].

Surface modification of the polymeric micelles with targeting ligands can be advantageous as well since these modifications prevent the nonspecific distribution of hydrophobic drugs loaded as core segments. Furthermore, the release of these drugs at the required sites improves the efficiency while reducing the dose requirements [101]. One good example of such modification is the loading of doxorubicin in the polymeric micelle. The metallic complexion is another approach in the preparation of polymeric micelles which are dependent on metal ions such as iron, zinc, and copper [102]. Most of the proteins or peptides require metal complexion to perform their functions in the biological environment. Hence, these metal complexion requirements are being exploited and researched for the delivery of polymeric micelles to achieve better theranostic efficacies [103].

Polymeric nanostructures

Polymeric nanostructures have been useful in enhancing the efficiency of therapeutic agents and imaging agents that have fascinated researchers in the recent past. Several polymeric nanostructures have been investigated for their ability to generate enhanced contrast differences in imaging modalities, including CT imaging and MRI [104]. Most of these polymeric nanostructures are either conjugated or chelated with a contrast agent, while others are incorporated with such agents in the polymeric matrices [104]. Iodinated oil, such as Lipiodol has been approved by FDA for clinical imaging, while mPEG-b-PCL based nanostructures loaded with iodinated oil were researched for their excellent stability and sensitivity in accumulating at the intended site generating contrast differences in CT imaging [105].

In MRI, polymeric nanostructures loaded with gadolinium and super paramagnetic iron oxide (SPIO) are found to have improved contrasting abilities. For example, Gadolinium loaded multiblock copolymers including poly-lactic acid – poly-ethylene glycol – Poly-L-lysine – diethylenetriamine pentaacetic acid (PLA-PEG-PLL-DTPA) mixed with Poly-L-histidine and PEG conjugated with biotin (PLHPEG-Biotin) were developed by Zhang et al. as a degradable MR contrast agent [106]. Vascular endothelial growth factor receptor antibodies conjugated with PLHPEG-Biotin facilitated the targeted delivery of gadolinium with improved T1 contrast compared to the commercial agents for MRI. On the other hand, SPIO loaded PEG-b-PCL polymeric nanostructures functionalized with folate showed enhanced contrast difference among diseased and normal tissues *in vivo* [107]. Due to the flexibility offered by polymeric nanostructures, researchers have exploited these nanostructures to enable multimodal imaging applications that would suffice the need for cross-referencing. For example, Weissleder et al. developed Dextran coated DTpa modified magneto-fluorescent nanoparticles with ^{64}Cu. This agent was efficient in generating contrast

differences in PET, MRI, and fluorescence imaging due to the presence of ^{64}Cu, iron oxide core, and Vivotag-680, respectively [108].

Polymeric nanostructures have been notably useful in the therapy of many cardiovascular diseases such as hypertension, atherosclerosis, and coronary artery diseases [109]. These nanostructures have proved to be the choice for stents and stent coatings. Cancer treatment with polymeric nanostructures has also gained pace due to their flexibility, allowing the incorporation or conjugation of multiple drugs and targeting ligands. Being the most preferred therapeutic intervention, peptide-based therapies for diabetes, cardiovascular disorders, and cancer have suffered from rapid clearance *in vivo*. This issue was overcome by encapsulating such peptide-based agents in polymeric nanostructures such as PLGA or PEG, which also enables improved circulation time [110]. Multi-drug loading was also made possible with these polymeric nanostructures wherein cross-linkers were used to form polymers of drug-conjugated monomers using the ring-opening metathesis polymerization [111]. A step forward, theranostic agents based on polymeric nanostructures have been developed, enabling better diagnosis and prognosis.

Dendrimers

Dendrimers are radially symmetric nonlinear, hyperbranched polymeric nanostructures. The architecture of these tree-like structures allows for a variety of functionalization with carefully tailored end groups at the periphery enabling modifications to their physicochemical properties [41]. Synthesis of these dendrimers can be carried out using the convergent, divergent, and click chemistry methods. Polyamidoamide (PAMAM) dendrimers are more commonly used for biomedical applications, more importantly in imaging applications [41]. The synthesis of these dendrimers start with a core molecule that is added further with other atoms or molecules called dendrons that form the hyper branches. The addition of each repeat unit results in the so-called 'generations' in the dendrimer. These generations are determined by the steric effect that control the future addition of repeating units [112]. Dendrimers are more widely studied for their application in medical imaging and drug delivery. The dendritic spaces of these dendrimers offer the ability to carry metallic nanomaterials that are delivered to the target site, leading to contrast differences from normal tissues. On the other hand, these dendrimers are also helpful as nanocarrier systems that carry a multitude of drugs such as anticancer agents. These dendrimers are also used as multifunctional nano-formulations due to their ability to carry multiple agents for imaging and therapy, thereby enabling nano-theragnostics [113].

Metallic nanomaterials

Noble metallic nanomaterials are gaining importance in the field of medical imaging due to their remarkable optical, chemical, and photo-thermal properties [114]. With the large surface-to-volume ratios offered by these metallic nanoparticles, more specifically, gold and silver nanoparticles, diagnostic imaging and therapeutic applications are possible. These metallic nanoparticles are also highly reactive to be functionalized with surface ligands for chemotherapeutic drugs, biomolecules, and other surface modifying structures [115]. In their nanoscales, metals exhibit a characteristic interaction with light leading to their oscillation at the electronic level enabling the localized surface plasmon resonance (LSPR). This free electron oscillation may occur via radiative or non-radiative decay mechanisms resulting in a strong scattering of visible light or thermal energy. Exploiting these behaviors of metallic nanoparticles, several researches are confronted to achieve excellence in biomedical imaging and therapy [116].

Gold and silver nanomaterials have been well known for their biomedical applications for several decades. Gold nanoparticles are widely useful due to their monodispersed size and shape characteristics which can be modulated by adjusting the reducing agent concentrations. Seed mediated and seedless growth are the two major approaches to prepare gold nanorods. These gold nanorods are being highly concentrated for their photothermal and NIR imaging applications. On the other hand, gold nanocages were prepared using galvanic replacement method exhibited a hollow interior. While silver nanocubes are widely useful as a sacrificing template since the reduction capacity of silver is comparatively less than gold. On account of the high atomic number, the application of gold and silver nanomaterials has been increasing in diagnostic imaging and therapy [117]. These increasingly noted applications

are a result of their non-covalent interactions with many biomolecules such as antibodies, proteins, peptides, and other macromolecules. It is, therefore, possible to biofunctionalized these metallic nanomaterials for targeted delivery leading to the use of LSPR in optical imaging and high atomic number leading to increased k-shells that can support x-ray attenuation in CT imaging. Nanoclusters of gold and silver are currently growing, and its application in nanomedicine with the advances in atomic precession controlled by newer nano-chemistries are emerging [118]. Other than gold and silver, nanomaterials of gadolinium, ytterbium, iron, manganese, and cadmium in various forms and combinations are being employed. When used in combination with other metal or metal oxide nanoparticles such as bimetallic nanoparticles, alloy nanomaterials, core-shell nanohybrids, and other such complex forms, these nanomaterials offer the intimate ability in acting as multifunctional agents for multimodal imaging and theranostics [119].

Magnetic nanomaterials

Magnetic nanomaterials have found a multitude of applications in the field of nanomedicine. Most of the magnetic nanomaterials used in nanomedicine are iron-based nanoparticles. Magnetic nanomaterials are the choice due to their key properties that are superior to their bulk counterparts. These characteristic properties of magnetic nanomaterials are given below.

Saturation magnetization **–** A magnetic nanomaterials' saturation magnetization is a unique property that is defined by the number of atomic magnetic dipoles and the magnetic moments of each of these dipoles. This parameter is achievable at optimal values in materials at nanoscale compared to their bulk forms [120]. Iron, cobalt, and doped ferrites offer a high saturation magnetization compared to others and hence are researched at their nanoscale for various applications. The larger size of these magnetic materials generally shows an increased magnetization due to the reduced surface effects [121]. However, due to high surface energy, the saturation magnetization is generally smaller in magnetic nanomaterials as the perfect alignments of magnetic dipoles are hindered. Hence, a change in the shape of these nanomaterials can drastically improve the saturation magnetization by interfering with the surface energy wherein the alignment of magnetic dipoles can be increased. One good example is that cubic magnetic nanomaterials offer higher saturation magnetization compared to spherical forms [122].

Superparamagnetism **–** This is a unique behavior exhibited by the magnetic nanomaterial wherein a paramagnetic action occurs in an otherwise ferromagnetic substance in its bulk form [123]. Defined by the magnetic anisotropy, each magnetic nanomaterial portrays a magnetic domain with the magnetization aligned in a specific direction. However, by interference from thermal energy, these magnetic alignments are disturbed over the anisotropic barriers. On the application of an external magnetic field, the magnetic nanomaterials show remnant magnetization, and this increases with the increase in applied magnetic field [124]. The superparamagnetic behavior of a magnetic nanomaterial depends on its particle size. The magnetic moments of the magnetic dipoles relax to zero when the external magnetic field is removed and thermal fluctuations take over. This is related to the Neel-Brown model for superparamagnetism. Magnetic nanomaterials are considered superparamagnetic if the corresponding relaxation time is less than 10^2 s at room temperature. Several factors, including surface charge, shape, size, and magnetic interactions of these magnetic nanomaterials determine their superparamagnetic behavior which is essential for these particles to be potentially used in imaging applications.

Magnetic moment **–** The magnetic moment from each dipole of a magnetic domain determine the behavior of magnetic nanomaterials. It is imperative that these magnetic moments present the average net magnetization at a given magnetic field strength, thereby offering an improved signal in MR imaging applications [125].

Magnetic susceptibility **–** Magnetic susceptibility is yet another characteristic property essential for the application of magnetic nanomaterials in nanomedicine. Magnetic susceptibility is determined by the electronic configurations of material, and based on these configurations, the net magnetization from the dipole moments in a domain varies [126]. These configurations allow to determine the magnetic properties of a material and are classified as diamagnetic, paramagnetic, superparamagnetic, ferromagnetic, ferrimagnetic, and anti-ferromagnetic materials based on their magnetic strengths [127].

Relaxivity – The most important parameter that needs to be considered for a magnetic nanomaterial to be useful for MR imaging applications is relaxivity. As noted with the conventional contrast agents for clinical MRI, magnetic nanomaterials interact with water molecules following the Solomon-Bloembergen-Morgan (SBM) theory [128]. According to this theory, water molecules interact with the magnetic nanomaterials, leading to a reduction in T1 and T2 relaxation properties. This capacity to reduce longitudinal or transverse magnetization is called as T1 relaxivity (r1) and T2 relaxivity (r2), respectively. Magnetic nanomaterials exhibit r1 and r2 values that determine their potential in generating contrast differences between tissues of interest either in T1- or T2-weighted MRI [129].

Recent nano-chemistries have led to modifications of magnetic nanomaterials in terms of size, shape, atomic interfaces, surface properties, and their biological interactions. With the availability of these nano-chemistries, researchers have been involved in upholding their applications in various nanomedicines, including diagnostics and therapy. Other than MRI, magnetic nanomaterials have been found to design strategies with applications in other imaging tools, including magnetic particle imaging (MPI), magneto-motive approaches, including magneto-motive ultrasound, and optical imaging [130]. These approaches clearly indicate that the magnetic properties are the key needed to ensemble the magnetic nanomaterials into nanomedicine.

Quantum dots

Nanoparticles in the form of quantum dots (QDs) based on semiconductor nanocrystals such as cadmium selenide and cadmium telluride are being employed. Their unique optical properties are being exploited to achieve biosensing and optical imaging applications. Typically, hundreds to thousands of atoms of group II–VI elements (e.g., CdTe, CdSe, CdS, ZnS, ZnSe, or ZnTe), group III–V elements (e.g., InP or InAs), group I–III–VI2 elements (e.g., $CuInS_2$ or AgInS2), group IV–VI elements (e.g., PbSe, PbS, or PbTe), or group IV elements (e.g., Si, C, or Ge) contribute a single quantum dot. QDs are large in comparison to conventional organic dyes but can be comparable in size to fluorescent proteins and other large biomolecules [131]. These semiconductor QDs, unfortunately, pose problems of toxicity leading to serious adverse effects and toxicity due to the presence of cadmium ions. One of the two following approaches is being employed to overcome the issues with cadmium-based QDs. In the first approach, the biostable QD is surface coated with a long last polymeric coating that would suffice the need for biocompatibility reducing the toxicity [132]. While the second approach, although being considered difficult, utilizes the advances in nano-chemistry and material science, thereby enhancing the fluorescence of cadmium-free QDs. Additionally, nanoclusters of noble metals are being considered and researched to develop *in vivo* OI applications. Gold and silver are employed for this replacement with QDs due to their extreme characteristics for tuning their optical properties when they are size tuned into nanoclusters, although these particles exhibit higher absorption. This can be achieved by modulating the size and surface properties of metal nanoparticles that can further lead to quantum confinement effects [133]. These effects allow for OI applications since the behavior of these metal nanocrystals appends the characteristics of QDs acting as fluorescent agents. Moreover, these metal nanoparticles can enable additional applications in therapy due to their excellent absorption and thermal properties. When quantum-confined in the form of nanoclusters, these metal nanoclusters offer optical imaging capabilities based on localized surface plasmon resonance (LSPR). Extremely large molar extinction coefficients, resonant Rayleigh scattering, and enhanced local electromagnetic fields result from an LSPR excitation of these noble metal nanoclusters [134].

Upconversion nanoparticles

In the recent past, a new class of nanoparticles called the Upconversion nanoparticles (UCNPs) have received importance over the conventionally used QDs. These are nanoparticles that are usually doped with rare-earth ions such as $Yb^{3+}/Er^{3+,}$ $Yb^{3+/}$ Tm^{3+} that absorb NIR light usually around 980 nm and emit a shorter wavelength with high energy according to the anti-Stokes' emission process [135]. NaYF4:Yb,Er coated with a cationic polymer, polyethylenimine (PEI) has been analyzed previously. The physicochemical properties, biocompatibility, and distribution in the tissues for application in *in vitro* and *in vivo* cancer diagnosis and imaging were evident for the above UCNPs

[136].

In the other study, UCNPs tri-doped with Yb^{3+}/Tm^{3+}/Er^{3+} combination was established for *in vivo* tumor targeting and imaging applications. Here, the tri-doped UCNPs were coated with a modified aminoPEG to allow for biocompatible circulation, and cyclopeptide [c(RGDFK)] was functionalized as a targeting ligand. The tri-doped UNCP can offer green, red, and NIR emissions when fine-tuned and the affinity of the cyclopeptide toward the $\alpha_v\beta_3$ integrin receptors, a contributor to tumor angiogenesis, enables targeting tumors along with reduced autofluorescence even at high penetration depth [137].

Surface modifications

The exploitation of nanomaterials' characteristics leading to their superior biological applications, however, has several limitations elaborated previously. Alongside, to improve their atomic precession and surface characteristics, several modifications are being employed [138]. These surface modifications empower the nanomaterials of concern to establish the required action in the biological system and can occur through several covalent and non-covalent interactions between nanomaterials and other biomolecules of concern [139]. Surface modifications on nanomaterials offer stability in the biological environment, which improves biocompatibility and biodistribution with a safe circulation half-life. These modifications also enable 'stealth' behavior, targeted delivery, and reasonable elimination from the system [140]. It is recollected that the surface reactivity of nanomaterials is exorbitant, and their expected outcome as an imaging agent or therapeutic efficacy depends on their surface modification. The purposes and examples of surface modifications are presented hereunder.

Most of these nanomaterials used for biomedical applications are not water-soluble. To overcome this concern, a variety of surface modifications are employed. More necessarily, the surface of the as-prepared core nanomaterial itself is modified or the addition of surface modifiers to offer free reactive functionalities such as amino or thiol groups are employed. These surface modifiers include a polymer such as polyethylene glycol (PEG), chitosan, and many more [141]. To be biocompatible, nanomaterials having a hydrophobic surface need to be encapsulated within a reasonably stable surface polymer [142]. Micelles are widely used to achieve this stability in the biological environment, thereby acting as a nanocarrier delivering the nanomaterial as a payload for diagnostic imaging [143]. Liposomal formulations are also engaged for this purpose since they resemble the cellular bilayer leading to biocompatible and biodegradable formulations that escape the immune response upon injection [144].

Nano-chemistries have also implemented changes to the properties exhibited by a material when doped with another. A classic example of this juncture is the modulation of magnetic properties exhibited by magnetic nanomaterials in various doped forms (example: iron oxide nanoparticles doped with manganese, cobalt, and nickel). When metals are prepared as bimetallic nanomaterials, they are highly useful in generating contrast differences in multiple modalities due to the variations in the surface through frustrations to the core metal [145].

Bioconjugation chemistry plays a significant role in establishing various theranostics applications, including single and multimodal imaging, biosensing, and photodynamic therapy. Surface modifications are also imbibed to serve as a nanocarrier-mediated, safe delivery of drugs and imaging payloads. These nanocarriers prevent the loss of payloads in circulation leading to a better delivery to the site that is targeted and reduces toxicity [146]. Dendrimers are a very good choice to act as a nanocarrier since they offer credibility with their surface functional groups that can be conjugated with small ligands for targeting or an anticancer agent for therapy [147]. The dendritic spaces on the other hand, can again be useful in entrapping nanoimaging payloads that are usually metallic or magnetic nanomaterials. These nanocarrier systems can further enable the application of nanotechnology for multifunctional theranostics through functionalization of targeting moieties by modifying the surface chemistry to the next level [148].

Targeted delivery of nanovectors – mechanisms

Nanovectors are a combination of multiple components in the nanoscale that could benefit diagnostic and therapeutic applications. In the context of this review, nanovectors combine the approach to multimodal medical imaging with the ability to target a cancer site. Hence, it is critical to

understand the mechanisms involved in targeting a nanomaterial-based contrast agent for imaging in multimodalities. In principle, tumor-targeting depends on the tumor microenvironment, including the tumor cell and cell organelles [149]. Generally, EPR plays a major role in accumulating the nanoformulation in metastatic tumor targets due to the presence of leaky vasculature. Additionally, lymphatic drainage is impaired or absent, enabling EPR to take over and accumulate large molecules such as polymers or small particles such as nanomaterials that are approximately 20 nm-500 nm in diameter [150]. On the other hand, several receptors play an important role in attracting various macromolecules and ligands in cancer growth. Taking advantage of these receptors, drug molecules or imaging agents can be delivered to the cancer target by functionalizing ligands using surface chemistry and biofunctionalization [151].

Issues and challenges

Nanovectors have extended their wings to a variety of biomedical applications in diagnostics and therapy, more particularly in cancer. Although nanoparticles offer competencies to tune their properties for these applications, several limitations still exist. Astonishingly, the potential characters for the utilization of nanomaterials stand as limitations as well for application in biomedicine. Engineering nanomaterials for *in vivo* diagnostic imaging has certain design considerations to overcome these limitations. A major concern is the toxicity issues that arise from these nanomaterials to living subjects. Chemical composition, size, and degradability in biological systems contribute to the toxicity effects of these nanomaterials. Biological degradation that occurs via any of the several pathways, namely, apoptosis, phagocytosis, necrosis, and or inflammation, is an essential factor that determines the removal of these nanomaterials. Excretion and metabolic clearance of these nanoparticles is yet another major limitation that needs to be addressed. Most of these nanomaterials find their way out of the system through renal clearance, while others enter the metabolic clearance via the liver and spleen. These clearance routes depend on the size of the nanomaterials being applied. On the other hand, the macrophagic phagocytic system (MPS) captures these nanomaterials, thereby fighting to eliminate them. Surface modifications can allow for improved nanomaterial properties to escape from the MPS and other pathways, leading to better circulation. Increasing the bioaccumulation at the target site and improving the circulation half-life of these nanomaterials requires attention when designing for imaging applications. These are again dependent on the particle size, surface charges, and shape of the particles, which allows for uptake by the reticuloendothelial system (RES) or extravasation through the fenestrations in the blood vessels. For example, when the size of the nanomaterials of concern is smaller, they are rapidly cleared renally while larger particles are metabolized in the liver, which further undergoes degradation. Functionalization of targeting ligands for a specific receptor extends the ability for a nanomaterial to reach the target site leading to improved accumulation, giving out better signals required by an imaging modality. More importantly, with the recently growing applications of multimodality imaging, nanomaterials' physicochemical properties are to be moderated so that these particles become able to generate signals for multiple imaging tools. As an example, iron oxide nanoparticles are well known for their T2 contrast ability, can enable T1 contrast as well when tailored to a paramagnetic substance such as manganese or gadolinium for dual T1-T2 contrast MR imaging applications. On the other hand, coating these magnetic materials with a fluorescent tag allows for multimodal imaging applications in MRI and fluorescence imaging.

CONCLUSION

In conclusion, nanovectors offer significant advantages over traditional small molecules based imaging. Multimodal imaging or theranostics is the most exciting field in biomedical research, where these nanovectors play an important role. With the multifunctionality, they offer more efficient targeting, better biocompatibility, and required circulation half-life. The other advantage is their tunability, like engineering can be done as per requirements. Nanotechnology will continue to contribute newer particles with novel and exciting properties to enrich the field of biomedical imaging, helping in the early diagnosis of life-threatening diseases.

ACKNOWLEDGMENTS

Authors acknowledge CARE for financial and infrastructural support. PG & PP acknowledge

CARE for fellowships too.

CONFLICTS OF INTERESTS

The authors declare no competing interests.

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