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Photonic and magnetic materials for on-demand local drug delivery

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ABSTRACT

Nanomedicine has been considered a promising tool for biomedical research and clinical practice in the 21st century because of the great impact nanomaterials could have on human health. The generation of new smart nanomaterials, which enable time- and space-controlled drug delivery, improve the limitations of conventional treatments, such as non-specific targeting, poor biodistribution and permeability. These smart nanomaterials can respond to internal biological stimuli (pH, enzyme expression and redox potential) and/or external stimuli (such as temperature, ultrasound, magnetic field and light) to further the precision of therapies. To this end, photonic and magnetic nanoparticles, such as gold, silver and iron oxide, have been used to increase sensitivity and responsiveness to external stimuli. In this review, we aim to report the main and most recent systems that involve photonic or magnetic nanomaterials for external stimulus-responsive drug release. The uniqueness of this review lies in highlighting the versatility of integrating these materials within different carriers. This leads to enhanced performance in terms of *in vitro* and *in vivo* efficacy, stability and toxicity. We also point out the current regulatory challenges for the translation of these systems from the bench to the bedside, as well as the yet unresolved matter regarding the standardization of these materials.

Contents

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1. Introduction

Despite the massive progress in recent years that modern medicine has made, conditions such as cancer, Alzheimeŕs and cardiopulmonary diseases are among the 10 most common causes of death worldwide [1]. Although many promising drugs have been identified, they tend to fail in the clinical stage due to several limitations such as high dose–response toxicity, inability to pass through biological barriers and rapid clearance by the body [2,3]. It is, therefore, necessary to develop drug delivery systems that allow controlling the release of the drug in the tissue of interest, keeping its concentration fixed in the required time frame. Among pivotal roles in drug delivery are spatial targeting, *i.e.,* the localization of the drug in the organ, tissue or cell of interest; and temporal targeting, *i.e.*, controlling the speed of delivery of the drug to the target. In this context, nanotechnologies present a revolutionary solution. In fact, formulations based on nanometric structures can be accurately designed to overcome all the limitations of conventional drugs, protecting them from rapid degradation, increasing their effectiveness and reducing the side effects related to non-specific biodistribution [4]. In this sense, three different targeting strategies have been developed so far to ensure the localization of nanoformulations in a specific location in the body: passive targeting, active targeting, and stimuli-responsive programmed specific targeting. Each strategy exploits a different mechanism to deliver and release the drug to the site of interest (Fig. 1). It must be acknowledged that these strategies can be used independently or in combination.

1.1. Passive targeting

Passive targeting takes advantage of the differential physiological condition of the tissue or organ of interest in the presence of a specific pathology [5]. This type of targeting is found, for example, in cases of inflammatory diseases and tumor pathologies, where the endothelia of blood vessels become more permeable than regular endothelia. In the case of malignant solid tumors particularly, the hierarchically organized healthy vascular network is extensively modified into a tumor-specific vasculature. This distinctive tumor vasculature is formed by irregular and disorganized blood vessels with large fenestrations (large pores within 0.1–3 μm in diameter) [6]. Furthermore, the reduced lymphatic drainage leads to a stagnation of blood plasma in the surrounding tissue. This phenomenon, known as the enhanced permeability and retention (EPR) effect, has been exploited for the accumulation of nanoformulations in cancer tissues. It is said that this accumulation of macromolecules can reach a 70-fold increase in the tumor microenvironment [7]. Thus, several nanoformulations using the EPR effect for cancer treatment have been approved by the Food and Drug Administration (FDA) as chemotherapy. Some examples are Doxil®, a liposomal delivery system of doxorubicin (DOX); Abraxane, an albumin-based nanoparticle delivery system of paclitaxel; and Vyxeos ®, a liposomal delivery system of Cytarabine and daunorubicin [8,9]. Generally, this phenomenon is driven by the physicochemical properties of the nanoformulations, being not only size but also shape, morphology and surface properties crucial factors that influence nanoparticles (NPs) circulation, biodistribution, clearance, and tumor targeting. To prolong systemic blood circulation and thus EPR-based targeting efficiency, NPs should be big enough to not be excreted by the kidney, but also small enough to not be rapidly recognized by the mononuclear phagocytic system (MPS) thus promoting their non-specific uptake. However, the exact size of NPs to avoid physiological biological barriers while increasing tumor accumulation is still unclear. Indeed, although a hydrodynamic diameter between 40 and 400 nm has previously been considered optimal to enhance the EPR effect [10], more recent studies showed that NPs smaller than 40 nm could result in much more effective tumor accumulation and penetration [11]. Thus, although EPR-based drug delivery systems can be tailored by modifying the chemical-physical characteristics of NPs (shape, size and surface properties), there are still no clear general rules for optimally exploiting the EPR. Furthermore, despite some clinical success, it is difficult to generalize a universal EPR-based nanoformulation design. This is due to the high heterogeneity of the tumor environment among the wide range of cancer phenotypes. Solid tumors, indeed, possess large variability of vascular permeability, lymphatic drainage, blood perfusion rates and extracellular matrix (ECM) density and composition [5]. Furthermore, the low uptake control, and the consequent off-target drug delivery, have been demonstrated by *meta*-analysis of pre-clinical studies. Said studies revealed that 99 % of the nanoformulation administered reaches off-target organs, accumulating preferably

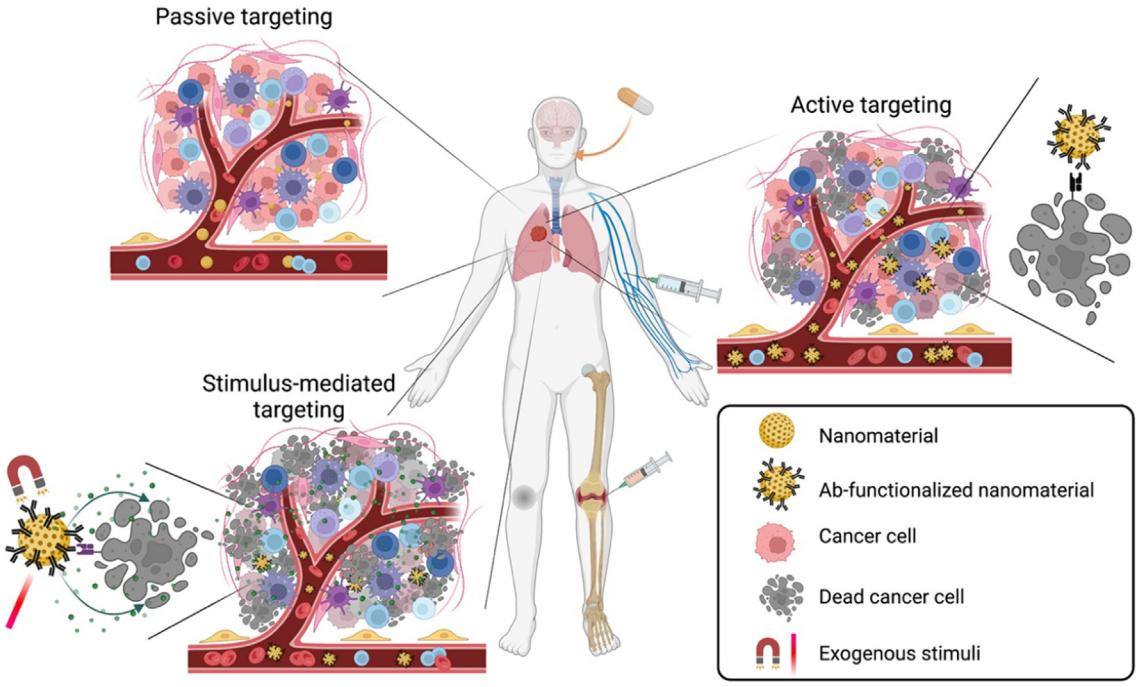

Fig. 1. Schematic representation of passive, active, and external stimuli mediated drug delivery. In all three cases, the nanocarriers reach the site of interest. Upon the arrival into the tissue, the nano-system with target molecules (active targeting) binds to the target cells through specific interactions, while passive targeted nanocarriers have a lower interaction with the target. Finally, external stimuli mediated drug delivery allows the release of the active pharmaceutical ingredients (API) only in presence of an exogenous input, *i.e.*, magnetic field, Infrared (IR), microwaves, ultrasound *etc*. Illustration created with BioRender.com.

in the liver and spleen rather than in the tumor [12]. In addition, pre-clinical models exhibit more sensitivity to EPR-based delivery strategies than that observed during clinical phases. Thus, when moving from the lab into the clinic, although accumulation of nanocarriers in human tumors by EPR occurs, its extent varies heavily between patients and tumor types. This leads to variable uptake and efficacy of nanomedicines [13].

1.2. Active targeting

While passive targeting relies on the physicochemical properties of the carrier, active targeting is based on the biological interaction between a molecule introduced on the surface of the nanomaterial and the target cell (ligand-target affinity). This usually triggers the nanomaterial internalization. Thus, nanocarriers functionalized with targeting molecules often induce an increase in the therapeutic efficacy by increasing their cellular uptake [14]. Active targeting can complement passive targeting by increasing the accumulation of NPs and their retention in target tissues. [15]. After reaching tumor tissues, the nanocarriers must be able to enter tumor cells efficiently to achieve intracellular drug delivery. This is because many of the commonly used active pharmaceutical ingredients (APIs) act on intracellular targets. Different biological moieties have been identified as suitable for functionalizing nanomaterials such as ligands, peptides, sugars, or antibodies, among others [16]. These ligands uniquely recognize specific surface molecules or receptors overexpressed in the organs, tissues, or cells of interest [17]. In addition to the methodology selected for the functionalization of the nanocarrier surface, the surface density of the ligand is also of primary importance for ensuring specific binding to the target cells. While a low density of the ligand reduces targeting efficiency, a high density leads to steric hindrance resulting also in a low targeting recognition [18]. One of the most targeted molecules studied in cancer cells is the epidermal growth factor receptor (EGFR). EGFR is a receptor protein penetrating the cell membrane involved in cell proliferation, apoptosis and angiogenesis. It is over-expressed in various types of cancer, including breast, kidney, ovary, and colon [19]. In the last year, it has been targeted using monoclonal antibodies, that act by blocking the ligand-induced EGFR tyrosine kinase activation. In addition, small-molecule tyrosine kinase inhibitors can be used to inhibit EGFR auto-phosphorylation by competing with the substrate [20]. Different nanocarriers presenting these molecules on their surface have been developed over the years to selectively induce the uptake of the drug of interest [21]. Folate receptors are also attracting increasing attention as target molecules for cancer treatment. These receptors are membrane folate-binding proteins overexpressed in both solid and liquid tumors, but with relatively low expression levels in healthy tissues [22]. They are involved in the uptake of folic acid (FA) through potocytosis [23], a receptor-mediated endocytosis for the uptake of small molecules. FA is a water-soluble molecule of the vitamin B type involved in cell growth, proliferation, and survival as well as the synthesis and repair of DNA. The small size of this ligand together with its low antigenicity and high affinity for the folate receptor make it an interesting ligand for the functionalization of nanomaterials [17]. Several scientific reports, indeed, report the use of FA conjugated liposomes, carbon nanotubes or polymeric nanomaterials with enhanced internalization and therapeutic efficacy.

On the other hand, a considerable number of publications questioned whether the active targeting strategy is sufficiently effective or even necessary for the treatment of solid tumors. This line of thought considers that the specific binding of nanocarriers to tumoral cells by active targeting usually occurs after they have diffused to the tumor by EPR-driven passive targeting [7]. In this sense, recent studies co-injecting targeted and non-targeted NPs with different sizes have shown that differences between clearance versus tumor penetration are highly dependent on the NPs size. Indeed, a time-dependent higher accumulation with deeper tumor penetration and prolonged tumor retention was observed when using active-targeted NPs. This contrasts with non-targeted NPs, which were found mostly in the edge of the tumor. However, these

observations were only observed using smaller NPs (<30 nm). For these smaller particles, active targeting is responsible for a significant decrease in the clearance from the tumor of unbound or "bystander" off-targeted NPs. In fact, the active targeting would decrease both their intravasation from the tumor back into the tumor blood vessels due to high interstitial pressure, and eventually their clearance by the macrophages. However, when using larger NPs (30 nm) that may hardly escape from the tumor microenvironment, their active targeting only slightly enhances the intratumoral delivery compared to their passive targeting [24]. It must be acknowledged that more thorough and systematic investigations on the parameters affecting active targeting efficiency are necessary. Surely the fact that no active-targeted formulations have currently been approved by the FDA for clinics is a consequence of the great variability in therapeutic efficiency observed so far. Another challenge lies in the complicated development and scale-up according to Good Manufacturing Practice (GMP) of pharmaceutical production of these nanocarriers, which are complex in terms of design and engineering [7]. However, despite these difficulties, an important advantage to consider regarding active targeting is the ability to target sites scattered throughout the body. Indeed, it makes it possible to treat diseases such as haematological malignancies and metastases for which EPR is not effective.

1.3. Stimuli-responsive targeting

More inclusive targeting strategies to overcome the limitations of passive and active targeting are being studied. These approaches do not rely on the fixed or passive accumulation capacity inherent to a given tumor and instead pursue a consistent delivery of the nanoformulations to a variety of clinical targets. These strategies aim to improve the delivery of nanomedicines across many solid tumor phenotypes, thereby maximizing their clinical applicability. For this purpose, they resort to the use of stimuli-responsive nanocarriers, and/or either by-pass or complement the EPR effect. Several articles have recently thoroughly reviewed the different strategies explored by researchers to bypass the EPR effect. This includes the use of hydrogels and implants to locally deliver NPs, cell-mediated delivery of nanoparticles, and delivery of immune-modulating payloads into tumors, among others [7,25]. This is also the case of those strategies that come under the umbrella of what is known as EPR-adaptative nanomedicine. This approach focuses on complementing the EPR effect by controlling the tumor blood flow, modulating the tumor vasculature and stroma, and killing the cancer cells to reduce their barrier function. In this sense, the wide variety of chemicals (e.g., vascular agents inducing an inflammation-like state, mild anti-angiogenic therapy, digesting enzymes of the ECM, hormonal effectors, etc.) or physical techniques (radiation, ultrasound, hyperthermia, and photodynamic therapy) explored with this aim have been recently and extensively overviewed [7,26]. Thus, in this review article, we focus on an extensive revision of the use of external stimuli-responsive nanocarriers. More specifically, we aim to review those studies that gain spatial–temporal control over drug delivery to improve site-specific targeted versus off-targeted effects of the drug carried by the responsive nanomaterial.

Stimuli-responsive programmed specific targeting is an emerging field of research that goes beyond the traditional design of nanocarriers. More recent studies have focused on obtaining a smart spatiotemporal drug release by engineering nanoformulations to respond to internal or external stimuli. In the first case, the drug delivery system reacts to biological stimuli such as pH, specific redox conditions or temperature changes. External stimuli, on the other hand, are able to control the activation of the nanomaterials remotely using exogenous means. This allows an activation independently from the local conditions, effectively allowing an ON/OFF triggering on demand.

1.3.1. pH

One of the most used endogenous stimuli for controlled drug release is pH. The physiological pH of healthy tissues and blood circulation is about 7.4. Significant variations occur in specific body areas such as the stomach (pH 2) and the intestines (pH 7). Furthermore, a low pH can be observed correlating with certain diseases, such as cancer, ischemia, and inflammation [27]. For pH-responsive materials, one type of design consists of the release of an entrapped API following structural changes that lead to the opening of pores. Other designs focus on releasing the drug that was covalently attached to acid-labile bonds. Said bonds become hydrolyzed at a determined pH value.

1.3.2. Redox

In recent years, drug delivery systems responsive to the redox state have also attracted great attention from the scientific community. Glutathione (GSH) is a very strong reducing agent present in most living organisms. Studies relating to the tumor environment have shown that the intracellular concentration of GSH in tumor tissues could be one hundred times higher than that of healthy tissues [28,29]. Thus, redox-responsive nanocarriers take advantage of the fact that certain bonds, such as disulfides (S-S), are cleaved in the presence of GSH. This linkage can be used to directly bind the drug of interest or to cross-link a gating or capping molecule on the NPs surface. In this manner, the redox conditions of tumors would allow triggering the controlled release of the cargo. Similarly, reactive oxygen species (ROS) are useful for smart drug targeting. In fact, in the presence of chronic inflammation, the basal levels of ROS are one hundred times higher than in healthy microenvironments.

1.3.3. Temperature

Another characteristic correlated to pathological conditions is the local increase in tissue temperature. Inflammatory states [30,31], infection sites [32], and tumors [33] register a significantly higher local temperature than healthy tissues. Thus, several therapeutic approaches are based on the use of thermo-responsive drug delivery systems. These systems are usually composed of a temperature-sensitive material that can undergo what is called a phase transition in response to a small change in temperature, going from a hydrophilic state to a hydrophobic one upon rapid dehydration. This structural change leads to the release of the drug into the surrounding environment. The biomedical application of these systems involves a design such that at body temperature values, the API is retained inside the material while allowing rapid release at temperatures above 40–45 °C [34].

1.3.4. External stimuli

Even though endogenous stimuli-controlled systems are designed to release the API under specific conditions, the great complexity of human biology, unfortunately, leads to unspecific drug release and off-target toxicity. Therefore, new efforts have been made to obtain a delivery system that could bring simultaneously control both in space and time of the drug release. Such a level of control needs the engineering of nanocarriers to allow drug release to be turned ON/OFF remotely. Thus, the possibility to develop a nanocarrier that can induce the release of the API by responding to an external stimulus is also an intensive field of study.

Compared to internal stimuli, external stimuli have an advantage in the field of drug delivery and, in particular, in cancer therapy. Internal (endogenous) stimuli are based on the tumor microenvironment (TME). Therefore, the control that can be

achieved over drug delivery depends on the patient, tumor type and stage. The use of external (exogenous) stimuli to trigger drug release, however, allows for manual control and modulation of the stimuli during the treatment based on individual requirements. Indeed, external physical stimuli such as magnetic or electric fields, acoustic waves, and electromagnetic radiation, can not only be specifically localized on the target to gain spatial control over drug release but also allow for an easy control of the exposition time and the intensity of the applied stimulus [34]. However, it is important to highlight that external responsive nanocarriers need the use of different types of equipment and specialized techniques to achieve the targeted stimulation needed to trigger drug release.

1.3.4.1. Magnetic field. One example of external stimuli is magnetic-triggered drug release. This approach involves the application of an alternating high or low frequency magnetic field. Magnetic fields can penetrate deeply into biological tissues without significant physical interaction. This allows both the visualization of magnetic materials through Magnetic Resonance Imaging (MRI) using a static magnetic field, and a controlled drug release in combination with Alternating Magnetic Fields (AMF)[35]. Generally, the use of AMF for triggering drug delivery is based on the ability of magnetic materials to convert electromagnetic energy into thermal energy. There are numerous literature reports that use systems composed of a magnetic material encapsulated within polymers capable of changing their structure in a temperature-sensitive manner. These are used, for instance, for the treatment of diseases such as cancer and post-surgical infection prevention [29,36].

1.3.4.2. Light. Light is another external stimulus actively used. The use of light has been reported in a wide variety of drug delivery systems with unique qualities that respond to a wide range of electromagnetic sources, such as lasers, lamps and light-emitting diodes (LEDs). This category of materials integrates nanostructures capable of responding to a wide variety of light wavelengths ranging from microwaves to infrared. The effectiveness of photo-triggered therapies relies on the type of light. During their design, one needs to reach a balance between the ability to penetrate biological tissues and the effect caused on the light-responsive nanocarrier. Short-wavelength light (ultraviolet, UV) has poor tissue penetration (up to 10 mm) but high energy. This can induce several effects on the organic molecules forming part of the nanocarriers. Some examples of these are covalent bond cleavage, conformational changes, and production of ROS. On the other hand, the use of long wavelength light (near-infrared, NIR) penetrates deeper (about 4–5 cm)[37]. In fact, by using certain nanomaterials, such as carbon-based, semiconductor-based and plasmonic metal-based ones, it is possible to convert this low excitation energy into heat [38]. Regarding therapeutic approaches, the most used wavelength frequencies are those that fall into the infrared range (700 nm to 1 mm). This is because NIR light presents a better safety profile to humans than UV light. The degree of penetration of NIR is generally enough for therapeutic purposes and capable of reaching considerably deep areas in the organism [37] In addition, another advantage of using light as a remote stimulus is its non-invasive nature, combined with its ease of use. In this sense, infrared-sensitive drug delivery systems have been prepared using heat responsive polymers. These can lead to an ON/OFF release of the drug cargo by opening-closing their structure under one-time or cyclic light irradiation. Furthermore, the reactive oxygen species produced through infrared irradiation can be exploited to induce physicochemical changes in the nanoplatform, leading to the release of the carried drug [39]. However, due to its relatively limited penetration, the application of light as a remote stimulus is restrained to where endoscopic techniques can reach.

1.3.4.3. Other stimuli. Although light irradiation and magnetic fields are the two most explored for drug release, others have also been studied. The main characteristic of these stimuli is their penetration in biological tissues and the low side effects related to their application. Among these, the use of ultrasound as a trigger mechanism for drug release has seen a significant increase in publications. Indeed, systems based on ultrasound are capable of penetrating into deep tissues of the body without being invasive or damaging [40]. In addition, this stimulus has great versatility, as it is possible to modulate its frequency, the number of cycles and, consequently, the exposure time. Most of the literature present reports using both biodegradable and non-biodegradable matrices, which subjected to short ultrasound cycles can release the drug of interest into the surrounding environment. In this context, different nanomaterials have emerged as promising to provide drug nanocarriers with responsiveness to different stimuli.

1.4. Magnetic and photonic materials vs exogenous stimuli

Among the unique properties of nanomaterials, their capability of absorbing energy, in the form of radiofrequency, light, and or magnetic fields, has raised great interest in drug delivery research. Usually, the stimuli application results in a change of the material structure and a spatial–temporal release of the drug. In other cases, the stimulus causes the rupture of the link by which the drug of interest is bound to the carriers (Table 1). Whatever the release of the drug, its spatio-temporal control allows reducing its systemic toxicity. This review focuses on the use of magnetic and photonic nanomaterials for the development of stimuli responsive materials for drug delivery. We considered that these materials have recently emerged as the most relevant in the field.

The concept of using magnetic materials for gaining remote control on drug delivery was first introduced by Widder *et al.* in 1980. He used microspheres that responded to the magnetic field to release Adriamycin®, also known as doxorubicin, an antineoplastic antibiotic drug from the anthracycline family, with a broad anticancer spectrum [41]. It is only in the last decade that the development of nanomaterials has made magnetic delivery one of the most promising methods of ensuring targeted and remote control of the release of drugs. Magnetic nanomaterials can be prepared from pure metals (iron, cobalt, nickel, molybdenum, etc.), their alloys, and also oxides [42]. The most used magnetic nanomaterials for drug delivery are composed of iron oxides with different oxidation states, namely iron oxide nanoparticles (IONPs). They possess high biocompatibility and low toxicity and can be easily manipulated using an external magnetic field. Magnetic nanoparticles, thanks to their dimensions (typical diameters below 20–30 nm), can cross biological membranes and thus can deliver drugs at the intracellular compartment targeted as site of action [43]. One of the determining factors for choosing the type of material is its magnetic properties.

It is well known that the optical, electrical, and magnetic properties of a nanomaterial are different from those of the bulk material. In particular, the magnetic properties of nanoparticles mainly depend on two different factors: i) **Regarding the size**: Macroscopic materials are composed of hundreds of magnetic domains separated by domain walls, which generate non-uniform magnetization. However, when the size of the nanoparticle is reduced, it presents a single domain, and its magnetizing energy is uniform [44]. By applying an external magnetic field, the nanoparticle has all the spins oriented in the same direction as field at issue. This allows a free rotation of the spins and therefore a free reversion of them once the magnetic field is turned off. This is a consequence of their superparamagnetic nature [45]. ii) **Regarding the chosen metal and its oxidation** state: The different metal elements have a different magnetization profile; iron, cobalt, nickel, and titanium

Table 1
Magnetic and photonic materials vs exogenous stimuli.

Material	Carrier	Agent	Stimuli	Mechanism of drug release/ delivery	Application	Reference
Magnetic	Liposome	Calcein, 5,6-carboxyfluorescein, doxorubicin, $MgSO_4$	Magnetic field	Enhanced permeability of lipid bilayer or disruption	Cancer therapy, Tissue engineering, regenerative medicine, pain killer therapy	[77,89,91,92,107,108]
Fe_3O_4, FePt, $CoFe_2O_4$, MoS	Hydrogel	Indomethacin, mitoxantrone, primary myoblasts, levodopa, methylene blue, doxorubincine, bupivacaine hydrochlorid, FITC-dextran	Magnetic field	Swelling/ deswelling of the polymer matrix due to heat production.	Tissue engineering, regenerative medicine, Parkinson's disease treatment, cancer therapy	[80,81,83,84,90,94,95]
		Doxorubicin		Thermosensitive cleavage	Cancer therapy	[97]
		Doxorubicin	NIR	Swelling/ deswelling of the polymer matrix due to heat production	Cancer therapy	[161]
	Nanosheet	Doxorubicin	NIR	Photothermal effect and structure modification	Cancer therapy	[160]
	Mesoporous nanoparticles	Melittin	Magnetic field	Non thermal structure modification	Cancer therapy	[104]
		Doxorubicin	NIR	Thermosensitive cleavage		[159]
		CO gas, ibuprofen		Structure modification due to heat production		[158,174]
		Ibuprofen	Microwaves	Structure modification due to heat conversion of microwaves		[174]
	Micro/nanocapsules	berberine, FITC-dextran, doxycycline	Magnetic field	Swelling/ deswelling of the polymeric structure	Local joints inflammation treatment	[87,88]
		Fluorescein		Thermosensitive cleavage for prodrug therapy	n.a.	[78]
	Micro/nanobubbles	Doxorubicin, Fluorescein	Ultrasound	Enhanced cavitation effect and rupture of micro/nanobubble structure	Cancer therapy	[196–199,203]
Photonic	Liposome	Cas9-sgPlk-1 plasmids	NIR	Enhanced permeability of lipid bilayer or disruption	Cancer therapy	[122]
AuNPs, AuNR, Au/Pt nanostars, ZnONPs, CuS, CNT TiO_2, AgNPs		Azo initiator (AIBA)		Structure disruption due to photothermic effect	Cancer therapy	[155]
		Verteporfin	X-rays	ROS generation trigger destabilization of the lipid bilayer	Cancer therapy	[315]
	Nanotubes	Ampicillin	Visible Light	Photocatalytic chain scission	Antibacterial therapy	[316]
	Micro/nanocapsules	Sorafenib tosylate	Visible Light	Light energy conversion into heat	Cancer therapy	[110]
		Doxorubicin		Light energy conversion into heat	Cancer therapy	[161]
		FITC-dextran	NIR	Structure modification due to heat production	n.a.	[162]
	Mesoporous nanoparticles	Rhodamine B	Visible Light	Nanovalve opening on particle surface.	n.a.	[126]
	Hydrogel	Bevacizumab	UV light	Swelling/ deswelling of the polymer matrix due to heat production	Ocular treatment	[317]

Table 1 (*continued*)

Material	Carrier	Agent	Stimuli	Mechanism of drug release/ delivery	Application	Reference
		Doxorubicin	NIR	Photothermal effect and ROS production	Cancer therapy	[156]
		Dexamethasone, indomethacin	Electric Field	Structure modification	Cancer therapy	[182]
	Nanofilm	Curcumin	Electric Field	Structure modification due to heat production	Cancer therapy	[181]
	MOF	Doxorubicin	Visible Light	Structure modification due to heat production	Cancer therapy	[318]
		Topotecan		Collapse of the structure	Cancer therapy	[112]

have a high magnetization in pure forms. However, their oxidized forms are those most used despite having a lower level of magnetization. In fact, metal oxides have a lower toxicity, and are, therefore, more suitable for biological applications.

These nanomaterials can respond to different exogenous stimuli for an efficient on-demand drug delivery systems. For example, the application of an external magnetic field can serve two different purposes: on the one hand, static magnetic fields allow to direct the nanocarrier to the site of action; on the other hand, alternating fields induce the production of heat, exploiting a phenomenon called magnetic hyperthermia. The main processes involved in heat generation are the Néel and Brownian relaxations. While the former is related to the magnetization hysteresis and is due to changes in direction of the magnetic moments, the latter is determined by the physical movement/rotation of magnetic nanoparticles (MNPs) [46]. The local heat generated by the nanoparticles can be used for the release of the drug of interest by changing the carrier structure or by breaking the bond by which the drug is linked to the carrier. It must be acknowledged that magnetic nanomaterials possess a unique versatility with respect to their responsiveness to different external stimuli. In addition to magnetic fields, they can also respond to ultrasound application and visible light irradiation. Although not extensively studied, several examples have been reported in the literature in which magnetic materials can induce a time and space directed drug release after the application of one of those two stimuli. This is achieved by causing a cavitation effect in the case of ultrasounds and by the modification of the carrier structure based on photothermia in the case of a light stimulus. Unfortunately, magnetic nanoparticles in general are prone to aggregation due to the delicate balance between repulsive forces. This is due to their surface/shell composition and the magnetic attractive forces between their cores. Thus, if the stealth capacity of the NP surface is not enough to reduce protein corona formation when administered *in vivo*, they suffer rapid MPS clearance [47]. These results not only in a very low accumulation in the target site, unwanted toxicity, and other adverse effects; but also in a significant reduction of their responsiveness to the corresponding remote stimuli. Thus, the integration of magnetic nanomaterials in other nanoformulations, namely liposomes, micelles, nanocapsules, and nanogels, is a widely used strategy to avoid these hurdles.

Photonic nanomaterials are the other big group of nanomaterials applied in remote drug delivery. Although the basic concepts of photonics have been known for the last 50 to 60 years, only in the last decade they have gained an increasing interest from the scientific community due to their wide range of applications in nanoscience and nanotechnology. Photonic materials can be defined, in general, as materials that emit, detect, manipulate, or control light. A photonic material, when designed at the nanoscale, offers opportunities for studying fundamental processes caused by the interaction between the radiation field and the matter on a scale much smaller than the wavelength of radiation. Plasmonic nanoparticles (PNPs) size-related properties are based on their interaction with light. This interaction occurs because the conduction electrons on the surface of plasmonic materials undergo a collective oscillation when they are excited by light at specific wavelengths. This oscillation is known as surface plasmon resonance (SPR), and it causes the absorption and scattering intensities of PNPs to be much higher than identically sized non-plasmonic nanomaterials. The optical response of the materials caused by this electron oscillation can be modulated by changing the physical and chemical properties of PNPs. Some of these properties are size, shape, composition, and even the surrounding environment of PNPs owing to its dielectric properties [48]. In addition, light scattering can be used in imaging techniques such as computed tomography and dark-field microscopy. On the other hand, light absorption is often used in photoacoustic and photothermal imaging, photodynamic therapy, or hyperthermia. Medical applications of photonic materials can be found since 1890, when the German bacteriologist Robert Koch discovered that gold cyanide was bacteriostatic to Mycobacterium tuberculosis *in vitro*. This subsequently led to the treatment of tuberculosis and rheumatoid arthritis with gold in the early 20th century. It was not until the year 2000 that the first study using gold nanoparticles for controlled drug delivery was unveiled, in a publication by Sershen and coworkers [49]. Their experiment consisted in entrapping a drug within a temperature sensitive hydrogel with embedded Au–Au_2S nanoshells. The authors demonstrated how, upon irradiation with NIR light, the local temperature was risen by the nanoshells, which induced the collapse of the hydrogel and thus the release of the drug [50]. In fact, different plasmonic materials can be found and used in many therapeutic approaches. Some examples of materials with plasmonic properties are silica, gold, silver, palladium, titanium, zinc, copper, aluminum, and bismuth. In addition, some carbon nanostructures could also be considered within this group, such as Carbon Nanotubes (CNT). Certain properties must be considered when a plasmonic material is selected for a biomedical application in general, and particularly for drug delivery system (DDS) design: its chemical/ biological stability, low cytotoxicity in a biological environment, and versatility in the functionalization of its surface with different biological molecules. The main property to consider when a plasmonic material is used as DDS is the efficiency in the generation of local heat upon light irradiation by the already explained SPR phenomena. Considering this, important parameters

that must be taken into account are the extinction coefficient (ε) and the photothermal-conversion efficiency (η). The former indicates the light-absorption performance of the plasmonic nanomaterial, while the later displays its ability to convert the absorbed light into heat. The possibility of joining DDS with an on-demand drug release improves conventional chemotherapeutic protocols. This allows minimizing off-target drug cytotoxicity and limited effectiveness owing to the lack of selectivity between cancerous and normal tissues present in traditional therapies. Fortunately, a remotely controlled DDS is not only advantageous in suppressing the adverse effects of targeting cytotoxic drugs toward diseased tissues, but also in reversing the low bioavailability of, for instance, most anti-cancer drugs [51].

In this review, we aim to highlight the advantages of using magnetic and photonic nanomaterials for the development of intelligent systems for controlled spatio-temporal drug delivery. First, we outline the carriers that have been developed to overcome the many drawbacks of magnetic and photonic materials for their direct use in remote drug delivery. Then, we focus on structural changes or molecular response of these materials to external stimuli, such as magnetic field, laser, microwaves and ultrasound, to enable controlled drug release. Finally, we overview of the status and current challenges of the application of these remote stimuli-responsive carriers in clinical trials, highlighting the problems not only at the biological level (toxicity, fate of the carrier in the human body, etc...), but also the legislative gap in the field.

2. Carriers

Over the past years, the attention of scientists has been driven to design nanoformulations for drug delivery. They pursue to concentrate the active pharmaceutical ingredient (API), direct it to the targeted delivery site and provide control over the timing and site of drug release. All that by incorporating stimuli-responsive nanomaterials within these nanoformulations. These nanohybrids structures help to maintain the colloidal stability of the incorporated stimuli-responsive nanomaterial in complex biological environments, as its aggregation negatively affect their response capacity. Besides, the development of integrated assembles and hierarchical structures of multicomponent functional materials allows improving both cellular internalization and nanocarriers penetration e.g., within the tumor. Engineering their design to also trigger switchable size, surface charge, controllable targeting molecules and/or various coatings provides a wide range of possibilities. Thus, the combination of the properties of each component leads to synergized nano-assemblies with remarkably smart drug delivery properties (Fig. 2). The rational design of these stimuli-responsive nano-assemblies usually involves the development of hybrids combining both organic and inorganic components [52]. It is also important to highlight that a nanostructured-based stimuli-responsive material for drug delivery and targeting must have specific characteristics, including e.g. biocompatiblility, biodegradablility, and sufficient average lifetime to release the

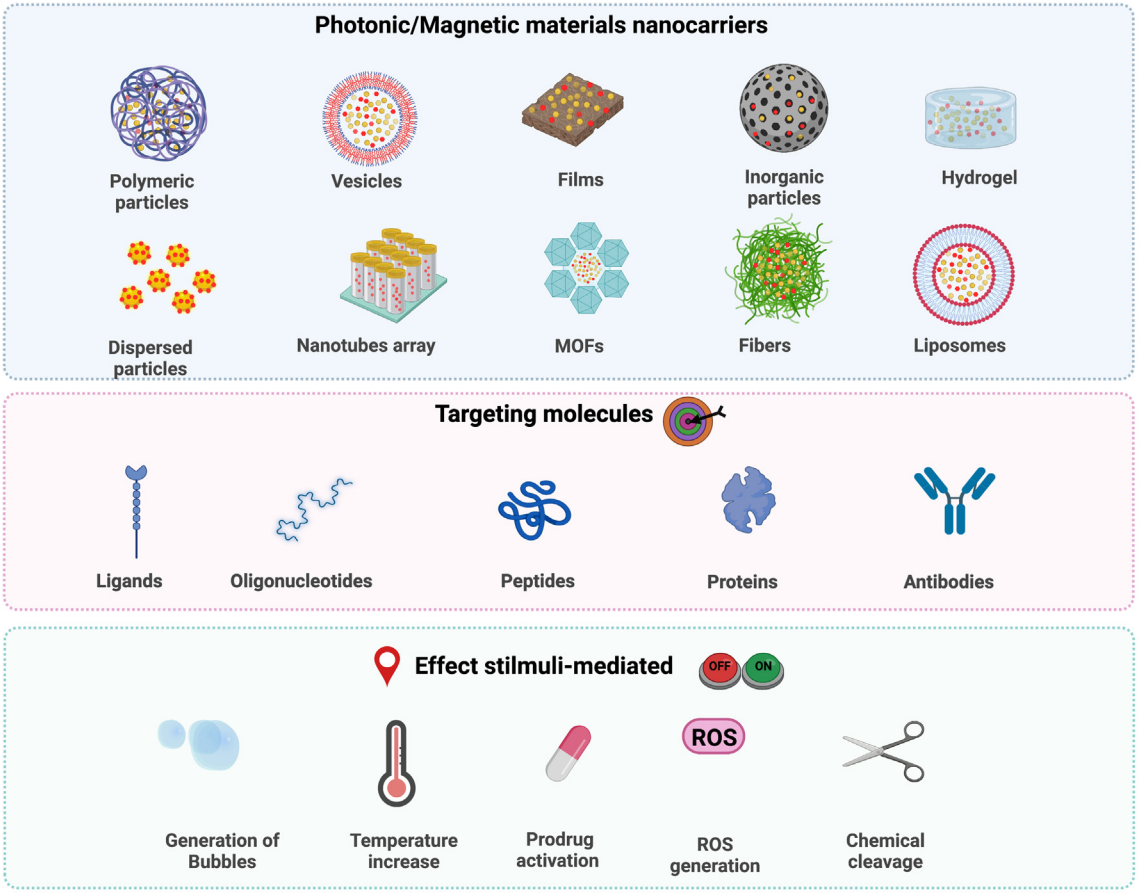

Fig. 2. Schematic structure of the different nanocarriers and their stimuli-related effects. According to the different administration route and target tissue, photonic and magnetic nanomaterials can be combined with smart materials to obtain different combinations of nanocarriers. These can be further modified to obtain an active targeting, by specific molecular functionalization of the surface. Then, the nanocarrier can be administer and can exert its effect on cells when stimulated by an exogenous input. Within the cell, the photonic and magnetic nanomaterials, upon the external stimuli, can generate different molecular and physical effects that can lead to a spatio-temporal drug release. Image created with BioRender.com.

drug in the desired site of action [53]. Ideally, it should also be easy to synthesize, and functionalized with simple surface modification protocols that ensure the site-directed binding of the biomolecules of interest. Different materials and synthetic procedures have been developed during the last decades.

2.1. Liposomes

The most common classes of FDA approved nanomedicines are lipid-based nanoparticles, generally liposomes. Liposomes are self-assembly lipid-based nanoparticles composed of synthetic or natural phospholipids that can be arranged in different structures, from unilamellar to multilamellar. Thanks to their easy formulation, they can be opportunely modified in size, lipid composition and surface charge, while maintaining high biocompatibility and bioavailability [54]. Liposomes are, indeed, a versatile system that can integrate hydrophobic, lipophilic, and hydrophilic APIs, even simultaneously, expanding their range of biomedical applications. The main characteristic that makes liposomes suitable for stimuli drug release is the transition temperature of phospholipids (Tc), which can be defined as the temperature at which it passes from a gel to a liquid crystalline phase [55].

2.2. Polymeric materials

A great versatility in terms of different nanostructures is represented by polymeric nanomaterials. Indeed, they can be represented by nanogels, nanospheres or nanocapsules [5657] All of these structures can be easily produced and help reducing the side effects of drugs by allowing the control of their vectorization and release. They can be designed using different polymers and thus their physicochemical characteristics can be modified to change charge, size, porosity, amphiphilicity, degradability, and softness. In addition, this nanomaterial ensures API protection from hydrolysis or oxidation [58]. The most used natural polymers are chitosan, cellulose, and alginate [59]. However, it is frequently reported in the literature the use of synthetic nanocompositions formulated with polyethylene glycol (PEG), poly (vinyl alcohol) (PVA), poly (ethyleneimine) (PEI), and poly (vinyl pyrrolidone) (PVP), all approved by the FDA as nonantigenic in nature [58].

2.3. Inorganic materials

Furthermore, only in the last years, research has focused on inorganic materials and in particular on mesoporous silica nanoparticles (MSNs). They attracted attention as they are biocompatible, easy to synthesize with a high control of both diameter (2–50 nm) and porosity and a thermally induced phase transition that changes their structure from linear to globular [60]. Moreover, their large accessible surface area (greater than or equal to 1000 m^2/g) can be easily functionalized making this material multifunctional [61]. In fact, MSNs are generally externally decorated with polymers *e.g.,* PEI, N-Isopropylacrylamide (NIPAM), N-(Hydroxymethyl) acrylamide (NHMA) or PEG to ensure their intravenous administration [62]. Apart from silica, graphene oxide and molybdenum disulfide are also promising inorganic nanomaterials. They are usually the main component in nanosheets, that confers them unique properties, such as quantum size effect, electronic and photonic confinement, and surface effects [63].

2.4. Metal-organic frameworks

Finally, metal–organic frameworks (MOFs) have proved to be a versatile multifunctional nanohybrid system for drug delivery. Generally, MOFs are composed of metal centers and organic ligands joined by coordination bonds in 2D or 3D structures of high crystallinity. Compared to other nanoformulations, MOFs present high drug loading, thanks to their well-defined porosity, low toxicity and side effects, and high colloidal stability in complex biological media. Furthermore, by choosing different metal and ligand compositions, it is possible to fine-tune their biodegradability, efficiency of drug loading and controlled drug release [64].

Throughout the following sections, we will exemplify the combination of photonic and magnetic materials within these nanocarriers to ensure a controlled stimuli-triggered drug release.

3. Stimuli

The use of external signals emitted from outside the body to control drug delivery from a nanocarrier is less affected by variations among tumor types, which provides to this controlled release strategy unique clinical advantages compared to the use of endogenous stimuli as triggers. The selection of the optimal remote triggering mechanism for a particular nanocarrier should consider not only the physiology of organ to be treated but also the nanocarriers' administration route. The different external stimuli have very different tissue penetration depth considering their clinically safe energy dosage (Fig. 3). Besides, the response of the activable nanocarrier could significantly change depending of its administration route (e.g. intravenous, hypodermic, intraperitoneal injection, localized implant, etc...), depending on the circulation time in blood of the nanocarrier, and its accumulation efficiency at the organ to be treated [65]. Several remote stimuli are being explored as triggering agents. The most common stimuli for drug delivery are the used of magnetic fields and light. Other examples of stimuli used for triggering could be X-ray, microwaves, electric pulses, and ultrasound. In this review will go through all of them.

3.1. Magnetic field

The magnetic field (MF) can be defined as a property or perturbation of space produced by the movement of electric charges or by the presence of electric currents. The intensity of the magnetic field depends proportionally on the intensity of the electric current. Mathematically, the magnetic field can be defined in terms of the amount of force exerted on a moving charge. If applying the Lorentz's law of force, we can express the force according to this equation F = qvB, where F is the magnetic force, q is the charge, v is the velocity, and B is the magnetic field. Magnetic fields could be classified in relation to the current from which it was generated into a **static magnetic field (SMF)**, if generated by a steady current, or into **alternating magnetic field (AMF)**, if generated by an alternating current.

In clinics, MF is hampered by a minimal interaction with ions, making the MF a non-invasive stimulus, with deep tissue penetration depth, and being able to be applied with high spatial-resolution (in the order of cm-mm). Albeit MF is generally considered safe, and its intensity and frequency do not have any direct influence on living organs, biological media could generate eddy currents due to the presence of ions in the media. This leads to heat generation on the fluids at the site of MF application and subsequent damages on the surrounding healthy tissues. Thus, frequency and amplitude of the magnetic field must be maintained below certain values to avoid this unspecific heating. First limits were set in 1984 by Atkinson and Brezovich that studied the tolerance to an MF, defining that the product of field frequency and field intensity shall not exceed 4.5×10^8 Am^{-1} s^{-1}[66]. Later, several other limits have been established ranging from 1.8×10^9 to 5×10^9 Am^{-1} s^{-1} [67–69].Taking in consideration this limitation, the use of magnetic fields in medicine has been extensively exploited in cancer detection [70], magnetic resonance imaging

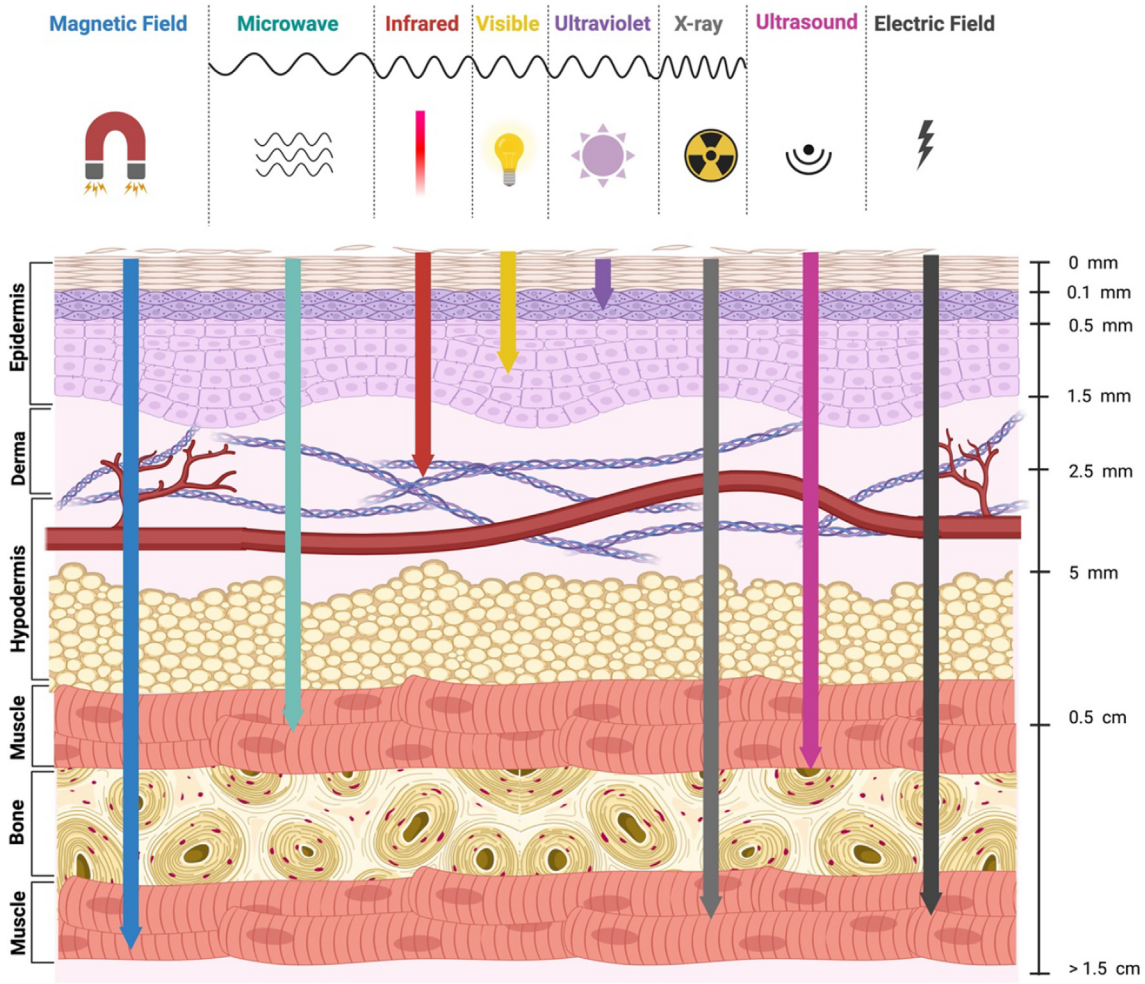

Fig. 3. Schematic illustration of the human tissue penetration depth of different external physical stimulus. Light in the UV–VIS range can reach only the superficial layer of the skin (epidermidis) while IR irradiation extents its effect to the derma. Microwave and ultrasound stimuli penetrate to the hypodermis and the muscles. Magnetic field and electric field and X-rays, instead, can exert their effects on deep tissues, such as the bone tissue. Created with BioRender.com.

[71], cardiovascular and neurological treatment [72,73], hyperthermia and thermoablation [74].

Generally, SMF is used to drive and concentrate the drug of interest in a specific tissue or organ exerting an attracting force onto magnetic nanomaterials without triggering any drug release. On the other hand, the medical application of AMF relies on the heating production to induce the cargo release. According to the frequency applied, AMF can be further divided into **low frequency MF** (LF-MF) or **high frequency magnetic field** (HF-AMF). In the first case, frequencies applied ranges between 1 Hz and 100 kHz, corresponding to a $H \times f < 4.85 \times 10^8$ A m^{-1} s^{-1}. Its use is preferred for delivery of bioactive molecules (antibiotics, enzymes, DNA), as less heat is generated by the application of LF-AMF, which minimize molecules undergoing structural damages. Indeed, changes on the permeability of drug delivery systems triggered by this stimulus are usually caused by an enhancement of MNP vibration. Magnetic fields in the range of 100 kHz to 300 GHz are referred as (HF-AMF), being currently considered as safe for clinical application as long as $H \times f$ does not exceed the above-mentioned limitations. Although these frequencies are applied in magnetic hyperthermia to promote dead in malignant tumoral cells induced by the local heating generated by the MNPs, they can be exploited to induce either changes in the carrier permeability or structure, or to weaken the drug-carrier interaction thus accelerating the diffusion of the loaded molecules. Unfortunately, there is evidence that triggering drug release with HF-AMF could damage the drug payload due to excessive heat generation, thus reducing the effectiveness of these stimulus for drug release. In this sense, **pulsed electromagnetic fields** (PEMFs) have been explored to combine the use of this stimulus with nanocarriers containing MNPs as an alternative and elegant solution for controlled drug delivery without loss of drug integrity. The use of PEMFs has the advantage that they are already employed in therapy while LF-AMF are mainly used in laboratory conditions and rarely applied in the clinic. The direct application of PEMFs on a target tissue is currently employed in clinic for the treatment of inflammation, bone fracture, arthritis, and in regenerative medicine [75,76].

To achieve spatio-temporal control, MF is mainly used as an extrinsic source to activate magnetic nanomaterials as hotspots to trigger drug release from the nanocarrier through different mechanisms: i) permeabilization of liposome bilayer ii) solubility/-conformational changes of thermosensitive polymers, and iii) cleavage of thermolabile bonds. The first approach relies on the use of magnetoliposomes, which consist of magnetic nanoparticles embedded in liposomes with a wide application in bio-imaging, cell signaling and drug delivery. As already explained, liposomes possess a high versatility for spatial–temporal drug delivery as they can carry both hydrophilic cargos, in the aqueous core, and hydrophobic molecules in the lipid membrane. Besides, their permeability can be tuned by their lipidic composition as it influences

the melting temperature (Tm) of the lipidic membrane being thus the payload released when heated above the Tm. Thus, the use of MF to activate MNPs as nanoheaters allows not only achieving spatio-temporal control of the drug release but also can reduce the drug leakage into the systemic circulation by the design of liposomes with higher Tm [77]. The second strategy is based on magnetic nanoparticles embedded in polymeric materials. Depending on the physicochemical properties of the organic matrix, the localized magnetic heating generated by the MNPs triggered by MF application could either degrade the matrix or induce a change in its porous structure thus releasing the embedded drug. To achieve the last strategy, different systems based on the breaking of thermo-labile Diels – Alder bonds or aliphatic azo linkers have been reported for triggering drug release [78]. As these bonds are stable at body temperature, the fact that their rupture is only possible at the high local temperatures reached by magnetic heating ensures that there is no drug leakage during systemic circulation of the nanocarrier. In the following paragraphs, we are going to elucidate examples of these three mechanisms in relation of the different types of magnetic field applied as external stimuli for on-demand drug delivery.

3.1.1. Static magnetic field (SMF)

As previously stated, the main medical application of SMF is to concentrate in a target tissue or organ the drug immobilized on magnetic material. One of the multiple examples reporting this application, consisted of driving a chemotherapeutic agent (BCNU®) immobilized onto MNPs to target a malignant glioma. The achieved magnetic driven vectorization of the intracranial injected NPs allowed to use lower concentrations of bound-BCNU, providing a more efficient tumor suppression than the observed when using free-BCNU [79].

However, the use of SMF to gain control over drug release has been much less explored. It has been mainly used to induce changes in the porosity of polymeric materials (nanohydrogels or nanospheres) containing magnetic nanoparticles thanks to their attractive force to these magnetic materials. For hydrogels, a wide number of publications had proven that the magnetoelastic properties of these gels could be used to control the release of both hydrophilic and hydrophobic drugs. Indeed, it is possible to found examples of the use of ferrogels (hydrogels containing magnetic nanoparticles) that are always in solid form or even injectable ones that turn solid at the human-body temperature after direct injection at the targeted site of action. Injectable ferrogels, such as those composed of Pluronic as gelling agent reported by Quin *et al*, are of great interest for clinical use as their direct injection at the pathological site avoids rising the tissue irritation and trauma caused by surgical insertion needed in the case of solid ferrogels [80]. These ferrogels were one of the first examples showing that the release rate of a drug from the hydrogel can be tuned and speed up with an external magnetic field (SMF of 300 mT). Several parameters could be tuned to modulate the drug release from ferrogels including composition, percentage and/or crosslinking of the gelling material, concentration of the embedded MNPs and the intensity of the SMF applied. In this sense, Cezar *et al* showed that the lowest is the amount of MNPs embedded within alginate hydrogels, the highest is the diffusion enhancement of the antineoplastic agent mitoxantrone under a SMF of 0.05 mT, whose application leads to a reversible deformation of the macro-porous structure of the system [81]. Another example of using a ferrogel to protects the drug from the environment and allows a sustained drug release under a magnetic field showed the encapsulation of Levodopa, a drug of relevance for Parkinson disease, that has a poor pharmacokinetic profile, and it is easily metabolized before reaching the brain [82]. Its encapsulation in a hydrogel containing MNPs stimulated the sustained release of the 64 % of the loaded Levodopa in 30 h by application of a SMF of 0.4 mT [83]. Although in these examples the application of a SMF increases drug release, there are also examples that report the opposite effect that consist in hindering the release of an entrapped compound by reducing the dimensions of the pores in the hydrogel network. This was the case when a SMF of 0.5 T was applied on a polysaccharide-based hydrogel composed of carboxymethyl cellulose and hyaluronic acid in which aminated $CoFe_2O_4$ were covalently attached to the gel fibers [84]. The same behaviour was also reported when a SMF of 3.5 mT was applied to magnetic nanoparticles encapsulated in alginate beads to achieve the on-demand release of the drug Berberine. Berberine is a drug that can be used for the treatment of different diseases, including cancer, metabolic and neurological diseases [85]. Despite its versatility, its application even at sub-acute concentration can damage the immune cells and cause gastric and hepatic side effects [86]. Thus, loading it into alginate beads reduces the toxicity of the molecule and, incorporating the MNPs leads to an on-demand drug release that not only minimize drug release during delivery process but also to a sustain release ensuring drug levels within the targeted therapeutic range for long time periods [87]. Considering these examples, the application of a SMF appears as an effective strategy to achieve controlled release of different biomolecules in time and space, thus prolonging their therapeutic effect while reducing the number of administrations. Unfortunately, to reach clinical application of these system, more *in vitro* and *in vivo* research must be still carried on confirming the feasibility of this stimulus for an effective drug delivery.

3.1.2. Low frequency magnetic field (LF-MF)

An elegant proof of the LF-MF as inductor of the drug release was proposed by Luo and colleagues [88], integrating a layer of magnetic nanoparticles into microcapsules and doxycycline as drug model. This derivative of the antibiotic tetracycline has been selected as its release can be easily monitored by the expression of a fluorescent protein (EGFP) that is turned on in the presence tetracycline or derivatives. Although the application of a magnetic field of 50 Hz has little effect on morphology of microcapsules, their permeability was significantly increased as a function of the application time, enhancing EGFP expression promoted by doxycycline triggered release. Indeed, the expression of the fluorescent protein proved that no damage occurred to the antibiotic whose structure remained intact and was able to activate the tetracyclin-On regulated gene expression system. Hence, authors demonstrated that by attracting these magnetic microcapsules with a magnet (SMF) to the site of interest, their internalization is favored and then the intracellular release of the antibiotic upon application of a LF-AMF was spatially confined to the targeted site while non-targeted sites remained unaffected. The increase in EGFP expression after applying LF-AMF, showed that the cargo is not damaged during its release, besides cell-toxicity was not observed during its application. Thus, this study showed the possibility to combine SMF with LF-AMF for targeted drug delivery and non-cytotoxic intracellular trigger of drug release.

In addition to magnetic microcapsules, the sustained released controlled by LF-AMF exposition (60 A/m and 96 kHz) was also achieved using liposomes where MNPs and the chemotherapy agent Camptosar® (CPT-11) was co-encapsulated [53]. Although this drug is one of the most tolerated ones for brain tumor treatment, its low selectivity over cancer cells give arise to chemical resistance. Thus, the functionalization of these magneto-liposomes with the antibody Cetuximab for recognizing over-expressed epidermal growth factor receptors on cancer cell surface, allowed triggering the release if CPT-11 was exposed to an AMF after their selective endocytosis by glioblastoma cells both *in vitro* and *in vivo*. The effect of the exposure time, frequency, and amount of MNPs loaded on LF-AMF drug release efficiency

was studied using magnetoliposomes loaded with carboxyfluorescein (CF) as model of hydrophilic drug and with hydrophobic magnetic nanoparticles physisorbed on the lipid bilayer [89]. Magnetoliposome response to the oscillating magnetic field resulted effective achieving a higher CF leakage when using long LF-AMF exposure time, high field frequency and high MNP concentration. Besides, a long lag time during which the drug release is very low was observed on those samples exposed to LF-AMF for 50 min at 5.2 kHz. The combined use of several physicochemical characterization techniques suggested that the slow release of CF at the first hours is due to the formation of local pores or defects at the lipidic bilayer, while the fast release that took place after 8 h of LF-AMF application can be related to a structural change. These results provided evidence that drug release triggered by LF-AMF could occur much faster and with higher efficiency through the modification of the membrane state rather than through the bilayer rupture. Besides, the observed lag time after LF-AMF application before the effective drug release would allow, for magnetoliposomes, to reach the target site without unspecific drug leakage during the transport but ensuring the drug release at the target site. Magnetic hydrogels, as the ones used for controlling drug release using static magnetic fields, could also be used for the application of LF-AMF. Indeed, Uva and co-workers reached the increase in the release of entrapped molecules when applying an LF-AMF with the same carboxymethylcellulose (CMC)-based ferrogels with which they managed to reduce the release of drugs by applying an SMF [84]. Indeed, they showed that the sequential application of alternate cycles of SMF and LF-AMF allow to reach an efficient sustained remotely controlled drug release for long time periods. However, an effect on drug release is not always observed when applying AMF as well as SMF. When using aminated Fe_2O_4 MNPs as crosslinkers of CMC hydrogels instead of Co-doped ones, the same authors reached a significant increase in the release of doxorubicin (DOX) when applying the same LF-AMF conditions while the application of a SMF did not influence its release. This could be related to aggregation issues during the hydrogel preparation as it was showed by electron microscopy analysis of the gel structure [90].

Although low frequency alternating magnetic field appears to be a promising therapy for cancer treatment, nanoformulations to control drug release with this remote stimulus must still be improved in stability to avoid unspecific drug release and must be carefully designed for intravenous administration. Besides, bulky, heavy, and often expensive coils or magnets are needed to generate the required magnetic-field strengths for triggering this stimulus, which limits the portability of this remotely controlled drug delivery strategy.

3.1.2.1. High frequency magnetic field. Different HF-AMF based on-demand drug delivery systems were developed over the years, involving the careful design of hybrid materials based on the use of hollow capsules, such as liposomes, or polymeric structures. In the following paragraphs, we highlight some of the most common and recent strategies for drug release induced by HF-AMF.

This is a stimulus widely explored to promote local heating within liposomes encapsulating MNPs either in the membrane or inside the water pool using them as carriers for magnetic-controlled delivery of drugs. Shaghasemi and co-workers demonstrated using magneto liposomes with different membrane melting temperatures (Tm) that drug release can be precisely magneto-thermally controlled from stealth liposomes with high Tm by applying HF-AMF [91]. The authors obtained liposomes with homogeneously dispersed superparamagnetic iron oxide nanoparticles in the membrane interior but with different lipid composition and thus Tm:1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Tm = − 2 °C), 1-myristoyl-2-palmitoyl-*sn*-glycero-3- phosphocholine (MPPC, Tm = 35 °C), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Tm = 41 °C) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC, Tm = 55 °C). AMF-triggered release assays showed that at a very low Tm (-2 °C), in which the carrier is already in the liquid phase, no drug release is observed once AMF is applied. On the contrary, with liposomes with higher Tm (41–55 °C) the accumulated release of the calcein, a self-quenching impermeable dye used as drug model, was of 90 % following the application of 3 consecutive AMF cycles. In addition to the liposome Tm, the size and functionalization of the magnetic nanoparticles used are extremely important parameters regarding drug release and are closely related to their position once integrated within the liposome structure. In fact, while there are no size limitations for incorporation into the liquid core, Amstad and colleagues report that there is a limitation of 5 nm in diameter for the incorporation of single hydrophobic NPs into the lipid layer [77]. The use of larger nanoparticles (10 nm) lead to the formation of large micellar structures, integration of magnetic clusters and reduced stability of the formed structures. It is interesting to note that the localization of the MNPs greatly influences the release of the drug, in fact, a more marked release is generally observed when they are localized within the lipid bilayer. Release yields rarely exceed 20 % when the magnetic material is incorporated within the aqueous core, while yields of up to 40 % can be observed after a single HF-AMF magnetic field pulse when these are incorporated within the lipid layer [77,91]. When MNPs are localized in the lumen, the bulk water needs to be heated strongly until reaching temperatures close to the lipid bilayeŕs Tm in order to trigger the release of cargo. This requirement not only significantly reduces release efficiency but also prevents the release of thermally sensitive cargo (chemicals, drugs, protein) as its thermal degradation and loss of functionality could thus occur while released [77]. In contrast, when MNPs are embedded in the lipid bilayer, the release of the drug can be attributed to locally dissipation of the magnetically induced heat leading to the localized achievement of Tm and the consequent increase in membrane permeability without the need for bulk water heating [77]. Several experiments exclude that the effect is related to liposome rupture, as DLS and morphological studies before and after AMF demonstrate an unmodified size of those samples subjected to AMF. In fact, a break would lead to the formation of large micelles and the exposure of small nanoparticles in the released medium. Indeed, because the liposome structure remained intact during AMF treatment, the repeatedly application of AMF-cycles allowed to control the dose and the release profile of the cargo over prolonged times at bulk temperatures close to the body temperature which is significantly below to the liposomeś Tm.

Although the controlled release of drugs from magnetoliposomes due to a local or nanoscale heating mechanism triggered by HF-AMF has clearly been demonstrated, the main challenge in this area is the development of synthetic strategies for the facile incorporation of the NPs without compromising liposome membrane integrity and avoiding the leakage of the cargo. In this sense, Fortes Brollo and co-workers reported in 2020 an innovative synthetic methodology combining magnetoliposome formation and drug loading in one step. Besides, they were able to control the location of the MNPs within the liposome structure and thus perform a comprehensive study on the effect of magnetic nanoparticle size and surface coating on the HF-AMF induction of DOX release [92]. They study the consequence on liposome structure and properties of tunning the size and spatial distribution of MNPs by changing their coating from positive (amino-propyl silane-APS coating), negative (Dimercapto succinic acid-DMSA coating) or hydrophobic (oleic acid coating). Only those MNPs negatively charged did not reduce the melting transition temperature of the liposome while maintaining good magnetic properties. Indeed,

these nanoparticles located only on the outer surface of the liposomes without altering neither their cargo capacity or the lipidic bilayer stability. However, these properties do instead change in the case when either positive or hydrophobic NPs are integrated within the liposome structure. Therefore, those liposomes coated with negative MNPs were selected to conduct *in vitro* drug release studies targeting MAA-MB-231 breast cancer cells. Cell survival was reduced up to 17 % (70 % reduction) after applying AMF (202 kHz and 30 mT) due to the achieved on-demand enhanced release of DOX.

Thermosensitive polymers were also used to design on-demand delivery systems triggered by HF-AMF. Injectable hydrogels integrating magnetic nanoparticles in the matrix network received great attention. A general advantage of injectable composites is that their use avoids invasive surgical procedures as they are applied directly to the desired location *in vivo* by extruding the liquid hydrogel components from a syringe. Furthermore, proteins, cells, drugs and nanoparticles can be easily integrated in the formulation by mixing them into the component solutions before injection as they become encapsulated by ionic interactions or physically entrapment within the hydrogel matrix while the in-situ gelation process occurs [93,94]. The integration of MNPs generally result in composite hydrogels with improved mechanical properties and increases biomolecule adsorption due to their large surface area. Besides, MNPs confers elastomer-like mechanical properties as reported by Campbell and colleagues when they are covalently integrated while acting as chemical crosslinkers of the hydrogel network structure [95]. The high volumetric concentration of magnetic nanoparticles in these injectable composites together with the fact that MNPs act as heat mediator source under the application of AMF, makes these materials of particular interest for externally mediated drug release. In this sense, Xie and coworkers designed a dual-drug-loaded chitosan injectable magnetic hydrogel for the synergistic chemotherapy of triple negative breast cancer by triggering the controlled co-delivery of DOX and docetaxel (DTX) [96]. It is well known that the synergistic combination of two or more chemotherapeutic drugs with different toxicity profiles and mechanisms of action could increase tumour regression by reducing the chances of developing multidrug resistance. Although previous studies reported the co-delivery of the two antitumor drugs, this article was the first to achieve their synchronous delivery and asynchronous release controlled remotely by HF-AMF. For this aim, magnetic nanoparticles were integrated in the injectable and self-healing chitosan gel as covalent crosslinkers of the matrix network while DTX-containing poly(lactic-co-glycolic acid) nanoparticles (PLGA-NPs) were embedded together with DOX during the gelation process. The application of AMF (282 kHz and 19.99 kA/m) for only 10 min has remarkable effects on DTX release but not on DOX release. This suggested that the global high increase in temperature of the microenvironment within the gel matrix triggered by magnetic heating does not involve a loss of the whole three-dimensional structure of the gel but only of the PLGA NPs structure. This asynchronous control in the AMF-triggered release of DOX and DTX when co-entrapped within these magnetic hydrogels make it possible to adjust the chemotherapy process and thus significant enhanced antitumoral activity *in vivo* on BALB/c mice with a synergistic effect on tumour growth. The use of different design strategies for obtaining these thermosensitive polymer/MNPs nanohybrids allows achieving very different drug release profiles [97]. This was clearly shown by the differential doxorubicin release curves obtained from two different hybrid nanocarriers even though the amount of drug loaded per mass of nanohybrid was similar in both cases. Indeed, it was shown that it is possible to increase on-demand the rate of release of the loaded drug by triggering conformation changes on a thermosensitive oligo (ethylene glycol) methyl ether methacrylate-based nanogel, where MNPs are entrapped by complexation with the carboxylic acid groups of the polymer matrix (DOX-MagNanoGels). However, when DOX is entrapped within molecularly imprinted polymers grown from the surface of individual iron oxide nanoparticles (DOX-MagMIPs), the passive release of the drug is drastically reduced thus allowing its on-demand release caused by hydrogen bond disruption when applying AMF, although of smaller amounts of doxorubicin. The differential drug release profiles of both nanocarriers had also differential effect on cell viability when AMF-triggered DOX release was tested in cancer cells. In both cases cell viability was significant lowered after AMF application, however it was reduced from 54 % to 30 % when AMF was applied to DOX-MagNanoGels while from 88 % to 60 % when applied to DOX-MagMIPs. These results confirmed that the passive release of DOX is drastically reduced in MagMIPs, and that although DOX is continuously released from MagNanoGels its rate of delivery could be significantly increased by AMF application.

Although very promising results have been reported, passive diffusion induces often unspecific leak of entrapped drugs. Thus, in addition to triggering or affecting the kinetics of release of entrapped drugs, the local heat profile triggered at the vicinity of iron oxide cores had gained interest for the remote control of prodrug delivery systems. These systems have been designed to ensure the chemical transformation of drugs into prodrugs to transport a biologically inactive derivative of the drug during its biodistribution, which can be specifically activated and delivered to its target tissue. Ideally, the prodrug should convert to the active drug as soon as the target is reached allowing for a specific temporal and spatial control of the release, and thus overcoming the lack of specificity of conventional chemotherapy [58,98]. Generally, three components can be identified in a prodrug delivery system: the drug, a cleavable linker, and a targeting molecule or nanoparticle. The choice of the linker play an important role for the release of the drug of interest. Indeed, it could be designed to be enzymatically/chemically cleavable so the active drug can be released under triggerable conditions cause by a stimulus or multistimulus either endogenous (hypoxia, redox environment, overexpressed enzyme, change in pH) [99–101] or exogenous (light, ultrasound, or magnetic fields remote application) [102–104]. External activatable prodrug-based nanocarriers have many advantages over internal stimuli owing to their potential to exert a precise control of the time and location of treatment [105]. Indeed, after the stimuli application, nano-systems undergoes a rupture of the linker-drug bond or the opening of a gatekeeper leading in both cases to the release of the drug. A smart example of the application of this therapy has been proposed by Lin and colleagues in 2021, by creating a superparamagnetic iron oxide nanoparticle (SPIONs) loaded mesoporous silica nanoparticles (MSNs) of 120 nm in which an esterase was encapsulated together with an anticancer peptide in their enlarged pore spaces [104]. The peptide was covalently conjugated via an ester linker to the pore wall and separated by a thermosensitive oligomer-based barrier from the esterase. This avoids its premature off-target release as the peptide remains inactive in the absence of HF-AMF stimulation. The removal of the separating barrier is triggered by the AMF-induced nanoscale heating from the SPIONs core because of the cleavage of the temperature labile C—N bonds of the azo moieties within the separating barrier. This allows the direct contact of the trapped esterase with the ester-linked peptide, leading to the ester bond cleavage and the consequent release of the peptide into the media. Remarkably, the higher is the frequency of the magnetic field applied, the greater is the peptide release. This was also assessed by the co-incubation of the obtained AMF-responsive nanocarriers and PANC-1 cells, were an extensive cell death (>90 %) was observed only after 30 min of AMF treatment. They also reported excellent biocompatibility and high tumour-

targeting efficiency of the nanocapsules, reaching an intensified anticancer *in vivo* efficiency by triggering moderate AMF-heating. The development of these carriers that respond to moderate heating allows to overcome the existing challenge in the field of damaging the cargo by AMF-induced overheating.

Another strategy explored to avoid overheating while ensuring pulsatile drug delivery over long time periods is to apply AMF not continuously but in pulses. As an example, Campbell and colleagues have designed an injectable, degradable in situ- gelling hydrogel nanocomposite material in which MNPS were covalently bound into the hydrogel network structure [95]. Although the resulting formulation was able to deliver pulsatile releases of the local anesthetic bupivacaine upon after 10 min AMF pulsed applications, the amount of drug released was too small and too short lived for an effective on-demand drug delivery therapy. Thus, the authors incorporated thermosensitive microgels containing the drug of interest inside the magnetic hydrogel previously developed [106]. The heat generated by MNPs while AMF is applied raises the local temperature above the microgels volume phase transition temperature (VPTT) triggering their deswelling and thus generating free volume in the hydrogel to enhance drug release. The microgels reswell refilling the hydrogels pores once the AMF is removed. This improvement on the hybrid composite design allowed them to improve the on-demand drug release regulation via pulsated HF-AMF application.

3.1.2.2. Pulsed electromagnetic fields. An example showing the feasibility of using PEMFs as stimulus to trigger magnetic-responsive controlled drug delivery involved the use of magnetoliposomes with high melting temperature (Tm = 52 °C) [107]. The MNPs were integrated either at the liposomal surface or by internalization inside the vesicles as individual entities or MNPs aggregates. Unlike when applying HF-AMF in which the increased permeability of the lipid bilayer is a consequence of promoting local heating within lipid vesicles encapsulating MNPs either in the membrane or inside the water pool, when applying PEMFs the increase in the bilayer permeability was shown to be triggered by mechanical actuation of the MNPs based on their vibration or rotation. Indeed, it has been observed a 20 % of release of the hydrophilic model drug used (carboxyfluorescein) after 3 h exposure without affecting the liposomes integrity. These results were among the first to prove that PEMFs could be an effective remote trigger and that high-Tm magnetoliposomes could respond to this stimulus at a temperature well below the main transition temperature of the liposomés bilayer. The mechanical motion of the magnetic nanoparticles locally destabilizes the lipid bilayer and causes its collapse and the subsequent release of the liposomes' payload. Similar results have been obtained in a study in which MNPs were functionalized to ensure the obtention of magnetoliposomes samples with a differential location of the MNPs either at the lipidic bilayer or at the lumen of the magnetoliposomes [108].

The use of pulsed electromagnetic fields still needs numerous fine-tuning before it can be considered an efficient on-demand drug delivery method, but it has significant assets that make it a promising stimulus for future clinical applications.

3.2. Light

Light, either UV, Visible (Vis) or NIR, is an ideal source of energy to be applied in DDS [109]. The main advantages of using light as a stimulus includes its simplicity, non-invasiveness, and amenability of modifications in wavelength, exposure time, beam diameter and intensity. Thus, it provides and easy control over the quantity, timing, and location of the drug release [110].

Light in the UV and Visible ranges, due to their poor penetration and absorption by tissues, is mostly suited for topical applications. On the other hand, light located at the NIR region of the electromagnetic spectrum has a deeper penetration ability in tissues and other biological structures, making it less harmful. This makes the NIR range a very interesting option in biomedicine [111].

The application of light as a stimulus for drug delivery has been a predominant field of research in the last decade [112]. The main reason for this is the fact that light can remotely trigger many physical, chemical, and biochemical processes without resorting to invasive approaches.

It must be acknowledged that light can play several roles in photonic nanomaterials: a) activation or use of the physicochemical properties of nanoparticles to **induce changes in the nanoplatform**, such as the heat generation used for the release of a heat-sensitive link that induces the release of a drug, or the activation of an enzyme to catalyze a prodrug into the active product; b) **affecting the surroundings** where the nanomaterials are located, facilitating the internalization of the nanoparticle by photoporation of the cell membrane or by altering the extracellular matrix; c) effectively **treating the target tissue, directly or indirectly** applying the physical properties of the materials to induce a change in the target, as for instance in synergistic phototherapy or photodynamic therapy. The photo-thermal triggering mechanism consists of the irradiation of the surface of a nanoparticle. This produces a localized surface plasmon resonance (SPR) in which the conductive free electrons collectively resonate in response to the incoming electromagnetic radiation. These results in amplified light absorption as well as scattering. Photonic materials have the ability to transfer this absorbed energy into heat with a high efficiency. Some common strategies of photo-induced drug delivery implicate chemical or thermal triggering mechanisms. Photo-chemical therapy mainly involves three different mechanisms: 1) the light is used to **irradiate the photo-responsive nanocarriers** inducing either the selective local heating for healing abnormal cells/tissues or changes in anchored drugs causing their delivery (e.g., by isomerization, oxidation) (aka. photothermal therapy, PTT) [113,114]; 2) **photocatalytic prodrug activation** into therapeutic drugs (photochemotherapy); and 3) the use of a **photosensitizer** (PS), which is a photoactivatable molecule or system, and molecular oxygen (O_2) mostly present in the target tissues (aka. photodynamic therapy, PDT) [115]. PDT implies that, when the photosensitizer is irradiated by a laser, it generates cytotoxic species as singlet molecular oxygen ($^1O_2{}^*$) and ROS. This is caused by a photodynamic process involving energy transfer from the PS in the triplet excited state to the surrounding molecular oxygen. Both $^1O_2$ and ROS are very reactive molecules, with a short half-life, taking effect only in the site of application. This oxidative stress generated is thus confined to the desired affected area, preserving the adjacent healthy tissue [116].

For the sake of simplicity, this review groups the different light applications into broad regions of the electromagnetic spectrum: visible, UV-light and Near-Infrared spectrum regions. These regions are commonly used in separated applications due to their different effects in biological structures. The **visible light** comprises the electromagnetic radiation in the range of 400–700 nm, corresponding to the spectrum which is visible to the human eye. Visible light allows the possibility of use gold nanoparticles for photodynamic, photothermal and photochemotherapies due to the surface plasmon resonance excitation caused by the light irradiation. **Ultraviolet light** comprises of wavelengths between 200 nm and 400 nm and can be divided into three main subcategories: UVA (320–400 nm), UVB (280–320 nm) and UVC (200–280 nm). This light is considered suitable for a limited number of therapeutic applications due to its low penetration capability. However, there are several approaches explored in the literature, from the structural collapse of the nanoplatform to photocatalytic

properties. It also shows a great potential for cosmetics and agriculture, where sunlight exposure can serve as trigger of the release. **Near-Infrared light** lies between ~ 700 nm and ~ 1,300 nm. It is one of the most promising types of light to be applied in therapy in general, and one of the popular ones regarding controlled drug release. The main reason is the low-absorptivity that biological structures present in certain ranges of this region (the so-called "biological windows", more in the following sections).

All these applications do not necessarily correspond to on demand drug delivery per se. Due to the myriad of studies that are constantly been published, trying to cover every study in photonic nanomaterials applied to therapy would be an exercise in futility. For that reason, the studies that use photonic nanomaterials were selected depending on whether the effect of the light stimulus was directly involved in the proper delivery and/or release of the therapeutic agent. Those studies that use photonic materials as synergistic agents alone, without a light-mediated activation of the therapeutic effect, where not reported in this review. However, comprehensive, and extensive reviews on the broader field of multimodality for imaging and combination therapy can be found elsewhere [117–120].

3.2.1. Visible spectrum

Several combinations of gold nanoparticles and carriers have been reported in the last years, exploiting different mechanism of drug release.Niikura K. *et al.* reported a study where water-dispersible gold nanoparticle vesicles (AuNVs), with an inner hollow structure, were used to encapsulate and release drugs [121]. Plasmonic gold nanoparticles were used because the ease to functionalize by thiol-modified biomolecules but also, because they have the possibility to generate heat after being then irradiated by using single wavelength light. In this work, the heat generated after light irradiation caused a fast and efficient drug delivery compared with a release performed with no irradiation. Light-triggered release of doxorubicin (DOX) were tested using *in vitro* model with HeLa cells. The results showed that only 5 min of irradiation by a diode laser (532 nm, 250 mW) of the cell culture medium was necessary to markedly increased the ratio of dead cells compared with cell culture media treated without laser irradiation, where DOX-AuNV treatment did not affect the cell viability. This reaffirmed the promising possibility of using AuNVs as a fast drug delivery carrier in combination with single wavelength optical fiber to perform localized therapies. The optical fiber helps the light penetration because it has a tissue poor penetration by herself and without it the technique could be limited. Similarly, Wang P. *et al* reported the development of a fashion multifunctional vehicle, based on a complex system formed by lipids and AuNPs to deliver Cas9-sgPlk-1 plasmid (LACP) to suppress Plk-1 gene expression in tumor therapy [122]. Lipid formulations were part of the selected agents to carry and delivery CRISPR/Cas9 system mainly due to the high cell loading efficiency and TAT peptide was also part of the formulation to ensure cell nucleus targeting [123]. The controlled heat generated (controlling the size of AuNPs and the laser irradiation conditions) by the localized surface plasmon resonance (LSPR) of gold nanoparticles was used by them only to trigger the release the therapeutic agent [124,125]. The photothermal effects of the formulation designed were assessed on melanoma induced mice by intratumoral injection of LACP containing green fluorescent protein (GFP)-fused CP, after 20 min of laser irradiation, 514 nm 24 $mWcm^{-2}$, the temperature of the skin above the tumor increased to 41.4 °C. This ensured that the therapeutic effect was related only to thermo-triggered release of CP and not to the photothermal therapy. GFP expression verified the success of CP transfection whereas cell apoptosis can indicate successful targeted gene (Plk-1) editing. Cytotoxicity studies and also real-time tracking assay ensured the apoptosis mechanism and also cellular uptake to perform gene editing. This complete and smart study opened the possibility to use easily synthetized nano-systems to further treatment of many diseases where a gene therapy was necessary, as for instance many types of cancer cystic fibrosis, heart diseases, diabetes, hemophilia and AIDS. Croissant *et al* reported the synthesis and successful use of nanovalves on MSNs as on-demand remotely controlled drug delivery system [126]. Gold nanoparticles were in this case embedded in the mesoporous silica matrix, Au@MSN@-Valve, allowed the triggering by light irradiation through a photothermal mechanism involving the plasmonic properties of a gold nanoparticle and the valve properties, which remain closed at physiological temperature and opened when the temperature increase. The irradiation of nanovalve Au@MSNs at wavelengths corresponding to the plasmon resonance of the gold core caused internal heating and the opening of the nanovalves, as the heat consequence, allowing the contents of the pores to escape. Rhodamine B was used as a model molecule to study the cargo loading in the mesoporous structure. The release of cargo molecules was monitored by using a probe diode laser (448 nm, 18 mW) to irradiate the upper part of the cuvette exciting the released dye (Rhodamine B) and a CCD detector was used to measure the fluorescence of the dye that escaped from the pores. The local temperature, rather than an increase of the temperature of the bulk solvent, was monitored during the irradiation to ensure that this one was responsible for the cargo release. Was not observed any temperature change during the irradiation time, and this proved that the heat necessary for uncapping the pore and releasing the cargo was only provided by the localized heat generated by the nanoparticles. This research group demonstrated the use of a novel Au@MSN nanomachines to control the release of cargo molecules at a desired time in a specified spatial location by using visible light' irradiation without premature leaking. These preliminary studies showed the possible potential of their DDS to, such as, kill selectively cancerous cells reducing the unwanted cytotoxicity of drugs in healthy cells but also using an effective hyperthermia.

Yin *et al* presented in this case the synthesis and characterization of another different king of gold nanoparticles, gold nanorods (AuNRs), with the capability to co-deliver anticancer drugs (DOX) and small interfering RNA (siRNA, against a G12D mutant K-Ras gene) [127]. Gold nanorods attracted the attention to be used as the triggered DDS mainly due to their high biocompatibility their optical properties such as localized surface plasmon resonance (SPR) and their photothermal effect [128,129], which are the properties used to perform de delivery. The use of synergistic combination of two biomolecules to destroy tumor cells and reduce the relapse of cancers were, already, studied and proved [130,131] but they improved this promising possibility with an on-demand drugs delivery upon irradiation with 665 nm light, which reduced the side-effect and increased the bioavailability. Panc-1 cells (CRL-1469) were treated with AuNRs nanoplex, fluorescence images corroborated the efficient internalization. Total RNA and cellular protein studies also probed the K-Ras inactivation by the siRNA. Once checked the possibility to co-delivery of DOX and siRNA by using an *in vitro* model cellular line, the prepared nanoformulation were also tested in animal models, tumor-bearing mice. Mice treated with AuNRs/DOX/K-Ras siRNA showed wide and clear tumor reduction. This mix of experiments demonstrated the promising, even in animals' model, delivery of two biomolecules with a synergetic effect and on demand activation by a single wavelength light.

Xu and coworkers published another visible-light-triggered drug delivery platform considering too plasmonic properties of gold nanoparticles (AuNPs) [132]. In this work the drug delivery platform was based on TiO_2 nanotubes arrays (TiNTs). The TiNTs-based platform was formed by two parts: one hydrophobic part containing AuNPs that acts as a cap in the top of the platform and the lower hydrophilic part that serves as drug storage.

The light irradiation caused in this case rather than an increasing in temperature a photocatalytic activation of AuNP-decorated TiNTs. The formation of $O_2^{\bullet}$, H_2O_2 and/or $OH^{\bullet}$ which could diffuse through the electrolyte caused the linker break at the lower part of the tubes and consequently the desired drug release. They proposed this platform as a powerful tool to perform a controllable antibacterial release under visible light. For that, the antibiotic Ampicillin (AMP) was linked to the structure and treated then under visible-light (xenon light source with a filter of $\lambda > 420$ nm, illumination intensity 50 $mWcm^{-2}$). The hydrophobic Au caps caused retention of the AMP loaded within the tubes in the dark, but after removing the upper cap, by visible light irradiation, the drug molecules covalently attached were released in a slow and controlled way. The release of AMP was also carried out in a bacterial culture test. *Escherichia coli* was used as a model target microorganism. Empty TiNTs platforms were compared with AMP covalently loaded TiNTs, obtaining a high bactericidal efficiency in the loaded platforms compared with a low efficiency for the non-loaded ones. Was concluded that this interesting platform could be used as a system to on-demand release, due to the chain scission caused by the gold nanoparticles, of drugs in its fully functional form, without photoinduced degradation.

In another different way Gandioso *et al* reported the use of a photoactivable pro-drug based in a metal complex where a targeted peptide vector was introduced [111]. The targeted peptide improved the pharmacological properties of the photoactivable metallodrugs such as aqueous solubility and cell uptake, as well as higher selectivity against cancer cells. Photoactivated metallodrugs are particularly promising, they are inert and nontoxic in the dark, but become highly active against a range of cancer cell lines upon irradiation with visible light. They proposed to use a Pt (IV) based pro-drug, trans,trans,trans-$[Pt(N_3)_2(OH)_2(py)_2]$ [133–135], where a cyclic peptide containing the RGD sequence (–Arg–Gly–Asp–) were conjugated. This RGD motif selectively recognize $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. These transmembrane glycoproteins are overexpressed in different tumor cells and they are also involved in tumor angiogenesis, which is very important process during tumor metastasis. Both reasons make them very important targets in medical chemistry [136,137]. The novelty of this complex resided in the use of a photoactivatable Pt (IV) pro-drug which became active after the irradiation with visible light. When the complex was localized within the tumor, after the light irradiation, it triggered the release of cytotoxic Pt (II) species with anticancer activity. SK-MEL-28 human malignant melanoma cell line was selected as a model to evaluate the internalization and the phototoxicity activity of the complex because its high expression of $\alpha_v\beta_3$ integrin [138], MBA-MD-468 breast adenocarcinoma cell line was used as positive control for a $\alpha_v\beta_5$ integrin (in this cell line the expression of $\alpha_v\beta_3$ integrin was considerably lower than $\alpha_v\beta_5$ integrin) and, in contrast, a DU-145 human prostate carcinoma cell line was selected as a negative cell line model since the expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins was considerably lower. The photocytotoxicity of the Pt–c(RGDfK) conjugate was determined upon irradiation with visible light (λ = 420 nm, 5 J cm^{-2}) in both cell lines and was observed higher toxicity of the complex in cancerous cells where $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins was overexpressed. They proved the potential of conjugated photoactivatable metal complexes, Pt (IV) pro-drugs to target peptides, to generate receptor targeted metal-based anticancer drugs with reduced toxic side effects and high selectivity opening up the door to a wide promising anticancer cytotoxic metallodrugs against tumors in a controlled and as consequent selective manner making the treatments more effective.

El-Hussein *et al*. studied the possibility to use another different king of metallic nanoparticles, silver nanoparticles (AgNPs), as a cytotoxic agent to be used as a photosensitizer in photodynamic therapy (PDT) alone and/or with combined chemotherapy [109]. Silver NPs were used as a unique system with the possibility to use it as photosensitizer generating ROS to affect the DNA and also to generate heat to affect cells, after laser irradiation in both cases. The study proposed by them induced PDT, in cell cultures, mediated by the laser irradiation of AgNPs using a 635 nm laser. The synthetized Ag NPs showed an absorption peak at 630 nm. Many parameters were employed to ensure the ROS production using human adenocarcinoma A549 cell line and also the DNA damage caused by the treatment was measured. This study proposed still need a future work to observe, among other factors, the role of other organelles in the ROS generation. Nonetheless, this was a good starting point to show the possibility of using silver nanoparticles in many on-demand treatments with two different applications using a single laser wavelength. Neri *et al*. proposed the use of Silver (Ag)-grafted PMA (poly-methacrylic acid, sodium salt) nanocomposite which showed good capability as a drug carrier allowing on-demand control of the dose, timing and duration of the drug released by laser irradiation stimuli [110]. They used plasmonic NPs (Ag) exploiting the intrinsical property of Ag to convert some part of the light energy into heat, and this heat was used as a trigger to provide spatio-temporal drug delivery control. Sorafenib is an anti-cancer drug approved for the treatment of several cancers, including thyroid cancer and advanced primary liver cancer. It is capable of inhibiting tumorigenesis and angiogenesis through activation of a receptor for tyrosine kinase signaling in the Ras/Raf/Mek/Erk cascade pathway [139,140]. Sorafenib was approved to be used in the treatment of specific tumors, such as thyroid cancer, primary kidney cancer and advanced primary liver cancer. Clinical applicability of Sorafenib was limited because this drugs also present a poor solubility in water media and its water solubility gets worsen when the pH of the water media increase [141]. Contrariwise its tosylate salt (SFT) showed lower side effects and bioavailability probably caused by the better solubility in biological media which improve their binding to albumin, which usually act as a drug transport [142,143]. A drug release experiment was performed by using three different systems: SFT loaded in Ag-PMA capsules by solvent evaporation (Ag-PMA SFT), SFT loaded in Ag-PMA capsules in a previously formed SiO_2 template (SFT-Ag-PMA) and PMA capsules without Ag (SFT-PMA). The capsules were irradiated with a laser at 420 nm, close to Ag SPR. Two different energy density P = 20 and 80 mW cm^{-2} were tested and also another different wavelength was used, far from Ag SPR: 632 nm to prove the better system to encapsulate the molecule with interest, its delivery and also to optimize the irradiation condition. After optimization was obtained a controlled release of SFT during a first period of time caused by the heat generated, but after that a longer release caused by the degradation of the polymer caused by the heating process. Despite the interesting results obtained, they concluded the necessity to carry on studies and also improvement in the particles formulation but also is necessary to study the drug retention and biodistribution after administration to improve the therapeutic outcomes.

3.2.2. UV-light spectrum

Nunzio *et al*. developed a methodology to prepare a UV-light triggered porous metal organic frameworks (nanoMOFs) with the novel possibility to use them as a DDS [112]. They showed the possibility to have MOFs with a large porosity, a tunable porous size, different shapes and functionalities. The internal part of the MOFs can be modified and adapted depending on the hydrophobicity/hydrophilicity of the molecules to be encapsulated. They synthetized Fe based MOFs able to absorb, by consecutive impregnations in aqueous solution, Topotecan (TPT). TPT is a cytotoxic drug that has gained broad acceptance in clinical use for the treatment of different type of cancers which show poor cellular uptake, due to its complex ionization chemistry, and a reversible hydrolyzation into

a relatively inactive and more toxic carboxylate from [144,145]. Nunzio and co-workers developed a fashion strategy to stabilize TPT in biological media by trapping it in a biodegradable Fe based nanoMOF. The irradiation using UV-light of 330 nm generated the MOFs structure collapse and the subsequent release of the loaded drug, they optimize and prove the possibility to load high concentrations of drugs and use them as a smart on-demand- drug carrier. The release was followed by emission at 540 nm obtaining, interestingly, 5-fold higher delivery efficiency when the process is induced by light than when it is not. They also studied the preservation of nanoMOFs' supramolecular structure after 3 h of irradiation to prove again the release mediated by the modification of the pore structure and not by MOFs degradation. Katagiri *et al.* developed another tunable UV-responsive material composed by inorganic/organic microcapsules formed by multilayers of PSS/PDDA coated with lipid bilayers and SiO2-TiO_2, prepared via the LbL colloid-templating technique and sol–gel chemistry [146]. This inorganic/organic capsule allowed the encapsulation of a low molecular weight dye (*i.e.* phenol red), which could be released upon UV irradiation. TiO_2 was selected as the UV-responsive component since it can decompose the organic materials by its photocatalytic reaction. The capsules were prepared by assembling a lipid bilayer onto a polyelectrolyte scaffold, a consequent adding of tetraethylorthosilicate (TEOS) and tetra-n-butylorthotitanate (TBTO) form the SiO_2-TiO_2 layer. The drug to be released was loaded previously to the UV sensible material, in this particular case phenol-red was used as a model molecule. After UV exposition at different intensities an increased amount of phenol-red was delivered to the media where the particles were suspended. Scanning electron images showed how the capsules change the morphology after UV irradiation. This system developed by Katagiri and coworkers still need some studies to conclude the possible applications but seems to be very promising methodology for cosmetic and agriculture where the UV light from the natural light can be used as a trigger to release low molecular weight molecules.

3.2.3. Near-Infrared spectrum

The biological windows or 'NIR windows' are a key aspect that influences most of the nano-photonic approaches for the last few years. NIR windows are narrow regions of the near-infrared region of the EM spectrum in which the organic structures such as cytochromes, melanin and other biomolecules have a minimum absorbance [147]. There are currently three biological windows described: NIR-I, II and III. They are generally considered to respectively lie between 700 and 950 nm, 1000–1350 nm, and 1550–1870 nm [148], which is still open for debate [149,150]. The irradiation at these regions of minimum biological absorbance allows significantly better penetration depths of biological tissues [151]. It also allows decreasing the power required for a given application to be effective, minimizing the damage of biological structures and the photobleaching of fluorophores that might be included [152,153].

The NIR-I has traditionally been exploited for a range of applications thanks to the large number of different compounds that interact with this range. However, at those wavelengths the tissues and other biological structures use to have autofluorescence, leading to increased background noise and lower penetration depth. In the case of the range between 1000 and above nm (traditionally considered the NIR-II or III), even though it has a slightly higher level of absorption by water and lipids, it does not present said autofluorescence, making it a significantly better candidate for certain applications [150,154]. Finally, it must be acknowledged that this range of NIR seems to be the trend in recent years in the design of *in vivo* applications of bio-probes in general [148]. This may be partially thanks to the observation that an increase in the wavelength can decrease the absorption and scattering of photons, improving the specifications of imaging agents absorbing in this range [148]. In the following paragraphs, some examples will be highlighted for each of the NIR windows. For the sake of simplicity, we will describe examples of applications of NIR-absorbing nanomaterials irradiated in the range between 700 and 950 nm (corresponding to the NIR-I) and then, in a separated section, those corresponding to longer wavelengths (1000 nm and above) for which the number of available excitable agents are considerably fewer [155].

3.2.3.1. Near-Infrared window I (700–950 nm). Hydrogels have a good potential for their applications but are hindered by their inaccurate drug release and allowed efficiency in terms of light absorption. One way around this is to use hydrogels combined with nanoparticles entrapped within them. One example of this is the recent work by Wu *et al.* which used a hydrogel with a NIR-absorbing dye (IR820) embedded within, together with mesoporous silica nanoparticles as doxorubicin carriers [156]. When the platform was irradiated with an 808 nm laser, the platform produced both heat and ROS. The ROS degrade the silica nanoparticles, releasing the drug entrapped in a much more controlled fashion, with a slow release aided by the presence of the hydrogel surrounding the nanoparticles. This treatment combined the controlled chemotherapy with phototherapy, allowing the authors to treat a mouse model of human oral squamous cell carcinoma, overcoming the inaccurate and low light absorption of other therapeutic approaches.

The increase in complexity of the various nanoplatforms has been rising up in recent years. One clear example of this is the work by Zhang and co-workers in 2020 [157]. In this work, they used multifunctional gold/platinum nanostars for synergistic tumor therapy. A targeting ligand, glucose oxidase (GOx) and NIR photosensitizer (IR780) were all linked by a thiol group that is cleaved by intracellular glutathione (GSH). GOx catalyzed intracellular glucose and consumed oxygen, generating hydrogen peroxide and enhancing the acidity of the tumor site. In the meantime, the platinum layer on the nanostars had a peroxidase-like activity and could catalyze the hydrogen peroxide producing toxic ROS. All this is in combination with the cleavage of the GSH-sensitive bond that releases the cargo. Thanks to this internal stimulus, the IR780 was released, which produced a PTT&PDT combined effect when irradiated with an 808 nm laser. This system not only allowed for a synergistic therapy of a mouse model of human gastric mucinous adenocarcinoma, but also provided real-time imaging capabilities thanks to the NIR fluorescence and specific targeting provided by the ligand.

An interesting approach for drug delivery is the self-assembly of the nanoparticles after they reach the target tissue. One example of this is the work by Tang *et al.* in 2018 which resorted to Mo(VI)-based polyoxometalates (POM) decorating hollow mesoporous organosilica nanoparticles [158]. They synthesized nanoparticles of < 50 nm with a $Mn_2(CO)_{10}$ payload, which are retained in the mildly acidic tumor microenvironment upon self-assembling into larger clusters thanks to the POM. The smaller size and later self-assembly benefits from the EPR effect and allows lower retention by the Kupffer cells and macrophages, extending the blood circulation time. This enhances tumor accumulation and retention. After the nanoparticles are retained, the irradiation of the tumor site with an 808 nm laser induces the release of the CO payload thanks to the conversion of Mo(VI) to Mo(V), as well as converting the light into heat for PTT effect, which in turn further enhances this decomposition. Additionally, the authors managed to detect the presence of the nanoparticles by photoacoustic imaging. All this work was carried out using a mouse tumor model of human glioblastoma. They claimed that this platform could also be used for other types of payloads.

Although not commonly used for their photonic properties, iron oxide nanoparticles (IONPs) can also be used as photothermal agents, since they have an absorption peak in NIR I. Wu and co-workers designed IONPs functionalized with a thermo-cleavable Azo linker that was used for doxorubicin drug delivery after NIR irradiation [159]. IONPs can indeed convert light to heat, rapidly reaching the rupture temperature of 43 °C, necessary for the release of the drug. With the proposed system it was possible not only to obtain an *in vitro* release of doxorubicin with a 3-fold increase compared to no-irradiated samples, but also an *in vivo* reduction of tumors, induced *in vivo* using a murine sarcoma mouse model, only when the laser was applied to the group treated with IONPs-doxorubicin. Thus, the authors demonstrated the combinatorial mechanism of the photothermal effects combined with chemotherapy.

IONPs have also been exploited as a photothermal agents in combination with molybdenum disulfide (MoS_2) nanosheets for doxorubicin release [160]. The magnetic material on one hand provides the possibility of directing the drug to the site of interest by applying a magnetic field, and on the other increases the photothermal capacity of MoS_2. This type of material has a tendency to aggregate. In order to overcome this, the authors resorted to the use of chitosan and carboxymethylcellulose to stabilize the structures under physiological conditions and in cell culture. This system showed a high loading capacity and a fast release under 808 nm laser irradiation both in PBS solution and on MCF-7 cells, a breast cancer cell line. The authors also observed an increase release under acidic conditions, which benefits from the mild acidic tumor microenvironment. Further studies *in vivo* with mMoS_2–CS/CMC revealed the efficacy of the combined photothermal treatment and the chemotherapy, with a decrease of tumor size and a prolonged retaining of the nanocomposite in the tumor of tumor-bearing mice.

An interesting approach for on-demand drug delivery was also reported by Wang *et al.*, using a similar material but in combination with a polymer [161]. They proposed the use of N-methyl pyrrolidone (NMP) to form an oleosol, containing PLGA, MoS_2 nanosheets and doxorubicin. The advantages of these systems rely both on PLGA and MoS_2. Indeed, while the polymer ensures *in vivo* biosafety, by undergoing an immediate liquid–solid phase transition upon contacting with water and fluids when dispersed in NMP, the nanomaterial acts as a photothermal agent converting the NIR irradiation at 808 nm into heat, leading to the drug release from the PLGA matrix. The implant formed with this technique resulted very efficient in terms of doxorubicin loading and release under NIR irradiation. In fact, the authors observed a concentration of DOX 3.6-fold higher at acidic pH of the irradiated samples compared to non-irradiated both *in vitro* and *in vivo*, suggesting two different mechanisms of action of the photothermal activation: i) expansion of the matrix and subsequent reduction of doxorubicin binding to it; ii) acceleration of drug motions due to heat generated by the MoS_2.

These are a few representative examples of the remarked approaches that involve the use of NIR-I light stimuli. Despite their obvious interest and good potential for future therapies, most of these approaches are notably far from their translation into the clinic. Future approaches should focus not only on materials that are already approved, but also on the use of experimental designs that are more regulation-oriented. This will be discussed in the coming sections.

3.2.3.2. Near-Infrared windows II and III (>1000 nm). Different formulations have been found in literature to be used as DDS upon NIR irradiation with wavelength up to 1000 nm. Angelatos *et al.*, in an early publication in 2005, reported preparation of light-responsive polyelectrolyte microcapsules [162]. Core-shell particles were prepared with poly(sodium 4-styrene sulfonate) (PSS) and Poly(allylamine hydrochloride) (PAH) with a procedure that creates an inner space free to load the molecules of interest [163]. The loading of biomolecules inside the PSS/PAH multilayer capsules was performed considering their reversible permeability, indeed, they switched from the "closed" to the "open" state by varying the pH of the bulk solution. They demonstrated the ability to load high molecular weight biomolecules whose release was possible thanks to the AuNPs loading inside capsule structure. Pulses of 10 ns with a 1064 nm light were used to induce the release and ensure the confinement of laser light energy to the capsule shell. Only for AuNPs loaded capsules, the laser irradiation produces a significant increased signal in the supernatant spectra. The authors further modified the system by lipidic shell addition, to confer stability and avoid leakage of the biomolecule, and antibody functionalization, for directed targeting. Although further studies are needed for assessing *in vitro* and *in vivo* effects, this system seems to be promising for on-demand delivery of macromolecules with a wide range of applications in biomedicine.

Silicene nanosheets (SNS), a new two-dimensional (2D) silicon allotrope, make good candidates for phototherapy thanks to their high near-infrared (NIR) optical performance and good biodegradability. Wang and co-workers reported in 2020 the possibility to deliver doxorubicin upon irradiation of their SNS with a 1064 nm laser (1 W cm^{-2}) [164]. The authors demonstrated that acidic pH increases DOX release, being a beneficial effect considering the acidic environment of tumors. In this case, bovine serum albumin was used to enhance stability, biocompatibility, cellular uptake and internalization of the nano-system as demonstrated by *in vitro* studies on 4 T1 cells, a breast cancer cell line from mouse. Laser irradiation induced the release of the anticancer drug thus reducing cell viability *in vitro* and tumor size *in vivo*, suggesting a promising chemo-photothermal combined treatment that enhances the therapeutic efficiency of the system.

Sun *et al.* recently reported the design and synthesis of a hybrid based on a uncommon photonic material used for DDS which is based on copper sulfide (CuS). CuS nanoparticles have good photothermal conversion properties upon irradiation using a 1064 nm [155]. The authors proposed the synergic use of photothermal and free radical generator system to reduce the viability of the KB cancerous cell line *in vitro* and in a mouse model. An Azo initiator (AIBA) was chosen as a free alkyl radical generator due to its high stability at 37 °C and the capability to generate radicals via heat-induced decomposition. A lipidic shell was used as carrier of both the CuS nanodisk and AIBA, and the system was further modified with folic acid as an active delivery molecule. The prepared nanosystem possessed a good biocompatibility *in vitro* even at high NPs concentration, and only the laser irradiation confers a cytotoxic effect, leading to cell death. *In vivo* experiments on mice confirmed the specific localization of the nanosystem to FA rich tumors. They also confirmed both the phothermic effect of the system proposed and the free radical formation under a 1064 nm laser irradiation. The possibility to on-demand deliver the drug by using NIR-II irradiation expands the applications even to deep tissues.

3.3. Others

3.3.1. X-rays

X-rays are electromagnetic radiation with wavelengths ranging from about 10 nm to 10 pm wavelength and high frequency. Although they are an indispensable diagnostic tool in modern medicine for the non-intrusive detection of bone fractures and diseased conditions such as cancer, few articles report this stimulus as the trigger for drug release.

Deng *et al.* designed X-ray triggered liposomes by co-embedding photosensitizers and gold nanoparticles inside a lipid

bilayer [165]. 1,2-dioleoyl-*sn*-glycero-3- phosphocholine (DOPC) and 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP) were chosen as lipid components in the liposome formulation because DOPC can load highly hydrophobic molecules and DOTAP can facilitate cellular uptake due to its positive charge. Gold was the selected metal due to its high atomic number and it's capability to interact with X-ray and with UV-light [166]. As a photosensitizer (PS) it was selected a clinical approved phodynamic agent for macular degeneration, the verteporfin (VP) [167]. The mechanism proposed to trigger the release was the generation of reactive oxygen species (ROS) as the key factor to oxidize the unsaturated lipids causing the disruption of the liposome structure [168]. The exposure of gold nanoparticles to X-ray leads to the direct production of ROS/$^1O_2$, whose levels are further increase by the interaction of the secondary electrons, produced by the metal interaction with the X-ray, and the PS. [169]. The ROS/$^1O_2$ generation from different liposome samples and the destabilization of the lipid bilayer was studied under X-ray radiation with different dosage (1, 2 and 4 Gy). Singlet oxygen green sensor (SOSG) was used for the highly specific detection of $^1O_2$ generated and a fluorescent dye was used to evaluate the liposomal content release. Once proved the possibility to deliver the liposomal content by using X-Ray irradiation as a trigger, these liposomes, functionalized with folic acid as active targeting agent to cancerous cells, were further loaded with an antisense oligonucleotide for *in vitro* assay of PAC1R gene knockdown in PC12 cells or DOX and etoposide (ETP) for *in vivo* assays in tumor-bearing mouse model (using HCT 1116 cells).

The promising results using X-ray-triggered loaded liposomes indicated that a combination of X-ray triggered chemo- and radiotherapy produced an enhanced effect with a great efficacy on cancer cell-killing and tumor growing during a large period of X-ray exposition. This study therefore proved, in a very efficient way, the efficacy of the on-demand triggered liposomes to be used as a promising DDS to carry drugs enhancing effectivity of the treatments and reducing the undesired side effects. The use of powerful X ray to perform the delivery allows higher penetration and consequently the possibility to treat depth tumors.

3.3.2. Microwaves

Microwaves are electromagnetic radiation with wavelengths ranging from about one meter to one millimeter corresponding to frequencies between 300 MHz and 300 GHz and can be considered a non-invasive stimulus able to penetrate deep into the interior of the body, to depths of 10 to 15 cm [170]. Thus, microwaves are a promising strategy for drug delivery triggers, they can act as a source to excite micropumps and microvalves or can be exploited to generate heat. The key parameter for a heating generation under microwave radiation is permittivity. It is a two-component value that describes materials electromagnetic energy storage capacity (real component) and its capability to transform electromagnetic energy to heat (imaginary component). The ratio between these two components described the dialectric loss tangent, defined as the material capability to adsorb and convert the microwave radiation into thermal energy [171]. Such property is related to atomic and molecular geometry. This thermal effect must be carefully managed as it can have adverse effects on tissues. Indeed, microwaves interact with dipole molecules, such as water, inducing rotations, vibrations and friction between them. The direct consequence is to heat aqueous media inducing the tissue damages. For these reasons, it is very important to choose microwaves frequency and amplitude in relation with the area to irradiate [172]. Thus, limiting for biomedical applications were set [173].

Although iron oxide nanoparticles present a low microwave absorption capacity, researcher have focused their attention on this material as microwaves absorber thanks to their good permittivity, saturated magnetization and high dielectric properties. Peng and co-workers increased the electromagnetic wave thermal conversion of magnetite by doping its core with magnesium. Furthermore, they introduced CuS crystals on the particle surface to control the system also by NIR irradiation [174]. The system presents a high ibuprofen loading and release under an electromagnetic wave of 2.45 GHz. It was possible to regulate in time the release of Ibuprofen, obtaining a higher release compared to NIR light irradiations, that was almost 1.5-fold less efficient.

Another interesting strategy was reported by Shi and colleagues, who proposed a mesoporous silica nano-system with a core of ZnO@Fe_3O_4 for the controlled release of drugs. As a proof of concept, they incapsulated into the pores of the nanocapsules the fluoresceine, whose release was controlled by the presence of a nano-valve composed by a short peptide. At physiological temperature the peptide is assemble and blocks the molecule inside the pore of the nano-system, but at 50 °C it disassembles allowing the diffusion of the drug. The synthesize system presents optimal microwaves absorbing property and an efficient thermal conversion adequate to generate a local heating for triggering the thermosensitive valve disassembly [175].

3.3.3. Electric pulse

Several publications focus on the assisted internalization of a drug co-incubated with the nanoparticles [176–179]. Other investigations resort to the use of electric fields to internalize the nanoparticles themselves inside the cells, or to release the drug in the surrounding environment upon excitation with said electric fields. Most of the examples described in this section are preliminary work, with a considerable potential for clinical translation.

One example of internalization of nanomaterials using electric fields is a study by Arab-Bafrani *et al.*, published in 2020. The authors show a method of internalization of gold nanoparticles with the help of an electric field [180]. They found that a current of 1.2 kV/cm in two pulses of 100 μs was able to give the highest cell viability of a colon cancer cell line (HT29) while obtaining a high internalization, higher than simply a passive internalization of the gold nanoparticles. This study did not use a drug conjugated to the nanoparticles that are internalized, but it is worth to be mentioned for its potential applications when designing novel nanoplatforms. This could be achieved using a drug conjugated instead of unloaded nanoparticles that are simply used as enhancers for the internalization of a free drug present in the medium.

One example of a design where the drug is loaded within the nanosystem itself is the work by Gunathilake *et al.* The authors proposed a proof of concept of an electric-field mediated drug release from a nanocomposite film [181]. Thus, they used a film generated by combining Poly(Lactic Acid) (PLA), Carboxymethyl Cellulose (CMC) and ZnO nanoparticles into films with curcumin as a poorly soluble drug model. Although the platform once built was macroscopic and the study did not use cell culture nor animal models, it laid the foundations of the use of electric stimuli for drug release in a nano-structured material. This is particularly important in the case of poorly soluble drugs. The authors found a linear profile of release upon the exposure of the materials to the electric field, with no noticeable passive diffusion when the electric field was off. As the authors claimed, this could be used for temporally precise therapeutic dosing.

Another more developed example was the work by Qu *et al.*, which resorted to the use of a conductive hydrogel which pore size was responsive to the current, the more stimuli provided, the more drug is released [182]. This system goes beyond the proof of concept since the authors also tested it in mouse fibroblasts and a rat model and confirmed the good biocompatibility of the material when subcutaneously implanted. However, since these hydrogels do not belong in the nanoscale, they fall beyond the scope of this

review. Testing new nanohydrogels with conductive and/or pores that are responsive to the current could be an interesting route for future investigations.

The electric stimulus has some potential for clinical translation. However, it has not been explored extensively either for drug delivery or for controlled administration. There are still several paths to explore, such as the use of nanogels instead of larger, standard hydrogels or the design of nanoplatforms that are in combination with a conjugated or entrapped drug.

3.3.4. Ultrasound

Ultrasounds (US) are acoustic waves that have frequencies higher than those audible to the human ear, generally above 20 kHz. In the clinical setting, high ultrasound frequencies are used for diagnostic purposes, while frequencies below 1 MHz are used for drug delivery. At these frequencies, indeed, the waves can penetrate deeply into the tissues without producing side effects, while guaranteeing a therapeutic effect. Another important aspect to consider is related to the intensity of the ultrasounds. The FDA in fact allows the use of ultrasounds with an intensity such as to lead to an increase of 1 °C in the local temperature of the tissue. Consequently, US spatial-peak temporal-average with intensities ranging from 10 to 720 mW/cm^2 are used in the field of drug delivery, even if it was possible to increase the intensity by decreasing the US frequency [183]. Similarly, the FDA has also regulated the mechanical index (MI), defined as the ratio between the negative pressure peak and the root of the frequency center. In fact, to avoid thermal effects and tissue damage, the Administration has imposed a maximum application MI of 1.9 [184]. Currently, ultrasounds are routinely used for ultrasound scans in diagnostic, but in the last years the application of this stimulus has been studied for therapeutic applications with drug delivery [185]. Three different cell membrane responses can be related to the use of ultrasound: sonoporation, cavitation and hyperthermia. In the first case, the application of US leads to localized permeabilization of the membrane, due to the generation of small pores that allow the nanoparticles to enter the cell [186,187]. Cavitation, on the other hand, is based on the use of micro or nano bubbles. These structures are generally composed of a core containing a gas (air, oxygen, sulfur, Sulfur hexafluoride or Perfluorocarbons) and a shell composed of polymers, lipids and/or surfactants. In the case of stable cavitation, the application of US leads to the controlled contraction and expansion of the gas contained in the core, in a cyclical manner, allowing a sustained release of the drug over time and causing vascular permeability [188]. In the inertial cavitation, however, a rapid and violent collapse of the structure is observed following the application of the US, which induces the formation of pores with dimensions in the range of 100–2000 nm, increasing the membrane permeability [189]. Finally, the application of high-intensity US can lead to a localized increase in temperature, also referred to as hyperthermia. The ultrasound waves act on the rotation and/or vibration of the tissue molecules, leading to the production of heat by friction, managing to reach local temperatures of 40–45 °C. The increase in temperature generates a greater fluidity of the lipid bilayer of the cells, resulting in an increased permeability of the membrane to the nanoparticles [190].

The outcome of ultrasound on carriers, instead, can be attributed to thermal or non-thermal effects. In the first case, heat-sensitive delivery systems (such as lipids or polymers) are used to respond to the production of heat, generated by the absorption of the energy of ultrasound waves, modifying their structure and releasing the drug of interest. In the second case, however, an acoustic pressure leads to a shear stress, which causes the loss of integrity of the nanocarrier [184].

FDA approved human serum albumin microbubbles as contrast agents back in 1994 [191]. In the last decades different studies reported the use of microbubbles as effective drug delivery systems. Lipid coated microbubbles were used as anticancer drug carriers, reducing drastically tumor cell viability after US application both *in vitro* and *in vivo* in a mouse melanoma model [192]. Microbubbles have been reported also for an increased permeability of the blood brain barrier (BBB), without permanent tissue damage [193,194].The BBB has an important impact on drug delivery to the brain. The fine control of the trans- endothelial transporter molecules makes the treatment of brain diseases, such as Alzheimer, schizophrenia, and cancers, very difficult [195]. They impede the access to the brain to the 98 % of small molecule (<600 Da) and all the macromolecules, reducing the possibility of an intravenous administration of the drug. In this context, Fan and co-workers reported the preparation of 1 μm microbubbles coated with a phospholipidic shell and a gas core, containing superparamagnetic iron oxide nanoparticles (SPIONs) for magnetically guided and US-induced release of doxorubicin in the brain [196]. The system presented IONPs bound in the lipidic shell through hydrophobic interactions and doxorubicin bound via electrostatic interaction. After magnetic targeting obtained by applying an external magnetic field, doxorubicin was successfully released in situ by microbubble destruction under US [196]. This work was later expanded by the same authors, this time using a rat model and binding the doxorubicin to the SPIONs, allowing real-time monitoring of the doxorubicin distribution in the brain tissue [197].

Unfortunately, incorporating iron oxide nanoparticles or drugs into the phospholipidic layer reduces the microbubble stability in bloodstream leading to an unspecific release. To prevent this, researchers focus on polymeric structures. PLGA has been reported in the literature as one of the polymers used for microbubble production. Using the same approach of Fan and co-workers, a PLGA system presenting IONPs in the shell and doxorubicin in the core of the microbubble was able to release the anticancer drug under low frequency ultrasounds [198]. A limitation of microbubbles relies on their dimensions, generally > 1 μm diameter, that confine them in the vascular space and possess low circulatory stability [199]. Thus, nanoscale nanocarriers have been designed to increase the stability in the bloodstream and to pass through the endothelial gaps of tumors or inflammatory tissues. Liquid perfluorocarbon (PFC) nanodroplets are one of the recent nanoformulation designed for drug delivery. The integration of magnetic nanoparticles in the nanodroplet allows to obtain vascular imaging and at the same time can promote drug uptake and delivery [200]. Magnetic nanoparticles act as stabilizing agents in the nanodroplets, extending their stability over time. Furthermore, the authors reported that the presence of iron oxide nanocrystals inside the droplet lead to a higher rate of conversion nanodroplets to microbubble in the presence of ultrasounds. Nanocrystals, indeed, reduce the vaporization threshold by acting as nucleation agents or by contributing to the super harmonic focusing effect. Into this system was also possible to encapsulates paclitaxel, a chemotherapy drug that works by inhibiting microtubules within cancer cells. A high encapsulation was obtained and a sustained released was achieved under ultrasound application, leading to a 45 % reduction of cell viability, compared to a 10 % reduction of paclitaxel alone.

Fingolimod (2-amino-2[2-(4-octylphenyl)ethyl]-1,3-propane diol (FTY720) is an immunosuppressive agent that can inhibit the SK-1 signaling pathway, interfering with cancer development. Originally approved by the FDA for the treatment of multiple sclerosis, in recent years has been reported as an efficient molecule for different cancer models [201]. Unfortunately, high doses FTY720 lead to cardiovascular side effects, making it difficult to use as anticancer drug. Thus, Guo *et al.* [202] encapsulated FTY720 together with IONPs and PFP into RGD modified liposomes to obtain a drug delivery system. Under a low intensity focused ultrasound, it was

possible to obtain an inhibitory effect of two different cell lines of human Hepatocellular carcinoma (HCC) and low toxicity on normal fibroblast 3 T3 cells. The drug directed release of FTY720, and its cellular uptake suggest that low intensity focused ultrasound can be an effective system for cancer treatment.

As for microbubbles, polymers can also be used for nanodroplet preparation. Doxorubicin was effectively incorporated into the core of PLGA-PEG-Glycol folate nanobubbles together with IONPs, using perfluorocarbon as gas inducing cavitation agent [203]. While the presence of nanoparticles leads to a higher uptake of DOX due to enhanced motion of the cavitation fluids, the authors reported that the concentration of IONPs is a critical parameter in the nanobubble formation. Indeed, when the nanoparticles exceed the 17.6 wt% two effects were observed: a drastic reduction of doxorubicin loading and the destruction of the nanobubble. Jin and co-workers also investigated the effects of different ultrasound pression on the drug release. This release rate was 2.5-fold increased when using 1 MPa compared to a 0.6 MPa US. This effect can be related to the shear force from acoustic radiation and the cavitation effect. The presence of magnetic nanoparticles, on one hand, increased the imaging capacity under US by not only being resonant with the US but also thanks to backscattering the US signal. On the other hand, they can help reaching the lethal dosage of doxorubicin in the tumor, by increasing drug release under US. In this study, the anticancer drug release was achieved by deformation or destruction of the nanobubbles, without any thermal mechanism involved.

3.4. Multi-stimuli

It is clear from previous sections that although exogenous stimuli aim to obtain a precise drug release, a sustained and controlled release (in space and time) is not always achieved. Thus, researchers have explored the possibility to combine two or more stimuli. Literature examples vary widely in terms of how they combine the different stimuli. Some examples combine multiple endogenous stimuli, which is out of the scope of this review. Other studies resort to the application of endogenous and exogenous stimuli together. Finally, some approaches revolve around the idea of combining multiple exogenous stimuli.

Usually, an exogenous stimulus, such as NIR or an alternating magnetic field, are used in combination to an endogenous stimulus, such pH. As already commented, an acidic pH is usually associated with tumors and inflammatory conditions. Thus, researchers have taken advantage of the local acidic environment to obtain a site-specific release of the drug, while the temporal control is given by the external stimulus.

For example, γ-Fe_2O_3 particles with a hollow structure and uniform size were used for encapsulating doxorubicin. 1-tetradecanol (TD) was used for sealing the hollow structure [204]. TD is a material that transit from solid phase to liquid phase at around 40 °C, which allowed a controlled release when a 45 kA/m and 186 kHz alternating magnetic field is applied. In this study, the authors observed that while acidic pH is not sufficient for inducing doxorubicin release, it enhances the drug release once AMF is applied, with a cumulative release of 63 %. When the system was tested *in vitro* using adenocarcinoma human alveolar basal epithelial cells (A549) the toxicity of the system was drastically enhanced upon exposure to an AMF, which was equivalent to that observed for free doxorubicin.

An equivalent result was obtained with graphene oxide opportunely irradiated with a NIR laser (808 nm) where a photochemical effect induces a better drug release in acidic conditions compared to physiological pH. This suggests that this system can be a valuable tool for cancer treatment or upon internalization, taking advantage of the low pH inside the lysosomes [205]. The authors successfully tested their system *in vitro* in a commonly used human cell line, HeLa.

It is worth noting that when resorting to the pH as a release endogenous stimulus, one must take into account the possibility of parenteral administration of drugs. This is in order to avoid that the different substances administered were altered in the digestive system before being absorbed. Consequently, this kind of approach forces the development of a nano-system that is stable at the intravenous level without a significant reduction of the therapeutic efficacy. Problems related to the fate of the nano-systems in the blood circulation are reported in the following chapter.

Similarly, the redox status of the cell can be exploited as endogenous stimulus in combination to NIR-triggered release of the drug cargo. For example, a complex system for the cytosolic gene delivery was reported by Wang and colleagues in 2014 [206]. They used gold nanorods bioconjugated with RGD peptide for active targeted delivery of DNA embedded in polyethyleneimine (PEI). To decrease the known cytotoxicity driven by the high cation density of this molecule, the authors introduced an intracellular di-sulfite linker that is stable in blood circulation. This linker is easily cleavable in an environment rich of glutathione (GSH). Photo-induced endosomal disruption under a laser of 808 nm, combined with the DNA protection provided by PEI, enhanced the gene transfection efficiency of green fluorescent protein. This allowed demonstrating a site-specific gene delivery *in vitro* in human glioblastoma cells, with negligible release observed without irradiation. It must be acknowledged that although promising *in vitro* results emerged from the study of the current literature, the complexity of the biological environment makes it difficult to achieve an effective and specific control based on GSH *in vivo*.

Considering the limitations in the use of an internal stimulus, recent research has attempted the use of two external stimuli both to ensure better control of the drug release over time, and to obtain a gradual release of the selected API. In this way, in fact, the onset of adverse effects related to high concentrations of drugs administered is avoided and the treatment of chronic diseases can be guaranteed without the need for repeated inoculation of the drug.

In 2018, Spadaro *et al*. used a PEGylated-PLGA random nanofibrous membrane loaded with gold and iron oxide nanoparticles and with silibinin, a promising anti-neoplastic agent [207]. This complex nanocarrier can be remotely controlled and activated by a laser or magnetic field to release biological agents on demand. The irradiation of the nanofibrous membrane by a low-intensity laser or activated by a magnetic field indicates a sustained silibinin release for at least 60 h, without any burst effect. Although, this system presents good results in terms of drug loading efficiency and release, further experiments are needed to study its toxicity and behavior *in vitro* and *in vivo.*

Lately, Gangrade *et al*. successfully applied electric field and NIR laser to a nanocomposite silk hydrogel system *in vitro* [208] and *in vivo* [209]. This hydrogel had embedded single-walled carbon nanotube (SWCNT) loaded with doxorubicin and decorated with folic acid (FA) [209]. Single-walled carbon nanotube are known for their strong absorption in NIR region, while FA is recognized by FA receptor-positive mouse breast tumor cells (4 T1), promoting the detection of the target cells and the uptake of the nanoplatform. The application of electric field, further permeabilizes the tumor for the deep infiltration of the nanocomposite into it. Once inside, NIR laser irradiation produces heat and induces the doxorubicin release. The authors proved that the simultaneous application of the NIR laser and the electric field induces selective apoptosis in the tumor target cells. The two exogenous stimuli have thus a synergistic effect of tumor growth inhibition.

In a recent work in 2021, Cao and co-workers reported a novel nano-in-micro platform responsive to NIR, magnetic, and pH stimuli. They accelerated the release of doxorubicin by embedding the

drug in a pH sensitive polymer together with graphene oxide and IONPs [210]. This hydrogel is based on poly(N-isopropylacrylamide) (PNIPAM) and alginate interpenetrating polymer. Under NIR irradiation and alternating magnetic field application reached temperature above the lower critical solution temperature, releasing doxorubicin following deswelling of the polymer. Interestingly, the authors show that a strong heating effect is induced by dual mode stimulation, with a dominant effect of the photothermal component. Furthermore, the introduction of alginate as a component endows the network of the hydrogel with pH-responsive features, inducing a higher release at pH 5.0. *In vitro* experiments in human breast cancer cells suggested a low toxicity of the nanocarrier and an effective release of doxorubicin, leading to a survival rate of only 36.3 % after dual-mode stimuli application. Therefore, the possibility to integrate different triggering mechanisms on the same nano-formulation opens the door to more accurate and custom-made treatments. Despite all the excellent results reported, research in the use of systems that use different external stimuli for drug release still has a long way to go before it can be applied in a clinical setting.

4. On-demand drug release in clinics: The state of the art

In the last twenty years, approximately 19,000 reports on the topic on controlled drug release have been published. Despite the expectation that these numbers may generate, the amount of nanosystems that apply to be approved by the FDA does not match the initial hype. Since 1970, FDA's Center for Drug Evaluation and Research (CDER) has received>600 applications (investigational new drug (IND), new drug application (NDA), and abbreviated new drug application (ANDA)) for human drug products containing nanomaterials – half of which were submitted within the last 10 years (NFT Report July 2020). The low number of applications correlates with the low number of clinical trials carried out so far with nanomaterials. If we search "nanoparticle", "nanopharmaceutical" and "nanomedicine" in the clinical trials database [211], we can find 126 trials in phase I, 240 trials in phase II and I/II, 48 trials in phase III and II/III, and 16 trials in phase IV. It is surprising to find that less of the half of the clinical trials in phases I and II are recruiting, active or enrolling by invitation. This means that most of the nanodevices are stuck in phases focused on finding the highest dose that can be given without causing side effects and its effectiveness. Another shocking data is that only five trials in phase II and three trials in phase III have available public results.

Throughout this section we will discuss the remain gaps between technological advances and clinical applications which could explain this scenario. As explained below, a major hurdle is the lack of a robust and flexible framework for safety assessment for the wide variety of nanomaterials that are being developed for therapeutical applications.

4.1. Nanoparticle behavior and interactions

A holdback in a better understanding of on-demand drug delivery nanodevices interaction with biological systems starts with the knowledge gap of each counterpart biological behavior and interactions (Fig. 4). From the very beginning in which a nanoparticle gets into contact with a biological media, it suffers surface modifications that may alter its biodistribution and pharmacokinetic [165,212,213]. Indeed, it is well known that they become surrounded by a layer of biomolecules known as "protein corona", as it is mainly formed by proteins adsorbed to their surface. Routinely performed assays to study the effects of these on-demand drug release nanoplatforms are *in vitro* and do not adequately inform about the biological behavior when the system is injected. The modifications in composition, size, and charge that nanodevices suffer when they encounter biomolecules and cells are usually not taken into account or studied. Wilhem and colleagues conducted a multivariate analysis to study the current delivery efficiency of nanoparticles to tumors and revealed that <1 % of the injected dose of the nanoparticles reached the tumoral tissue [12]. Their work also revealed that the nanoparticles with higher delivery efficiency were inorganic, with hydrodynamic diameters smaller than 100 nm, with neutral zeta potentials and rod-shaped. Throughout the following section, we will discuss a comprehensive review on how the bio-nanointeractions can hamper the fully potential of nanomaterials as drug-delivery systems.

4.1.1. Protein corona (PC)

Once the nanomaterial has been administered regardless the route of administration (*i.e.* inhalation, injection, ingestion, or dermal application) inevitably encounter a complex physiological media. A major factor that directly affect the behavior of nanodevices is the protein corona. The corona that forms when nanoparticles encounter biological media is composed of a complex range of adsorbed biomolecules such as proteins, glycans and cytokines [214]. This layer has been shown to mediate basic biological processes of administered particles such as cellular uptake [214], immunological response and toxicity, plasma circulation time [215] and clearance [216]. Monopoli *et al.* already discussed how the interactions between nanoparticles and cells may be ruled by the extrinsic properties defined by the protein corona rather than the intrinsic properties of the bare nanoparticle [217]. Certain components of this corona, called opsonins, induce the nanomedicines uptake by the mononuclear phagocytic system (MPS), and consequently their clearance from the bloodstream.

Portilla *et al.* studied the influence of PC biodynamics in the cellular processing of MNPs. Three different polymers were used to coat the MNPs: amino-propylsilane (APS), dextran (DEX) and dimercaptosuccinic acid (DMSA) to produce a positive, neutral, and negative surface charge respectively [218]. They showed that the proteins in the respective coronas were similar in terms of composition and abundance for the three coatings. In addition, a great number of such proteins have affinity for divalent ions. Despite there are many studies that claimed the close relationship of the coatings and protein binding, they demonstrated the implications of the iron ions in the proteins' enrichment of the corona [219]. Cell internalization studies carried out with macrophages and tumoral cells revealed that not only the coating, but also the hydrodynamic size of the PC influence the internalization pathway. PC formation in photonic materials is also widely studied. Like magnetic materials, its biological behavior cannot be predicted on one single parameter but the entangle of different physicochemical properties. Gold NPs synthetized with five different coatings were used to prove the effect on the size and composition of PC formation, however, particle size and shape is also known to directly affect PC composition [220,221]. Therefore, the binding of plasma proteins to nanocarriers can clearly influence their biodistribution and therapeutic efficacy. The implications of protein corona in the therapeutic outcome of nanomaterials are beyond the scope of this review and has been thoroughly discussed elsewhere [212,222,223]. However, we think it is important to highlight that to achieve an effective clinical translation of nano-based drug-delivery systems this is a critical aspect to study and considered.

4.1.2. Mononuclear phagocytic system

Nanomedicines can be formulated for several administration routes (e.g., oral, dermal, pulmonary, ocular, and parenteral, among others), and the initial biological barriers to overcome will depend

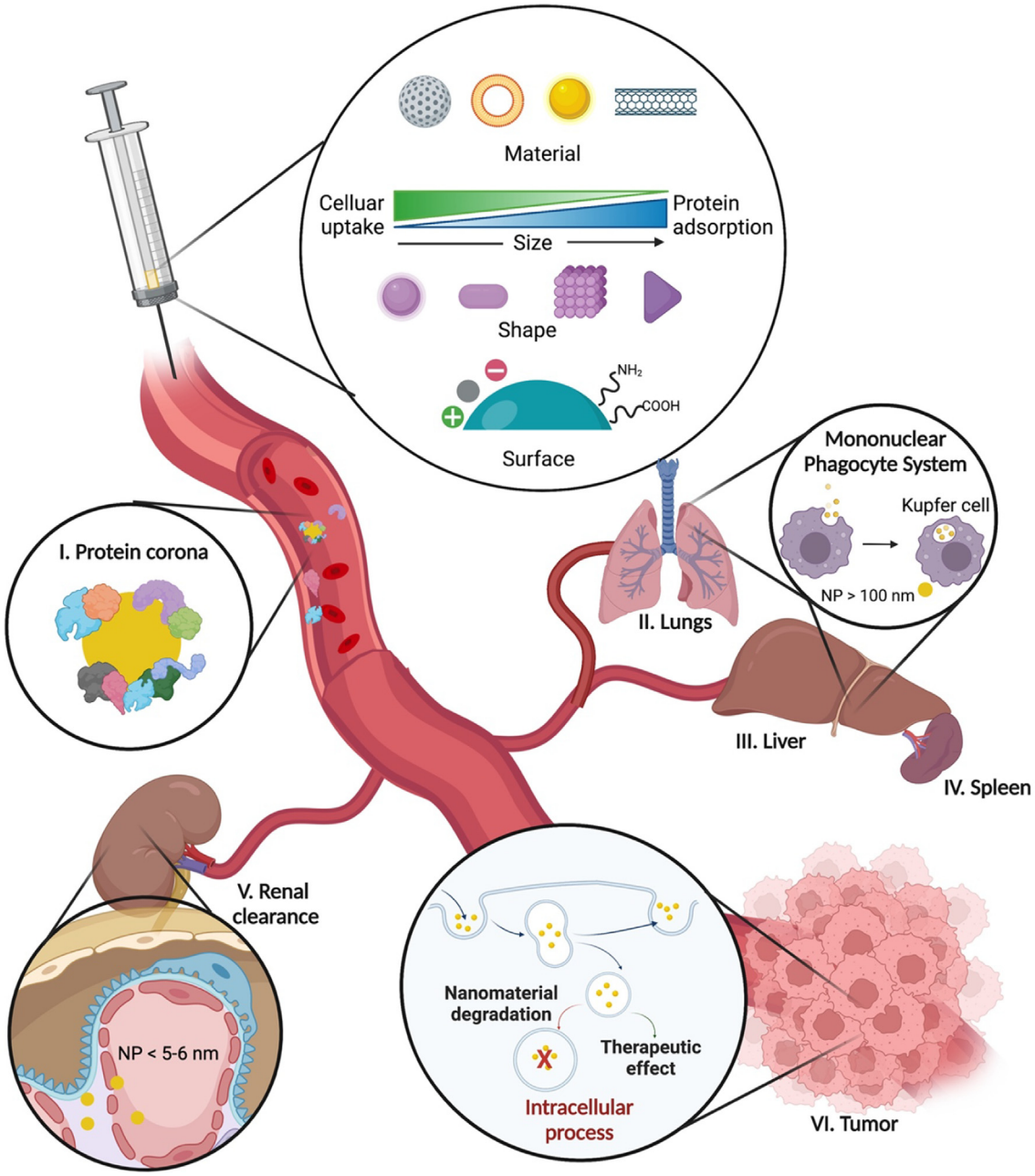

Fig. 4. Schematic illustration of nanomaterial fate and trafficking after intravenous administration. Several systems are involved: Blood with protein corona formation (I), clearance organs of the mononuclear phagocyte system: lungs (II), liver (III) and spleen (IV), renal clearance (V) and tumor accumulation (VI). Created with BioRender.com.

on the route. Indeed, the administration route directly affects the clearance by mononuclear phagocytic system (MPS) and filtration by the liver and spleen that quickly sequesters most of the drugs [224].

An easy way to imagine de MPS is to imagine a diffuse organ mainly formed by macrophages and monocytes and distributed in the liver, spleen, lymph nodes, and bone marrow, among other organs. Multispectral optoacustic tomography (MSOT), a technique that combines the high contrast of the optical imaging with the high resolution of ultrasound imaging, confirmed a rapid clearance of PEGylated Cy7-SPIONs from circulation and accumulation in liver and spleen within minutes post-administration [225]. These organs possess features like those driving EPR and tumor accumulation, including high blood vessel density, fenestrated vasculature, and the prominent presence of phagocytes [226]. The scientific community is making efforts to understand the crosstalk between these phagocytic cells and nanoparticles, which will suppose a huge step forward its clinical translation. Highlighting some of the already known factors, thoroughly reviews discussing the size-dependent behavior of nanoparticles in the circulation time and biodistribution have been published [12,224]. In case of MPS clearance, small sizes are less likely to be taken by macrophages; however, they are more likely to be excreted through urine (renal filtration cutoff size: 5.5 nm) and get trapped in the liver (vascular fenestrations in liver cutoff size: 50–100 nm). Feng et al. studied the biodistribution of 10 nm and 30 nm core MNPs coated with PEI and PEG. The 10 nm PEG-coated MNPs achieved the highest tumor uptake, however, independently of size and charge all accumulate in liver and spleen [227].

Others well-known trends are charge and protein corona composition. To investigate the role of this nano-bio interface on internalization behavior of macrophages, the group led by Chen *et al.*

preincubated gold nanorods (AuNR) functionalized with seven different surface chemistry constituents: positively charged cetyltrimethyl ammonium bromide (CTAB), poly(diallyldimethyl ammonium chloride) (PDDAC), polyethyleneimine (PEI), and aminopolyethylene glycol (PEG-NH_2); negatively charged polystyrene sulfonate (PSS) and carboxipolyethylene glycol (PEG-COOH), and neutral metoxipolyethylene glycol (PEG-OCH_3) [228]. They subsequently exposed them to macrophages in serum-free medium and found out that surface charge played the major role in macrophages internalization; being the positive charge AuNR the ones more efficiently internalized. However, after incubation in human plasma and hence in presence of protein corona, those AuNR that contained abundant complement cascade protein corona (C3a and C5a) showed more uptake due to their recognition by the complement receptor CD35. However, those that had a greater apolipoprotein fraction in their corona were internalized due to their recognition by the scavenger receptor CD36. We will not go into detail on the different mechanisms by which nanoparticles can be removed from circulation by the MPS, as this is a topic that is outside the focus of this review. However, we would like to emphasize that the mechanisms of cellular uptake cannot be generalized, and depending on the cell line used, some receptors will stand out over others.

Different strategies are being studied to avoid MPS and improve the circulation time of nanoplatforms. The widely used strategy, the stealth effect, consists of grafting a stealth-coating layer in which the gold-standard polymer used is the poly (ethylene glycol) (PEG). The hydrophilic shell of PEG prevents the nonspecific adsorption of opsonins and antibodies onto the NPs and avoids the uptake by the phagocytic cells prolonging the circulation time in the body [229]. However, many studies and clinical trials have reported the presence of antibodies anti-PEG, that not only hampers the therapeutic effect but also generates immune responses [230]. There are systematically reviews that thoroughly discussed the major causes involved in the immunogenic reactions associated to PEG that treat the safety of PEG-coated nanomedicines [231–233]. The use of "self peptides", as for instance the CD47, is an interesting approach to evade MPS clearance of nanocarriers. CD47 is a glycoprotein expressed on mammalian cell membranes whose extracellular domain could interact with signal regulatory protein alpha (SIRPα) expressed in phagocytes surface inhibiting phagocytosis. Thus, CD47 is a putative marker of "self" that gives phagocytes a "don't-eat me" signal [234]. Tang *et al.* demonstrated that liposomes labelled with a mouse "self peptide" can adhere to macrophage membranes and, thus, inhibit uptake by macrophages for>24 h *in vitro.* In addition, a delay clearance of liposomes by the liver and spleen for>48 h *in vivo*, prolonging the circulation time of subsequently injected poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs) was shown [235]. Another strategy is the use of agents that block phagocytic uptake, such as the clinically approved proton-pump inhibitor esomeprazole (ESO), as pretreatment before the injection of the nanocarrier of interest. Belhadj *et al.* conducted an *in vivo* experiment in which they confirmed that ESO pretreatment greatly decreased liver and spleen distribution of the developed nanobased drug carrier [236]. Unlike stealth nanoparticles, MPS blockade provides a limited window of time to prevent/decrease nanocarrier clearance as consecutive injections of the blockade agent could eventually lead to systematic toxicity.

4.1.3. Intracellular trafficking

After systemic administration, if the targeted tissue of a given nanomedicine lies beyond the vascular wall, it must have the ability to cross the endothelium to reach the extracellular matrix (ECM). It is noticeable that for several illness (e.g., cancer, diseases involving acute inflammation) is possible to achieve a heterogenous distribution of the nanoparticles by passive targeting. The disruptions that compromise the permeability of the microvessels favor the transvascular transport of the nanomaterial. In addition, nanocarriers must be able to cross the interstitium, the space between cells and tissues which is surrounded by cell membranes and blood vessel walls. This transport is influenced not only by the physicochemical properties of the nanocarriers, but also by the physiological and physicochemical properties of the interstitial space (e.g., interstitial pressure, structure, composition). Indeed, the interstitium of tumors not only differs greatly from that of healthy tissues, but also among different tumor types. Although in all tumors the leaky vasculature and dysfunctional lymphatics leads to interstitial hypertension, the ECMs composition, architecture, mechanical strength, and degree of compaction varies considerably between different types. Indeed, while pancreatic cancer is surrounded by a fibrous wall that hampers drug to penetrate, glioblastoma has a less compact consistency [237,238]. Thus, the influence of tissue stroma on the nanocarrier diffusion and distribution within the targeted tissue has also to be considered for a successful therapy design. This migration of the nanocarriers could also be favored due to the remote application of an external stimulus. As an example, several articles have showed that magnetic nanoparticles could act as hot spots upon exposure to AMFs. Indeed, using collagen-based 3D cell culture models it has been shown a significant enhancement of MNP uptake by cells and a disruption of the collagen matrix induced by the local temperature rise around MNPs during the AMF application [239].

If cellular uptake is needed for the therapy, more barriers should be considered. This process generally occurs via endocytosis, but when the therapy is triggered by the application of an external stimuli, also direct entry of the nanocarrier to the cell cytoplasm could be favored by several mechanisms (*i.e.,* translocation, lipid fusion, electroporation or alterations in the bilayer membrane of the cells). Upon endocytosis, however, nanoparticles are typically confined within intracellular vesicles (*i.e.,* endosomes, phagosomes, or macropinosomes) without an immediate access to the cytoplasm. Noticeable, this route of entry plays a critical role in immune adverse reactions, such as, induction of cellular inflammation since endosomal vesicles are important sites for certain toll-like receptors (TLRs) and major histocompatibility complexes. TLRs recognizes pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). They have an essential role in innate immunity and mediate in different cellular response to nanocarriers exposure [240].

Upon endocytosis, many nanomedicines must reach a specific intracellular location, such as the nucleus or the cytosol, to perform its function. Several strategies have been developed to overcome the endosomal barrier, including triggering pore formation in the endosomal membrane, pH-buffering effect ("proton sponge" effect), fusogenic mechanism, or photochemical disruption of the endosome membrane. However, as it has been recently reviewed by Smith *et al.*, a greater understanding of the properties that govern endosomal escape is crucial for the development of more effective drug delivery systems to reliably deliver cargos to the cytosol [241]. Nevertheless, if the nanocarriers are not able to escape the endosome, they may enter the recycling pathway. Carriers are routed toward lysosomal degradation and/or undergo exocytosis. To add more complexity to this cellular transport, accumulative evidence is indeed revealing that the transcellular transport route (entry into one cell, exocytosis, and re-entry into another) is also important in mediating the intercellular exchange of nanoparticles [242]. Thus, even if the nanocarrier could not escape from endosomes, the particle (or its cargo) may still arrive at its site of action and perform its function in another cell.

The different outputs depend, not only on the physicochemical properties of the nanocarriers, but also on the engineering of their

surface and on the effect of remote stimulus. As an example, several articles have showed that if MNPs are located inside lysosomes, the energy released upon AMF exposure may cause lysosomal membrane permeabilization [243]. Therefore, in the case of nanocarriers responsive to remote stimulus, their external activation could indeed favor a different intracellular pathway than the one originally destined due to its physicochemical characteristics. Even though little research has been published about the intracellular trafficking pathways, thoroughly reviews on endocytosis, cell fate and nanotoxicology of nanomaterials has been published and can be consulted if it is of interest to delve into this topic [240,244,245].

It is also important to consider that the nanoparticle physicochemical properties and aggregation states can change during intracellular trafficking within the intracellular vesicles. This implies that changes to their responsiveness to the remote stimuli used for their activation could be expected. Etheridge *et al.*, using IONPs as a model system, demonstrated a 50 % reduction in Specific absorption rate (SAR) when simulating tissue environmental conditions using PBS and 1 % agarose mixture (common tissue phantom) [246]. Liu *et al.* studied the aggregation states of DNA-decorated gold (fPlas-gold) nanoparticles during intracellular transport [247]. They concluded that in the early stages of endocytosis, fPlas-gold nanoparticles appear mostly as single particles and they clustered during the vesicular transport and maturation. These dynamic rearrangements can not only negatively affect the outcome of the nanocarriers when activated by external stimuli but can also have a positive impact. In this sense, while Mulens-Arias *et al.* characterized the structure and density of AuNP rearrangements triggered by endosomal uptake and lysosomal confinement, they proved the existence of a plasmon coupling-like process when AuNPs are internalized. A 2nd plasmon resonance band was shifted to the NIR region when the nanoparticle size and fractal dimensions of the intracellular cluster increased. The intracellular plasmon-coupling phenomenon translates to an efficient heating efficiency with the excitation at 808 nm in macrophages, endothelial cells, and colon cancer cells. This phenomenon resulted in an advantage since the NIR-transparent canonical AuNPs were transformed into NIR-absorbing clusters in the tumor microenvironment [248].

4.1.4. Nanotoxicity

After the cellular uptake and cell trafficking, the next major issue is nanotoxicity. Nanotoxicology began to gain importance in order to have a better understanding of the toxic and undesired effects that these materials can generate in living organisms that hampers their application. The study of this field opened a new window for achieving reliable safety evaluation and proper regulation of nanomaterial production, use, and deposition [249]. It is well-known that nanomaterials behave in a different way that their bulk material due to e.g., the small size, large surface to volume ration, high percentage of atoms and molecules in the surface, surface forces, unique chemical and physical properties, and high and fast adsorption and absorption of molecules (gas or liquid phases)[250]. Moreover, the great heterogeneity in terms of chemical composition, impurities, functionalization strategies, surface properties (surface charge/hydrophobicity/ligands), size, shape, agglomeration, of nanomaterials also affect their behavior. Focusing on gold and magnetic nanomaterials, systematic reviews can be found in literature, which thoroughly discussed their *in vitro* and *in vivo* toxicity [251–255].

There are several studies that suggest that one of the main molecular mechanisms driven magnetic and gold toxicity is oxidative stress, an imbalance between the production of reactive oxygen species (ROS) and antioxidant mechanisms. It has been proved that MNPs induce toxicity on rats' lymphocytes, on neuroblast cells or on lung fibroblasts, among others, via ROS generation in a concentration and time-dependent manner [256–258]. Regarding gold nanoparticles, Li *et al.*, performed a systematic study to evaluate the size-dependent toxicity of this material [253]. The study reported that the *in vitro* toxicity is both dose- and size-dependent; hence, those of higher concentration and smaller size would cause more cytotoxicity. Moreover, the intracellular ROS production determination indicated that AuNPs with smaller sizes induced more oxidative stress and caused more subsequent damages to the cells, which was coincident with the cytotoxicity result. ROS generation is mainly attributed to the presence of pro-oxidant functional groups on their reactive surface or due to nanoparticle-cell interactions [259]. In case of iron (Fe^{2+}), it can give and receive electrons; thus, it may participate in Fenton's reactions. Fenton's reaction consists of the reaction between Fe^{2+} and peroxide hydrogen (H_2O_2) generating $OH\cdot$ radical inside the cells. The generated oxidative stress will completely alter the cell homeostasis leading to protein misfolding and aggregation, lipid peroxidation, DNA degradation and breaking and genome mutations. All these events will eventually lead to cell death [254]. Among the different cell death mechanisms e.g., apoptosis, necrosis, and autophagy [245]; this last mechanism is gaining more and more evidence as the major cell death mechanism-derived from nanomaterials [260,261]. Briefly, autophagy is the catabolic process involving the sequestration of the cytoplasm within double-membrane vesicles in which autophagic targets are degraded to maintain cell homeostasis [245]. In fact, autophagy is associated to some process of nanotoxicology derived from nanoparticles' inter- and intracellular transport [262,263]. However, little is known about the underlying mechanism. Man *et al.*, used polythilenimine coated MNPs (PEI-MNPs) as a model to report autophagy induction via activation of both NF- κB and TGF-β signaling pathways in cancer cells. *Jin et al*, demonstrated that two clinically used superparamagnetic iron oxide nanoparticles (SPIONs) specifically induced macrophage autophagy through activation of TLR4 pathway [264]. The modulation of autophagy by nanomaterials can be exploited in the biomedical field for e.g., in cancer treatment. PEI-coated MNPs were reported to activate two pro-inflammatory pathways involved in autophagy in HeLa cancer cells [265]. However, this crosstalk between autophagy and nanoparticles must be appropriate tuned to avoid intrinsic cytotoxicity [262,266,267].

Noticeable, the study of nanotoxicity is not trivial. It is determined not only by the physicochemical properties of the material, but also by the cell line or model used and the assay, which sometimes leads to controversy between authors [251]. Another major factor is the exposure to external stimuli. A study published by Bae *et al.*, reported reduced cell viability, apoptosis and cell cycle aberrations on hepatocytes *in vitro* and *in vivo* when MNPs were exposed to static magnetic field [268].

As it is very well discussed by Tirumala *et al.*, there is an urgent need in development novel methods to assess nanoparticles safety and ensure their arrival to the clinic, as well as proper regulatory standards for assess safety [269]. This last issue will be further discussed deeply. Examples of new nanotoxicity assessment methods are already under development. It is well known that miRNAs are associated to disease states, and Han *et al.* have taken advantage of these biomarkers to develop a functional nanoprobe to evaluate the potential toxicity of nanoparticles through detecting multiple miRNAs in nanoparticle-exposed living cells [270]. They have constructed three oligonucleotide-based sensors that recognized specific nucleotide sequences based on fluorescence resonance energy transfer (FRET) mechanism. With this tool they were able to in situ detect two miRNAs (miR-21 and miR221) involved in oxidative stress, inflammation, and tumorigenesis.

4.2. Models to evaluate biocompatibility and nanotoxicology

In vitro assays for testing materials for on demand drug release seem to be retained in 2D cell cultures. One of the main drawbacks of this system is its inadequate representation of the physiological complexity, as the cells lack the interaction with each other and with the ECM according to their specific cellular organization in the targeted tissue [271,272]. Few articles have been published in which 3D cell models have been used to test effectiveness on achieving remotely-triggered drug release from nanosystems. Such is the case of Moreira *et al.* who encapsulated doxorubicin in a nano-in-micro particles and tested their therapeutic efficiency using 3D cell cultures. Nanoparticles consist of a gold core mesoporous silica shell and the microparticles were made of salicylic acid in poly (lactic-co-glycolic acid). They showed that the use of the developed pH and temperature responsive nano-in-micro spheres allow to reduce the size of the HeLa spheroids up to 48 % by NIR-triggered drug release. [273]. Nguyen *et al.* also used 3D culture model (breast cancer spheroids) to show the feasibility of a spatio-temporal- controlled drug release in response to NIR laser irradiation of what they called macrophage-based microrobots. These microrobots were prepared by co-loading SPIONs and doxorubicin-containing thermosensitive nanoliposomes (TSLPs) into macrophages (MΦs) isolated and differentiated from spleens of BALB/c mice. Due to the tumor-homing ability of MΦs and their loading with SPIONs, the obtained microrobots presented a dual tumor-targeting function regulated by both chemotaxis and an external static magnetic field (magnetic targeting). MNPs also promoted photothermal heating in response to NIR irradiation, which trigger DOX release from the TSLPs. Besides, *in vivo* experiments confirmed that after a single intravenously injection of the microrobots, the combined application of the magnetic field (to enhance tumor targeting) and NIR laser (to trigger DOX release) markedly inhibit the growth of tumors with a subtherapeutic dose of DOX. [87]. However, most published studies used two-dimensional (2D) cultures or 3D hydrogels with static mechanical properties and static incubation with the cell culture media. Unfortunately, these cell culture platforms do not capture the dynamic landscape of a dynamic stiffening tumor microenvironment. These limitations have led to the improvement of traditional cell culture systems to mimic the complexity of native systems, through the development of new techniques for co-culturing multiple cell types and the development of complex *in vitro* models known as organs-on-a-chip. [274]. Organ-on-a-chip devices are a prodigious alternative to conventional 3D gels or spheroids for testing nanodevices. It is a promising technique that can mimic a specific human model that has functional responses on the level of organs or tissues, which may accelerate the safety evaluation and biological behavior of nanocarriers [275,276].

The scientific community is dedicating many efforts to the development of robust, cost-effective, standardized, and reproducible 3D heterotypic human cell-based culture models. All of that for unbiased high-throughput screening of any novel nano-based therapeutic strategies. However, the use of simple models to assess toxicity and efficacy of materials for biomedical applications is gaining more attention in the scientific community. The invertebrates (e.g., *Hydra vulgaris, Drosophila melanogaster*, *Caenorhabditis elegans*...) [277,278] and vertebrates other than rodents (e.g., *Danio rerio* aka zebrafish) [279–281] are starting to be used for nanomaterial risk assessment. Table 2 summarized the advantages of these models for safety and efficacy studies of nanocarriers with potential biomedical applications.

Hydra is a diploblastic animal with an outer ectoderm and an inner endoderm separated by a non-cellular mesoglea. At one end of the body is a mouth surrounded by a ring of tentacles. *Hydra* is highly sensitive since all the cells are in contact with the aqueous environment and the permeation of toxic substances into the animal is facilitated. *Hydra* reproduces fast and is easy to culture in the laboratory becoming a cost-effective model. Therefore, *Hydra* can be used to study the toxicological impact of nanomaterials on morphology, reproduction, and regeneration capabilities. Since the genome of Hydra is already sequenced, molecular mechanisms involved in nanotoxicity can be elucidated [282]. Also, it is important to mention that *Hydra foxO* genes, a family of transcription factors involved in responses to oxidative stress significant parallels in regulation to those of bilaterian animals [283]. Different nanomaterials have been tested in Hydra *e.g.*, diatomite porous biosilica [284], silver [285], gold [286]and magnetic nanoparticles [287].

As for vertebrate models, although the similarities and differences between zebrafish and humans have to be fully investigated, the high degree of metabolic and physiological conservation between the two organisms makes it a potentially ideal model. In addition, the optical characteristics and rapid life cycle of this animal make it attractive for high-throughput screening of molecules, drugs and nanocarriers. [288]. For example, this animal model has been used to test mesoporous silica and metal nanoparticles [280,288,289].

These models offer promising alternatives prior to *in vivo* assays to strengthen preclinical studies and improve the success rate that nanotherapeutics currently have in clinical phases. The extrapolation from animal to human remains challenging. The use of biomimetic models for unbiased testing of nanomedicines efficiency along with more detailed assays to assess safety and pharmacoki-

Table 2
Characteristic of invertebrates and vertebrate animal models used for nanomaterial risk assessment.

Hydra vulgaris	*Caenorhabditis elegans*	*Danio rerio*	*Drosophila melanogaster*
Small size	Small size	Small size	Small size
Fast reproduction	Short life span	Rapid development	Short generation time
	Hermaphroditic.	High number of embryos	Short life span
	Synchronized isogenic populations		Large number of offspring
	Highly amenable to genetic manipulation	Highly amenable to genetic manipulation	Highly amenable to genetic manipulation
Easy to culture and cost-effective	Easy to culture and cost-effective	Easy to culture and cost-effective	Easy to culture and cost-effective
Transparent body	Transparent	Optical transparency. Transparent embryos	
Able to regenerate		Physiology and metabolism conservation with human	Good conservation of basic signaling pathways and cellular processes
6071 genes shared with humans	4571 genes shared with humans	70% of orthologue genes to humans	5696 genes shared with humans
No animal licensing laws	No animal licensing laws		No animal licensing laws

netics (absorption, distribution, metabolism and excretion (ADME)) properties of stimulus-responsive nanotherapeutics are an urgent need. As it can be seen in Table 3, inbred mouse strains have been preferred over outbred stocks, with strains such as C57BL/6 and BALB/c as the most used. However, in order to obtain results that are maximally generalizable across conditions and populations, defined outbred stocks from heterogeneous backgrounds are more appropriate and much more cost-effective research subjects.

Contrary to the general opinion, the adoption of outbred mice as research subjects might improve future experimental replicability. In addition, inbred mice fail to reproduce the variability and complexity of each disease patient population. Mind that each patient will be conditioned by age-associated, hormonal, and pathological processes that may affect not only target organs and, thus, biodistribution routes, but also, metabolism which indeed will alter detoxification, biodisponibility and pharmacokinetics processes. However, current studies of nanotoxicity in animal models with a brain, cardiovascular system, liver, digestive tract, reproductive system, and skin diseases are unsystematic [290]. Another challenge is that *in vivo* assays should be performed with a drug dosage and a route of administration that ensure a good relativity among the *in vitro* experiments, animal evaluation and human trials. In addition to that, the duration of the *in vivo* experiments should correlate the period of therapy to assess not only acute but also chronic toxicities that could arise. Finally, a major challenge is still the translation for mice to human of the immune system reactions. Little is known about the processes involved with those NPs recognized by the immune system. Few works have showed that proteins adsorbed onto the surface of nanoparticles can activate macrophages *via* surface receptors, resulting in the secretion of pro-inflammatory cytokines [291]and others have seen that NPs can be sensed by Toll-like receptors (TLRs), Pattern Recognition Receptors (PRRs) on the surface of phagocytic cells [292,293]. TLRs comprise a family of evolutionary conserved pattern recognition molecules that have an essential role in mammalian innate immune defense. It has been described that TLR orthologues are expressed differently in mice and humans due to variations in the expression of TLR transcripts in different cell types and different transcription regulation on cellular activation [294].

4.3. Regulatory challenges

Considering the lack of photonic and magnetic materials in the clinic, we think appropriate to give a general view of the nanomaterials' regulatory challenges. The first clear definition of nanomaterial was recommended by The European Commission (EC) based on the European Commission Joint Research Center and on the Scientific Committee on Emerging and Newly Identified Health Risks. The definition was stated based uniquely on the size, without con-

Table 3
Experimental mice models most frequently applied with photonic and magnetic nanocarriers for external stimuli drug delivery.

Nanovehicle	Mouse model	Route administration	Time Treatment	Targeting	Reference
FluidMAG-CMX-Dox	BALB/c mice. HeLa cells injected subcutaneously	Intratumoral	21 days	No active targeting	[319]
Liposome with acid-coated magnetic Fe_3O_4 and CPT-11	BALB/c nude mice. U87 cells injected orthotopically	Intravenous	21 days	Anti-EGFR monoclonal antibody (Cetuximab)	[53]
Fe_3O_4 and DOX in PLGA nanomatrix	BALB/c mice. CT26 cells injected subcutaneously	Intratumoral	15 days	No active targeting	[320]
PLGA-DOX-MoS_2 nanosheet	BALB/c nude mice. 4 T1 cells injected subcutaneously	Intratumoral	40 days	No active targeting	[161]
DOX-containing chitosan/carboxymethylcellulose functionalized mMoS_2 nanocomposites	BALB/c mice. 4 T1 cells injected subcutaneously	Intravenous	14 days	No active targeting	[160]
DOX-containing CuS NPs, styrene (St), N-isopropylacrylamide (NIPAm), methacrylic acid (MAA) and Gd(AA)3phen copolymer	BALB/c mice. 4 T1 cells injected subcutaneously	Intravenous	14 days	No active targeting	[321]
Neuron growth factor encapsulated in electromagnetized carbon porous nanocookies	SD male rats	Surgery	30 days	No active targeting	[322]
DOX, DTX and iron oxide NPs encapsulated in chitosan hydrogel	BALB/c mice. MDA-MB-231 cells subcutaneously injected	Intratumoral	84 days	No active targeting	[96]
COF-Au-DOX nanosheet	BALB/c mice. 4 T1 cells injected subcutaneously	Intravenous	14 days	No active targeting	[318]
DOX-loaded single-walled carbon nanotube embedded hydrogel	BALB/c mice. 4 T1 cells injected subcutaneously	Surgery	21 days	No active targeting	[209]
DOX and CDDP- loaded titanium nitride (Ti_2N) MXene-based nanosystem	BALB/c nude mice. MCF-7 cells injected subcutaneously	Intravenous	28 days	Bombesin	[323]
$CaCO_3$-coated gold nanostars with Ce6 into PBMCs-derived NK cells	C57BL/6 and BALB/c nude mice. A549 cells were inoculated to the abdomen.	Intravenous	25 days	NK cells	[324]
ICG and R837- loaded hydrogel	BALB/c mice. 4 T1 or A375 cells injected subcutaneously	Intratumoral	35 days	No active targeting	[325]
Amoxicillin and ibuprofen loaded CP/OD hydrogels	Kunming mice	Subcutaneous	35 days	No active targeting	[182]
Chloroquine phosphate (CQ)-loaded photosensitive nanoMOF coated by heparin	4 T1 cells bearing mice (strain no detailed)	Intravenous	14 days	No active targeting	[326]
POM-anchored hollow mesoporous organosilica nanoparticle (HMON)	BALB/c mice bearing U87MG cells	Intravenous	7 days	No active targeting	[158]
DOX-loaded silica nanoparticles embedded in IR820/ methylcellulose hydrogel	BALB/c mice and BALB/c nude mice. Cal27 cells injected subcutaneously	Peritumoral	21 days	No active targeting	[156]
Gold/platinum star-shaped core conjugated with a GSH-sensitive disulfide bond, atargeting ligand (rHSA-FA), IR780 and glucose oxidase (GOx)	MGC-803 cells bearing nude mice (strain no detailed)	Intravenous	15 days	Folic acid probes	[157]

sidering the hazards and risks derived from the constituent particles. Nanomaterial definition compromised natural, incidental, or manufactured materials containing unbound, agglomerated and aggregated particles provided that for 50 % or more of the constituent particles are in the size range of 1 nm to 100 nm [295]. This regulatory recommendation was thought as a tool for regulatory bodies to stablish their own guidance describing nanomedicine legislation regarding quality, safety, efficacy, and risks assessment [296].

Although the EMA introduced the term nanomedicine as systems for clinical applications, with at least one nanoscale component, resulting in specific properties and characteristics that can be defined for the intended use (e.g., route of administration, dose) and associated with the expected clinical benefits (e.g., preferential distribution at the organ/tissue level) [295], the FDA has not still established regulatory definitions of "nanotechnology," "nanomaterial," "nanoscale," or other related terms. Based on the definition of "nanotechnology" stablished by the National Nanotechnology Initiative Program which highlights (1) one dimension in the nanoscale range and (2) exhibit properties attributable to its dimensions; the FDA criteria for evaluating the safety, efficacy, public health impact, or regulatory status of nanotechnology products focus exclusively on the unique properties and behaviors that the application of nanotechnology may confer [297,298].

The inconsistency in the regulatory framework is more evident when we try to categorize these nanosystems as medicinal products or medical devices. A challenging decision that must be considered by the scientific community since the regulatory regimes for market authorization are substantially different. According to the current EU directives, the mode of action is the key point for a nanomedical product to be considered as medicine or medical device. With medical devices, the mode of action is physical (mechanical or chemical), while a medicinal product acts by pharmacological, immunological, or metabolic means [299]. FDA distinguishes three product areas according to whether the product has a chemical (drug), mechanical (device), or biological mode of action. (*i.e.*: biological product applicable to the prevention, treatment, or cure of a disease or condition of human beings). FDA-regulated medical products meet the definition of "drug" as articles that are intended to affect the structure or any function of the body of human beings or other animals [300]. Besides, the FDA points out that "devices" does not achieve their primary intended purposes through chemical action and do not dependent upon being metabolized. For example, both magnetic and gold nanoparticles can be injected into a tumor site and exposed to electromagnetic energy. The absorbed electromagnetic energy will be converted into thermal energy and this local heat is transferred to the surrounding cancer cells. The heat transfer, as opposed to a binding interaction with the nanoparticle, causes the cancer cells to die. Therefore, this effect is not achieved through chemical action and nanoparticles will be considered as devices. However, when talking about multifunctional drug delivery systems that release their API to the diseased tissue or organ under the presence of an external stimuli (*i.e* magnetic fields, ultrasound, light), the combination of drugs and devices in the same nanosystems has to be considered under the regulatory framework. Besides drug–device combination, it is also possible to have biologic–device combinations (such as an endothelial cell growth factor-loaded nanocapsule) or even drug–biologic-device combinations (such as an antibody-functionalized chemotherapeutic drug-loaded nanoparticle). This more complex nanosystems entail additional challenges in their regulation since they can exhibit more than one mechanism of action. Both the FDA and the EMA are considering nanoengineered products as combination products [301,302]. In this scenario, the primary mode of action, defined as the way the product achieves its major therapeutic effect, is the main criteria for categorizing and reviewing a combination product. The FDA has stablished the submission of an abbreviated new drug application (ANDA) for the approval of a nanomaterial as long as the applicants can demonstrate that the nanosystem is bioequivalent to the reference-listed drug. According to the FDA, the existing health and safety tests that are used to assess the safety of normal size materials (*i.e.*, "traditional bulk counterparts") are generally considered adequate to assess the health effects of nanoproducts. Under this statement, if the macroscopic version of a material is considered as safe, then, the nanomaterial is safe as well. It bears noting that bulk materials and their corresponding nanoscale versions are not synthetized in the same way and, hence, different traces may be found in the final product that can induce toxicity in the body, and that cannot be determined in the bulk-material. In addition, physico-chemical properties of nanosystems are ruled by their size, charge, shape and polarity, among other properties. Besides, due to their unique size-related properties (*i.e.* magnetism, fluorescence...) interferences with the therapeutic outcome could take place. Therefore, it seems plausible that the pharmacokinetics and pharmacodynamics of a nano-system will never be the same as its bulk counterpart. The urgent need for a specific regulatory framework for nanosystems is clear, and in this sense, several critical perspectives could be consulted to deepen this topic [303–305].

Among the materials on which nanoparticles currently approved for clinical use are based, large majority are polymeric (Copaxone®, Genexol®, Oncaspar®), liposomal (Doxil®, Caelyx®, Mepact®, Myocet®), nanocrystal (Emend®, Rapamune®) or metal-based (NanoTherm®, Sinerem®) materials. A list of globally marketed nanomedicines approved by the FDA and the EMA can be found in the review recently published by Halwani [306]. These materials seek functional effects such as increased bioavailability, decreased dosage, or increased potency of a drug product, decreased toxicity of a drug product or improved delivery of a functional molecule. Contrary to the simplify design of the approved nano-systems, the advances in nanotechnology, along with researchers' ambitious, lead to more complex systems with multiple components in ideal spatial arrangements. This complexity in structure, form and size affects the biological behavior and hence the clinical application, which bring to light the still unmeet challenges regarding the characterization and categorization of these systems.

4.4. Seeking standardization protocols and risk assessment policies for nanotherapeutics

Soares and colleagues already discussed the importance of developing standardized methodologies and reference materials to harmonize the characterization of nanomaterials [296]. It is important to know the limitations and strength of the techniques used while evaluating the characterization and biological behavior of nanomaterials. Scientists in the nano field are great aware of the absence of reference materials to calibrate analytical tools for comparative analysis not only among batches, but also among different groups. Moreover, different physical and chemical properties in the ligands' batches alter the properties of the final nanocarrier. All of these and other factors such as sample preparation or even interpretation of the results, underscores the need of standard protocol to provide reliable information. A key point of discrepancy is the characterization of nanomaterials. In order to analyze the size of a given nanoparticle, authors commonly used dynamic light scattering (DLS), scanning and transmission electron microscopy (SEM and TEM respectively), nanoparticle tracking analysis (NTA), UV VIS spectroscopy or size exclusion chromatography. Electron microscopy is the technique that provides the most accurate estimation of size and homogeneity of nanoparticles. On the

other hand, DLS allows having the average measurement of the hydrodynamic diameter of the NP and the solvent molecules that diffuse at the same rate as the colloid in a liquid environment [307]. Regardless of the technique used, factors such as morphology, sample preparation or aggregation states will directly affect the measurements.

Considering cytotoxicity assays, commonly used approaches performed in the laboratory consists in the evaluation of e.g., the metabolic activity of mitochondria dehydrogenase enzymes (MTT, XTT, WST-1, CellTiter Blue®), the ubiquitous intracellular esterase activity (Calcein AM), the integrity or permeabilization of cell membrane (Trypan Blue, Propidium Iodide), oxidative stress levels (DCFDA) or phospholipid content in cell membrane (Annexin V). However, as Hoskins *et al*., discussed, nanoparticles may interact with these assay systems resulting in overestimated viability results and irreproducibility and inconsistency among them. [308]. Kumar et al. conducted a comparative study of six different cell viability techniques (CellTiter Blue®, DCFDA, MTT, Calcein AM, Propidium iodide and their own developed system called Bodipy. FL.L-cystine (BFC)) to identify the most reliable and reproducible one in two cell lines (Ln229, a glioblastoma cell line, and MDA-MB231, a triple negative breast cancer cell line). Bodipy.FL.L-cystine (BFC) is a fluorescent L-cystine molecule previously developed for labelling proteins and nucleic acids, and for measuring intracellular Trx/GSH levels [309]. The combination of CellTiter Blue® with their BFC assay was found to be the most efficient and accurate system to study cell viability. On the other hand, spectroscopic assays based on MTT and Calcein AM lacked reliability by not showing a consistent dose-dependent effect. [310]. Another important factor to keep in mind is the time required to perform the assay readout to report meaningful results. The majority of cytotoxicity assays evaluate the cellular fate after exposure to the nanosystems, without informing of the cellular physiological state. Therefore, understanding the toxicity of nanomaterials and establishing reliable methodologies for assessing nanotoxicity deserve further investigation. New techniques such as atomic force microscopy (AFM), a well-established characterization technique used for the topographical study of materials, is asking to be applied to biology. Hoskins *et al.* explored the potential of this technique to elucidate the interaction of Fe_3O_4 core NPs coated with PEI or both PEI and PEG in three human cell lines including neuroblastoma (SH-SY5Y), breast cancer (MCF-7) and macrophage-like (differentiated U937) cells. Their data indicate that the change in cell morphology after nanoparticle exposure may be the result of a different aspect of cellular stress not measurable by conventional endpoint cellular toxicity assays [311]. Briefly remark that quantification of nanocarriers internalization *in vitro* and biodistribution also are influenced by the technique (atomic spectroscopy, isotopic labeling, and nuclear analytical methods) [312]. Comprehensive reviews on novel methods and approaches for characterization, toxicity and efficacy evaluation of nanomaterials have been thoroughly discussed elsewhere [249,269,307].

In order to provide clarity in the methods and tools needed to ensure the safety, efficacy, quality, and performance of these systems, the FDA initiated a Nanotechnology Regulatory Science Research Plan in 2013. In response to this, the Nanotechnology Characterization Lab (NCL) was established. By employing a standardized analytical cascade, it oversees the physicochemical, immunological, pharmacological and toxicological characteristics and efficacy both *in vitro* and *in vivo* of nanodevices (Fig. 5).

This introduces that a clinically useful formulation should possess the properties of reproducibility, scale up possibility, and verifiability. Three properties that are not considered enough in the laboratories, where effort is dedicated to probe the application of the nanodevice to be published. A major step in the clinical translation is the identification of the critical steps in the manufacturing process comprising reagents and technical equipment. Accordingly, more attentions should be paid to the development of advanced approaches that can precisely control over the preparation process, for the purpose of generating nanoplatforms with required features, high batch-to-batch reproducibility, and industrial scale-up feasibility. In addition to standard production meth-

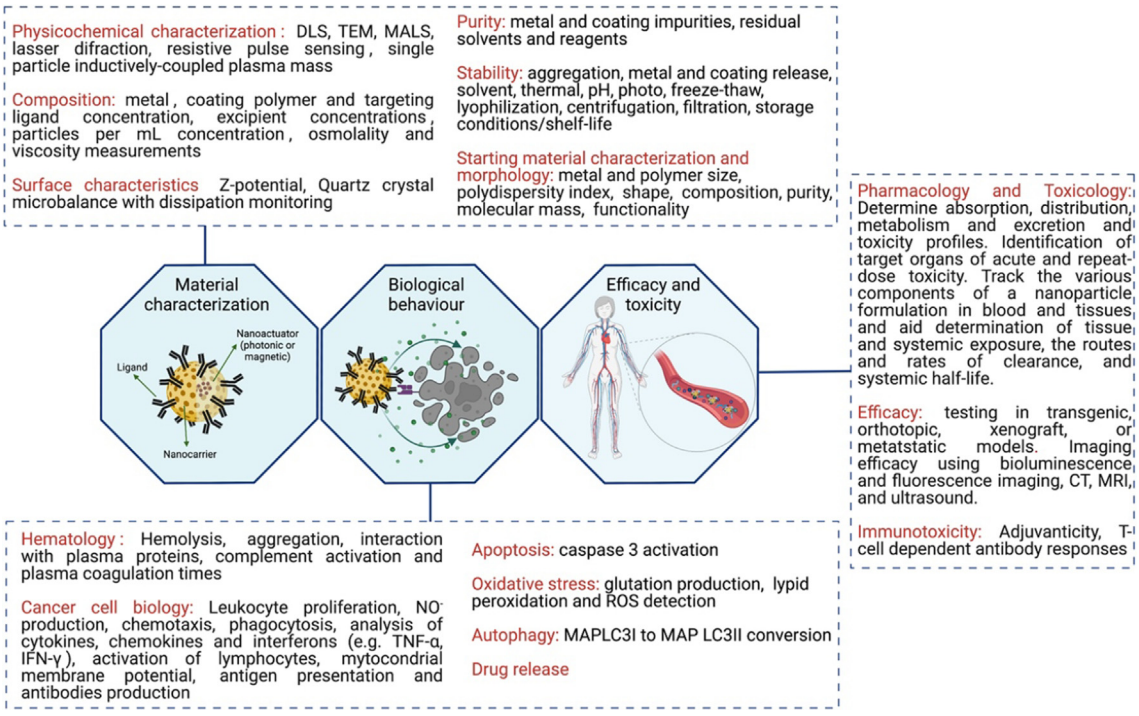

Fig. 5. FDA standardized analytical cascade for nanodevices. The protocol involves a series of chemical-physical characterizations of the nanomaterials, followed by *in vitro* biological studies, and by *in vivo* pharmacological and toxicological analyses. Created with BioRender.com.

ods, an accurate stimuli dosage control may finally accelerate the translation of smart drugs from the bench to the bedside.

Besides, another pressing need is to find and validate new approaches that can extrapolate acute *in vitro* outcomes to predict chronic *in vivo* effects. To this purpose, a few European initiatives focused on risk assessment of nanomaterials for their therapeutic use have been launched. These initiatives are focused on exploring, developing and testing different combinations of nano-specific safety studies. Among the including concepts highlight the use of predictive toxicology based on high-throughput screening techniques, well-designed nano-specific alternative test strategies (ATS) in agreement with the 3Rs rule (refine, reduce, and replace animal testing) by expanding the use of *in vitro* and in silico strategies, and the inclusion of rational design of nanomaterials from the earliest stages of development (safer-by-design concept). The latter concept implies that nanomedicine safety should be considered as an integrated pathway from the very early stages of research and innovation to the final stages of product validation. This is clearly a different concept from the classical safety assessment paradigm that seeks to address potential risks and regulate the therapeutic product downstream, close to its full development and market entry [313]. In this context, it is important to highlight that through joining the efforts among several EU H2020 addressing risk assessment and management of nanomaterials (e.g., RiskGONE, Nanorigo, Gov4Nano), a European Risk Governance Council (ERGC) is planned to be established as a science-based advisory and governance body for engineered nanomaterials safety. The planned ERGC will be also responsible of providing communication with stakeholders and civil society based on high-quality scientific evidence supporting a clear understanding of risks, their assessment and management within wider societal considerations [314].

5. Final remarks and future perspective

Nanotechnology is experiencing its golden age in recent years with the development of materials with new chemical-physical properties and the possibility of overcoming the limits of previous technologies. Great examples of this phenomenon are some of the vaccines prepared for the recent SARS-CoV-2 pandemic, which are based on nanoformulations using liposomes for mRNA drug delivery. In the last few decades, scientists over the world have focused their efforts on designing new complex systems that can overcome some of the most common drawbacks of traditional medicine: toxicity, inability to pass through biological barriers and rapid clearance. In particular, the possibility to include a nanoactuator, such as a photonic and magnetic nanomaterial, has open the door of a drug delivery controlled not only in space but also in time. This provides good prospects to create an optimized therapy for patients. Indeed, the application of an external stimulus on these nanomaterials allows for a sophisticated release of the cargo, thanks to chemical-physical and molecular changes in these smart carriers. The photonic and magnetic nanomaterial-based nanocarriers exhibit better outcomes compared to the already approved drugs, both in terms of efficacy and side effects. New strategies of pro-drug therapy activation rely on the incorporation of photonic or magnetic materials that can selectively generate heat under external stimuli, leading to the release in situ of the cargo.

Although basic science has made extraordinary progress by creating a plethora of smart DDS for spatio-temporal controlled release of the cargo, only a few carriers have entered clinical trials. The translation to a clinical medicine is still hampered by several factors: uncertain fate of the nanomaterial in the body, lack of standardization protocols and the presence of several regulatory frameworks. Nevertheless, the European regulatory organisms are moving on to create a comprehensive compendium for enhancing the reproducibility and the safety of these nanomaterials for clinical application. A uniform legislation will help in an easier translation of stimuli-responsive DDSs from the development stage, towards the complex phases of clinical trials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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