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Advanced Micro-Nano-Bio Systems for Future Targeted Therapies

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Abstract: This article aims at highlighting the most recent and promising research trends, the open challenges and the possible routes to follow in the field of targeted therapy. A highly interdisciplinary viewpoint has been used, trying to evidence and discuss the different opportunities deriving from recent evolutions of nanotechnology, polymer science, robotics and biotechnology. The most used vectors for nanomedicine applications are described, together with the different action strategies reported in the literature, such as passive targeting, site-directed targeting and remotely triggerable drug delivery. Special emphasis is given to magnetically triggered systems and ultrasound-responsive materials, identified as the most promising paradigms. Key competences and system integration strategies derived from robotics are also introduced, focusing the attention on the crucial issue of achieving high controllability of the vector at the micro- and nano-scale. Finally, biocomponents are described, highlighting their potential as functional sensing elements or smart mechanisms to be integrated on board of advanced micro-nano therapeutic devices. The conclusion aims at depicting the importance of novel and improved targeted therapy strategies, to be coupled with the emerging world of predicting and personalized medicine. To this aim, a real merging of skills and approaches, derived from the aforementioned research fields, is recognized as highly desirable and rich of opportunities.

Keywords: Drug delivery, micro-nano-bio systems, microrobotics, nanocarriers, nanomedicine, nanorobotics, nanotechnology, targeted therapy.

INTRODUCTION

According to a definition of the U.S. National Institutes of Health (NIH), a targeted therapy is "a type of treatment that uses drugs or other substances to identify and attack specific types of cancer cells with less harm to normal cells". Although cancer represents the main focus (being also embedded in the definition), targeted therapies can also find important applications in the treatment of many non-cancer-related diseases, from cardiovascular, to infectious and chronic inflammatory ones.

In any case, the key parameter to be considered is the Therapeutic Index (TI), defined as the ratio between the drug dose that produces toxicity in 50% of the population (TD_{50}) and the minimum drug dose that is effective for the desired therapy for 50% of the population (ED_{50}):

$$TI = \frac{TD_{50}}{ED_{50}} \quad (1)$$

A higher TI can be achieved by increasing the drug dose that produces undesired toxicity or reducing that for effective therapy. A proper drug targeting simultaneously has both effects, thus significantly increasing the TI of a specific compound.

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Traditional cytotoxic chemotherapy typically has a narrow therapeutic index: responses are often partial, brief and unpredictable [1], due to low compound solubility, toxicity to healthy cells, scarce accumulation at the tumor site, etc. These circumstances clearly highlight the need of more targeted procedures, with the challenging aim of achieving a "magic bullet" (a drug that selectively attaches to diseased cells but non-toxic to healthy ones), already postulated by Paul Ehrlich almost one century ago [2, 3].

Nanotechnology recently opened new exciting opportunities in the field of targeted therapy. The possibility to fabricate, characterize and control things at the nanoscale, formerly suggested by Richard Feynman's speech in 1959 [4], appeared to be concrete in 1981, with the first journal article on nanotechnology [5]. In the last two decades, a broad range of nanomaterials has been developed for targeted therapies. The unique features of these vectors such as large surface area, structural properties and long circulation time in blood compared with small drug molecules have allowed to increase the TI of anti-cancer drugs [6, 7]. In addition, incorporation into nanosystems allowed to reintroduce into clinical practice drugs that were no longer used, due to poor dispersion ability and suboptimal pharmacokinetic profiles [8].

The first objective of nanomedicine (defined as the design and development of therapeutic agents with diameters ranging from 1 nm to 1 μm) has concerned the development of passive nanocarriers. At the beginning, these systems mainly served as "drug containers" able to efficiently disperse therapeutic molecules in their structures, to assure a

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favorable blood half-life and to minimize the immune system response. They mostly relied on passive drug targeting, consisting of a preferential accumulation of nanocarriers at the tumor site, due to the enhanced permeation and retention (EPR) effect [9]. However, many of the carriers developed to this purpose show physico-chemical properties that allow for versatile modification possibilities. Thus, active targeting emerged, by employing specific affinity ligands able to recognize and bind to cancer cells or to the angiogenic endothelium surrounding the tumor, thus triggering drug endocytosis [10].

The described nanomedicine paradigm recently evolved towards responsive nanosystems, to be triggered by means of altered microenvironment conditions or by remote energy sources. This opened the way to more flexible drug delivery strategies, based on an internal trigger (e.g. a smart material affected by changes in its environment) or, more interestingly, on an external one, provided by an operator. Passive triggering can be achieved, for example, by synthesizing pH-responsive materials, able to deliver their drug payload in correspondence of relatively acidic microenvironments (such as those surrounding cancerous tissues) [11]. Active (remote) triggering requires to design *ad hoc* materials, responsive to one or more physical stimuli, such as light, magnetic fields, ultrasound, electrical fields, radio frequency radiation and microwave radiation [12]. Remote triggering is particularly interesting, as it enables on-demand drug release with repeated and reproducible dosing, with a potentially high increase of the TI of many compounds (Fig. **1**).

It has to be highlighted that passive triggering is a "transversal" mechanism, which is always present in targeted therapies. Indeed, it allows drugs to be released from the carrier and to result available for the cells. In the case of passive targeting, the extracellular conditions normally act as a trigger. In the case of active targeting, the altered chemico-physical conditions at intracellular level normally constitute the trigger for drug release. In the case of active triggering, passive triggering mechanisms are still on the stage, but they are undesired as they lower the efficiency of remote drug release control. Thus, chemical strategies are needed to provide the nanocarriers with a high resistance to physiological environmental shifts, making them responsive only to external triggers.

All the above mentioned research trends are strongly grounded on very specific disciplines, such as organic chemistry, materials science and pharmaceutics, in addition to micro/nanotechnologies. In parallel, the last decades were also characterized by the thrive of highly interdisciplinary research lines in which micro and nanotechnologies were associated with robotics, computer science and biotechnologies. These efforts aimed at developing miniaturized controllable devices (micro and nanorobots) able to perform operations at the nanoscale [13, 14]. This represents an ambitious objective that would have clear benefits for the treatment of many pathologies: nanorobots may aid in cancer therapy, controlled drug delivery, circulating diagnostic systems, single cell surgery and tissue repair [15]. Several technological difficulties in miniaturizing all the required components (sensors, actuators, control and communication systems and energy sources) hampered the development of complex nanomachines, so far. However, encouraging results are emerging and the nanorobotics community is rapidly growing around this interdisciplinary paradigm [16].

The integration of bio-components in miniaturized therapeutic elements is an aspect that concerns crosswise both smart materials for targeted drug delivery and miniaturized robotic devices. In the former case, specific engineered proteins, antibodies, aptamers, and other biological molecules can be integrated in synthetic carriers, to enhance their functionality [17]. In addition, whole living cells can be also in-

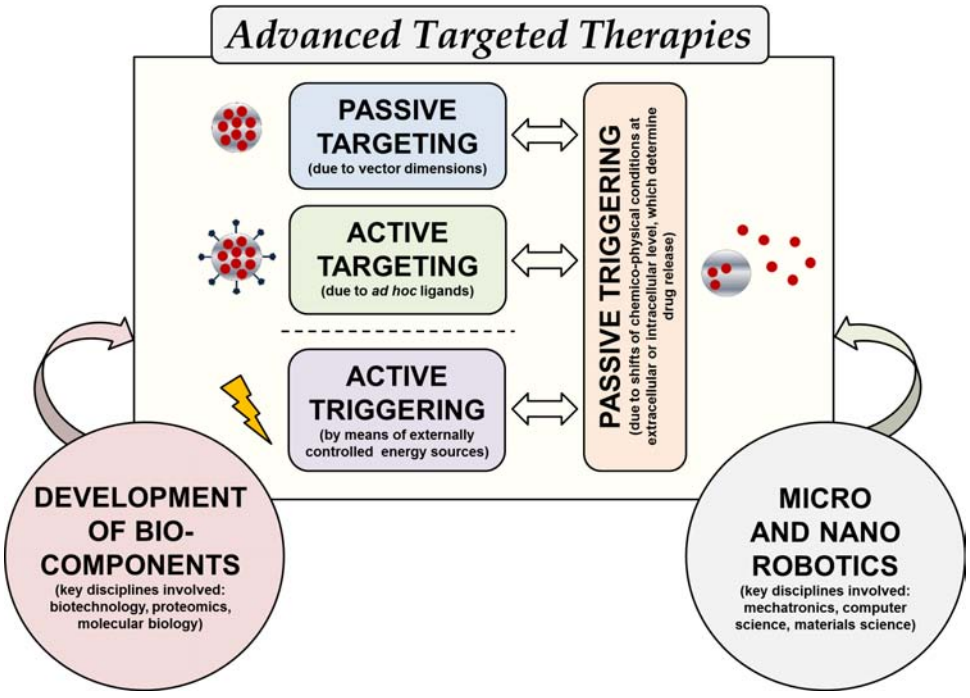

Fig. (1). Building blocks of advanced targeted therapies, included possible contaminations due to interdisciplinary efforts involving the fields of micro/nanorobotics and biotechnology.

cluded, e.g. to produce and locally deliver therapeutic or regenerative factors [18]. Cell-deriving exosomes recently emerged as effective means to deliver beneficial factors to target tissues [19]. In the latter case, molecular biology and biotechnology can be employed in order to provide miniaturized robots with bio-sensors (e.g. proteins able to recognize certain molecules and to induce conformational changes or to trigger other signals) or embedded miniaturized actuators, such as flagellated magnetotactic bacteria [20]. Future efforts in biotechnology are promising: therapeutic nanosystems will be increasingly built by exploiting existing biological components, especially if our ability to engineer them, thus finely tuning their physico-chemical properties, will progress.

Several review papers can be found in the literature, focused on one or few of the research lines described in this Introduction, and treating these topics in a rather specialized fashion. This work aims at providing a concise yet complete overview of the key scientific advances achieved in the last five years in the targeted therapy field and at highlighting the still unsolved issues and unknown aspects, thus possibly serving as a basis for a multidisciplinary approach to such research field. The focus will be centered on passive and site-directed nanocarriers, on remotely triggered nanosystems, on controllable micro/nanorobots and on the integration of bio-components. Finally, it will be highlighted that a convergence of the mentioned research areas may enable the emergence of novel paradigms, based on advanced micro-nano-bio systems, for safe and high-efficacy targeted therapies scarcely conceived, yet.

NANOCARRIERS FOR PASSIVE AND ACTIVE DRUG DELIVERY

Nanovectors

The most currently used nanosystems in anti-cancer pre-clinical and clinical studies [21] are depicted in Fig. (**2**).

Nanoparticles have a diameter of ~100 nm and can be made of different polymeric or non-polymeric materials. The most biocompatible and used in the clinics are polymeric nanoparticles. They can be distinguished between nanospheres, in which the drug is dispersed throughout the particle, and nanocapsules, in which the drug is entrapped in a cavity, surrounded by a polymeric membrane [22]. As all colloidal systems, nanoparticles are rapidly opsonized and cleared by macrophages. Thus, once injected intravenously, 90% of them is taken up by the liver and the spleen within few minutes. This drawback can turn in an advantage if macrophages or liver Kupffer cells constitute the target [23]. On the other hand, opsonization can be reduced by providing nanoparticles with hydrophilic surfactants adsorbed on their surface or block/branched copolymers: poly(ethylene oxide) (PEO) and poly(ethylene glycol) (PEG) have been widely used to this purpose [24]. In addition, nanoparticles show a high tailorability: they can be easily functionalized by ligands or other functional molecules, in order to enhance/modulate their properties. Thus, they represent promising and versatile tools in cancer therapy and, more in general, in drug delivery [25].

Polymeric micelles are amphiphilic copolymers that self-assemble in aqueous environment, thus forming spherical colloidal systems with the drug embedded in a central core. They are usually smaller than 100 nm and they are characterized by a hydrophobic core and a highly hydrophilic surface (named corona), which is of crucial importance to avoid the reticulo-endothelial system (RES) uptake, by preventing the interactions between the core and the blood components [21]. Micelles can be functionalized by conjugating ligands to the ends of the hydrophilic segments: peptide and sugar moieties on micelle surface have been used to effectively target receptors of specific cell types [26]. A plethora of different polymeric micelles showed up in the last years, from crosslinked triblock systems [27] to colloids composed of hyaluronic acid and phospholipids [28]. Novel formulations continue to emerge, with the aim of enhancing drug encapsulation and stability, self-associative properties and biocompatibility.

Liposomes are spherical vesicles formed by one or several phospholipid bilayers surrounding an aqueous core, in which drugs can be entrapped [29]. Unmodified liposomes undergo rapid clearance from the blood stream due to sequestration by macrophages of the RES. Long-circulating

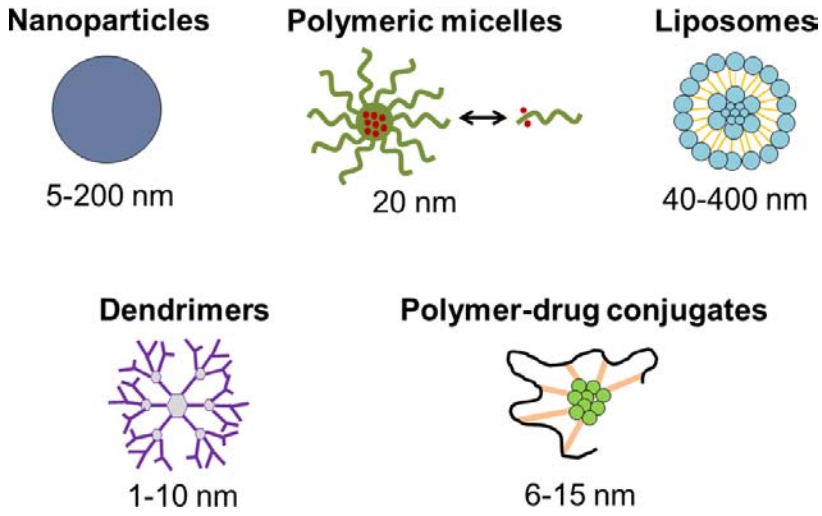

Fig. (2). Nanocarriers widely used, at present, in pre-clinical and clinical studies. Reproduced with permission from [21]. Copyright (2010): Elsevier.

liposomes can be achieved by grafting PEG or other hydrophobic chains on their surface, or by including phosphatidylinositol and gangliosides in their formulation [23]. Liposomes are really versatile structures: liposome-cell bio-interface features (binding, uptake, retention, etc.) and liposome diffusivity are the key parameters that affect the efficacy of such nanovectors. Recently, it has been demonstrated that cationic liposomes can be designed by playing on such parameters, thus allowing their diffusion within three-dimensional tumor spheroids [30].

Dendrimers are highly branched macromolecules with a controlled three-dimensional architecture. The central core is achieved through a series of polymerization reactions and the nanostructure grows in concentric layers, to produce stepwise increases in size up to ~10 nm. Drugs are normally attached to surface groups by chemical modifications [31]. At present, dendrimers are the only known synthetic nanovector category that allows mathematically defined control and systematic engineering of its nanostructure. As a consequence, dendrimer–drug conjugates are generally considered to be more adaptable to a greater range of nanomedicine administration routes and targeting strategies than the other nanoparticle categories [32]. In addition, they can be used as stabilizing agents for non-polymeric nanoparticles (*e.g.* gold and silver ones) [33].

Finally, polymer-drug conjugates are macromolecules constituted by a polymer backbone to which drugs are conjugated *via* linker regions [34]. These systems were developed with the main purpose to improve cell specificity of low molecular weight anti-cancer drugs, by drastically affecting their pharmacokinetics. N-(2-hydroxy propyl) methacrylamide (HPMA) copolymer has been extensively used as conjugate of doxorubicin and other anti-cancer drugs, demonstrating its efficacy in significantly enhancing drug concentration at the target site (up to 50 folds higher) in comparison with non-conjugated therapeutics [35].

The landscape of therapeutic nanovectors is not limited to the described systems: it extends from carbon nanotubes to nanobubbles and nanogels, with a wide range of opportunities in terms of dimensions, tailorability and physico-chemical properties, reviewed in several specialized articles [36-38].

Passive and Active Targeting

As mentioned, several therapeutic nanosystems developed so far are passive and exert their action on neoplastic tissues by simply exploiting the EPR effect. Indeed, tumor microvasculature is leaky and characterized by abnormal branching and enlarged interendothelial gaps (~400 nm). Such gaps allow an extravasation of nanosystems from the surrounding vessels into the tumor [39]. Passive drug targeting relies on circulating nanovectors that passively extravasate in solid tumors and accumulate at the desired sites due to the balance between vascular hemodynamic forces and diffusion mechanisms. Here, the drug is released into the tumor extracellular matrix and then it diffuses towards the cancer cells. Drug release can be determined, for example, by a sudden pH shift or other physico-chemical mechanisms triggered by the tumor microenvironment. Nanovectors can be also directly internalized by malignant cells and release drugs at intracellular level (Fig. **3A**). This method is rather simple, but its efficiency is affected by a high heterogeneity of the EPR phenomenon, which varies dramatically between different tumors and from patient to patient [40]. Few passive polymeric nanoparticles are undergoing clinical trials, at present [8]. Most of them are provided with a PEG coating, able to enhance biocompatibility and circulation half-life [41].

Site-directed nanovectors are characterized by affinity ligands, which permit to the therapeutic carrier to selectively bind to tumor cells (Fig. **3B**). The ligands thus enhance the uptake of drugs/therapeutic molecules into target cells, by promoting internalization of the nanovectors by receptor-mediated endocytosis.

Few tumor-directed nanosystems (mainly liposomes and polymeric nanoparticles) have been developed so far [42, 43]. These systems have some pitfalls, such as poor tumor penetration, hard-to-predict therapeutic responses and complexity of nanosystem formulations. Indeed, the majority of tumor-directed nanovectors have failed in delivering their cargos to the target cells, due to their inability to overcome the several membrane layers and biological barriers between endothelial and cancer cells. These obstacles are constituted by pericytes, smooth muscle cells, fibroblast layers, high tumor cellular density and high tumor interstitial fluid pressure [6, 44].

To overcome the aforementioned issues, endothelial cell-directed nanovectors have been developed [45, 46]. These systems selectively bind to the angiogenic endothelium (*e.g.* by exploiting arginylglycylaspartic acid (RGD)-based ligands) *via* integrin receptors and release their drug content into endothelial cells (Fig. **3C**). This inhibits the growth of blood vessels that supply the tumor, instead of directly targeting tumor cells.

Remotely Triggered Nanosystems

Independently on the type of nanovector used, traditional drug delivery strategies are monotonic: drugs are released from the encapsulating nanomaterial with a certain kinetics, possibly once reached the desired target. In many applications, a direct control of drug release kinetics by an external operator would be highly desirable, in order to precisely determine the timing, duration, dosage and location of drug release. On-demand drug delivery can be achieved by means of remotely triggerable nanosystems, able to respond to different "wireless" physical inputs that can be easily managed by an operator. To this purpose, several physical effects can be exploited. Triggerable systems sensitive to near infrared radiation (NIR), ultraviolet and visible light, electricity, electrochemical processes, radio frequency radiation and microwave radiation have been reviewed in [12]. In this section, we will focus on nanosystems responsive to magnetic fields and ultrasound, in addition to drug/gene delivery based on electroporation. These are in our opinion the most promising paradigms, due to the maturity of the triggering technologies.

Magnetically Triggered Nanomaterials

Magnetic nanomaterials essentially consist of a magnetic core (usually showing a superparamagnetic behavior due to

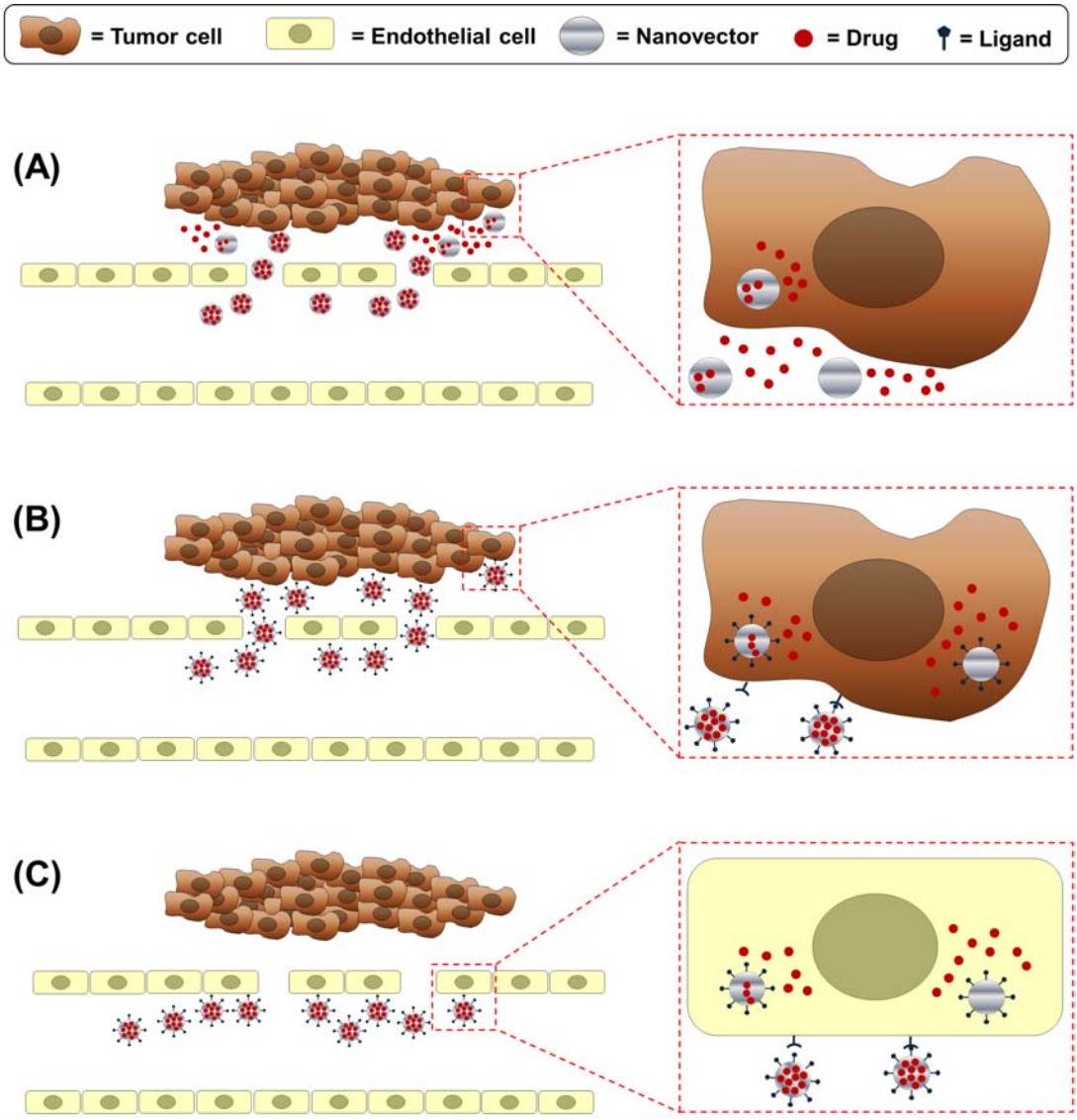

Fig. (3). Drug targeting strategies. (**A**) Passive drug targeting: the large gaps between endothelial cells, due to the presence of neoplastic tissues, facilitate the extravasation of nanovectors into the tumor microenvironment. Here, due to altered conditions (pH, temperature, etc.), the drug is released into the extracellular matrix and it partly diffuses into tumor cells; (**B**) active drug targeting based on tumor-directed nanovectors provided with proper affinity ligands: the nanovectors passively extravasate and concentrate in the target tissue *via* EPR effect. Nanovector/drug uptake and internalization is enhanced thanks to receptor-mediated endocytosis; (**C**) active drug targeting based on endothelial cell-directed nanovectors provided with proper affinity ligands: instead of penetrating into the tumor microenvironment, the nanovectors bind to angiogenic endothelial cell surface receptors. The drug diffuses into endothelial cells and inhibits the growth of blood vessels. Thus, tumor cells are not directly targeted, but their growth is hampered by acting on the blood vessels that supply them with nutrients.

its reduced dimensions), made of a metal oxide such as Fe_3O_4, surrounded by a polymeric (*e.g.* polystyrene, chitosan, polyethylene glycol), inorganic (*e.g.* gold, silica) or polysaccharide (*e.g.* dextran) coating required to stabilize the structure, to enhance its biocompatibility and to provide a support for drug binding or absorption. These structures can be simple particles [47], which are the most investigated systems currently employed also in the clinical practice, nanocylinders [48], or more complex structures enriched with bio-components or carbon nanotubes [49, 50].

Magnetic nanomaterials for targeted therapy are advantageous compared with other nanovectors, as they are visible under magnetic resonance imaging (MRI), are able to heat up when subject to alternating magnetic fields, and can be magnetically dragged to be collected in a target region. All these features allow to exploit magnetic nanostructures as multifunctional therapeutic platforms [51] allowing to reach a target region and to perform phototermal therapy [52], hyperthermia/ablation [53, 54], targeted drug delivery or gene therapy with a visual feedback of the procedure provided by MRI.

Important works have been reported in the recent literature, describing the fabrication and testing of magnetic nanoparticles for drug delivery, gene therapy or hyperthermia. Validation was performed mainly *in vitro*, but also *in vivo*, by using rat models. For example, dextran nanoparti-

cles (~200 nm in diameter) have been intravenously injected and accumulated at the tumor site by exploiting an implanted permanent magnet left in place for 4 h. Particles were then internalized *via* endocytosis and degraded, thus enabling the release of therapeutic genes [55]. Concerning hyperthermia, many studies aimed at identifying the best set of conditions allowing to get higher specific absorption ratios (SARs) and *in situ* heating capabilities without damaging healthy tissues. It has been demonstrated that combining a hard magnetic core and a soft magnetic shell in a 15 nm particle, energy conversion into heat capability can be maximized for *in vivo* applications (on mice) [56]. Other groups investigated the contribution of coatings, exposure times, alternating magnetic field parameters and nanoparticle concentrations on the heating process [57, 58].

Despite enabling therapeutic procedures that would be difficult or unfeasible with other technologies, magnetic nanomaterials show some drawbacks, mainly related to the intrinsic behavior of magnetic fields. Indeed, magnetic gradients decrease very fast with distance, thus making really challenging to go beyond a 10-15 cm distance between the external magnetic field sources and the target site [59]. To address this limitation, many groups investigated the possibility to implant a permanent magnet close to the target [55], but both the invasiveness of the procedure and the need to use smaller magnets, not always able to keep the nanocarriers close to the target withstanding the blood flow, make this solution controversial.

One of the main challenges of this research field consists of increasing the magnetic properties of nanostructures without affecting their biocompatibility, thus to enhance the overall controllability and heat production efficiency. Long-term toxicity and biocompatibility issues are obviously crucial and scarcely investigated, at present. To address them, a switch from animal trials (mainly based on rats) to human clinical trials will be needed. Furthermore, since we are not currently able to retrieve magnetic nanostructures once administered, it will be necessary to develop biodegradable and highly compatible ones, allowing the recycle of Fe in normal metabolic pathways. As an alternative, nanovectors able to be fully excreted by means of the kidneys could be used. However, biodistribution, excretion capability and toxicity of the magnetic nanosystems strongly depend on the working district [60, 61] and still represent a controversial issue.

Ultrasound Triggered Nanomaterials

Ultrasound (US) is probably one of the most emerging and effective tools for remotely triggerable target therapy due to its non-invasiveness, safety, spatial precision (in the case of focused US), relatively low cost and ease of use [12]. The additional possibility to both diagnose and treat at the same time, for certain applications, is another intriguing feature of this technology.

Remote US triggering requires *ad hoc* designed micro/nano materials, which must be responsive to a specific ultrasonic stimulation regime. Typical applications regard regenerative medicine, gene therapy and, to a larger extent, drug delivery. Although the most studied application is tumor target therapy, there are many other clinical settings in which US-mediated drug delivery systems can be used. Relevant examples include neurological pathologies, in which US are used to reversibly trigger the disruption of the blood-brain barrier (BBB), thrombotic diseases, in which US can dissolve vascular obstructions and trigger the release of specific drugs and other non cancer-related pathologies (*e.g.* chronic inflammation) [62].

The interaction between ultrasonic stimulation and drug carriers can enhance the TI (described in equation 1) through two different mechanisms which usually act synergically at the target site: (1) increase of drug release from the carriers and (2) increase of tissue uptake thanks to sonoporation mechanisms (based on a transient increase in cell membrane porosity and permeability) [63]. Sonoporation can be further enhanced by using specific contrast agents (*e.g.* microbubbles) which promote the mechanical action of acoustic cavitation [64-67].

The possibility to simultaneously visualize and control in real-time the efficacy of therapeutic agent delivery to the pathological site (theranostic capability) can further increase the efficiency of the therapy [23, 68, 69].

The design of US-responsive drug carriers must be grounded on the type of stimulus they respond to. Indeed, different physical phenomena can be exploited, by applying distinct exposure conditions (US wave parameters such as frequency (f), intensity (I), duty cycle (DC), therapy duration (TD) and pulse repetition period (PRP)) and inducing different bioeffects. It is of common use to classify these effects in two main groups, namely thermal and non-thermal (mechanical) effects [68, 70].

Thermal effects are associated with a deposition in the tissue of part of the energy carried by the US wave. The Thermal Index (ThI), which is defined as the ratio between the transducer output power (W_p) and the power required to raise the tissue temperature by 1°C (W_{deg}), provides a quantitative estimation of the potential temperature elevation of tissues irradiated by an US wave.

$$ThI = \frac{W_p}{W_{deg}} \quad (2)$$

There are few examples of studies that use thermo-sensitive carriers designed to release drugs above specific temperatures (*e.g.* thermo-sensitive micelles and temperature-sensitive liposomes). However, the dominant physical mechanisms used to trigger drug release from US-sensitive carriers are the mechanical ones.

Among mechanical effects it is worth mentioning radiation force, acoustic streaming and acoustic cavitation. This last phenomenon involves the formation, oscillation and possible collapse of gas bubbles within the tissues. Two different cavitation regimes can be identified, bringing to different bubble dynamics: stable (non-inertial) cavitation, which is characterized by small oscillation of the bubble radius about this equilibrium in response to a relatively low intensity pressure field, and transient (inertial) cavitation, which is characterized by strongly nonlinear oscillations up to collapse in response to higher intensities [71].

The mechanical action of acoustic cavitation can promote, at first, a drug release from US-sensitive drug carriers

at the target site. At the same time, the collapsing microbubbles can further enhance cell membrane permeability, consequently increasing the overall tissue uptake.

The Mechanical Index (MI) represents a relative indicator of the likelihood of mechanical bio-effects and is defined as the ratio between the peak negative pressure (the minimum acoustic pressure in the insonated area, PnP) and the square root of the nominal frequency (f) of the sound beam. Although there are several limitations in using the MI in certain applications, this number has been generally adopted as a safety index for medical US systems. It is now accepted that, with MI less than 0.4, the probability that mechanical bioeffects occur is very low [72].

$$MI = \frac{PnP}{\sqrt{f}} \quad (3)$$

In recent years, numerous studies have shown that US-mediated drug delivery is an effective and promising means to achieve a targeted therapy. US-responsive drug delivery systems mainly include the use of micro/nanobubbles, liposomes and micelles.

Microbubbles (MB) are widely used as contrast agent for US imaging, but their tendency to oscillate and collapse in response to US (due to acoustic cavitation), makes them suitable also for therapeutic modalities [73-75]. Recently, preclinical studies have demonstrated the therapeutic benefits of exploiting US-targeted drug-loaded MB destruction for the treatment of different tumors (brain, liver, pancreas, breast, etc.) [76], but also for many other medical applications such as thrombolysis [77], angiogenesis therapies [78-80], induction of an immune response [81], transdermal drug delivery [82] and disruption of the BBB [83].

Due to the typical size of microbubbles (1-10 μm), their medical applications are mainly restricted to cardiovascular targets. When there is the need to pass through the vessel epithelium or to penetrate inside the single target cell in order to deliver the drug at the intracellular level, nanovectors with sizes smaller than 1 μm must be used. To this purpose, liposomes [84-88], polimeric micelles [89-92] and nanobubbles [93, 94] have been developed and tested. Although the mechanisms involved in this process are not completely understood yet, it has been argued and partly demonstrated that thermal and/or mechanical effects induced by US can generate transient pores in the nanovehicle membrane, through which drugs are released.

Several *in vitro* and *in vivo* studies have been conducted in recent years; the results are very promising and clinical trials will be initiated shortly. Geers *et al*. [95] designed a safe system for US-guided targeted drug delivery to cancer cells. They demonstrated that US (f = 1 MHz, DC = 20%, I = 2 W/cm^2, TD = 15 s) triggered the release *in vitro* of doxorubicin from liposome-loaded MB. Husseini *et al*. [96] achieved the same goal by using folate-conjugated micelles stimulated by US (f = 70 kHz, continuous wave, variable intensity, TD = 10 s, PRP = 20 s) and verified that cavitation effects played a primary role in the drug release process. Heath *et al*. [97] showed that a US therapy (f = 1.0 MHz, MI = 0.5, DC = 20%, TD = 5 min, PRP = 0.01 s) mediated by MB increased cell permeability and enhanced drug uptake by carcinoma cells in both *in vitro* and *in vivo* experiments. By monitoring in real-time the treatment, the efficacy of US-mediated drug delivery systems can be substantially increased: Ranjan *et al*. performed *in vivo* image-guided experiments of targeted drug delivery and observed a precise spatial delivery of doxorubicin to tumor tissues by using temperature-sensitive liposomes in combination with a magnetic resonance-guided high intensity focused ultrasound (MR-HIFU) system [98].

In this section the attention has been mainly focused on recent strategies for US-mediated drug delivery, but US-based targeted therapies also include other exciting applications, such as those related to targeted regenerative medicine. On this subject, in addition to therapies based on a direct US stimulation, it has been recently shown that nanoscale piezoelectric materials, stimulated by US, can provide beneficial stimuli for the differentiation/regeneration of cells and tissues [99].

It has been demonstrated *in vitro* that highly piezoelectric nanoparticles such as boron nitride nanotubes (BNNTs), once internalized and stimulated by outer non-focused US sources, can promote neurite outgrowth and nerve regeneration [100] or overexpress key genes of skeletal muscle development (such as those encoding actin and the different isoforms of myosin) [101]. It is still controversial if such effects are due to mechanically or electrically mediated intracellular changes, but it has been argued that actin polymerization and actin nucleation can have an important role [102].

Although a large number of *in vitro* and *in vivo* studies have been performed, with promising results, some of them do not appear rigorous or they do not report information on the acoustic exposure and dose [103]. Therefore, an effective translation to the clinical settings still has to be achieved and a clear roadmap still has to be defined. Indeed, many issues remain to be faced in this field [104].

The major open problems, which hamper a further development and impact of therapies based on US-mediated drug delivery, are listed in the following:

(1) *Formulation of proper drug carriers and drug incorporation procedures*. Further research and technical efforts will be needed to achieve suitable materials, with finely tuned physico-chemical properties and responsiveness, and to effectively load drugs into them (within the carriers or attached to the carrier membranes). The achievement of optimized theranostic nanovectors is also a challenge, which would enable a real-time feedback on the therapy.

(2) *Optimization of acoustic stimulation parameters*. Many efforts still have to be made in this direction, in order to optimize the treatments by providing correct US doses and avoiding as much as possible side effects.

(3) *Understanding of the phenomena involved during US-nanomaterial-cell interactions*. A deep knowledge of the interactions occurring between acoustic energy, nanomaterials and cells, towards the achievement of the desired bioeffect, is still lacking. On the other hand, it is crucial to improve the safety and efficiency of treatments, as well as to devise future therapies based on similar paradigms.

Electroporation-based Drug/Gene Delivery

Electroporation is based on short high-voltage pulses that allow nanotherapeutics to overcome the cell membrane barrier. The strategy consists of applying an external electric field, which just surpasses the cell membrane capacitance, and that is able to transiently and reversibly induce a breakdown of the membrane. This transient condition implies a permeabilized state that can be used to load cells with a variety of diagnostic and therapeutic agents, from ions to drugs, dyes, tracers, antibodies, vaccines, nanoparticles and oligonucleotides [105]. Differently from other techniques, electroporation has been demonstrated effective not only *in vitro* and *in vivo* on animal models, but also in clinical trials on humans. Protocols for specifically targeting certain tissues, but also to efficiently perform patient management, are being object of research efforts from almost ten years [106, 107]. Although the main focus has been on chemotherapeutic delivery and gene therapy [108], electroporation has been also proposed for different therapies, such as periodical insulin administration [109].

Two key factors in electroporation procedures are specificity and safety (related to the use of low-voltage stimuli). Bulk electroporation techniques have been extensively used in the past decade, but they were rather non-specific and it required high voltage, thus resulting in variable efficiency and low cell viability.

Recent literature reports several examples of nanosystems developed for enhancing electroporation efficacy and safety at cell level. Alumina nano-straws were used by Xie and colleagues to achieve hollow nanowires interfaced with cells on one extremity and connected to a microfluidic chamber on the other one. Cell engulfment on such nanosystem permitted to considerably reduce electroporation voltage in comparison with traditional ones and to increase homogeneity over a large area [110]. Vertical nanopillar electrodes were also recently proposed. Although this technology was intended to be used for recording extracellular and intracellular action potentials with high signal-to-noise ratios, it is also promising for advanced low-voltage electroporation procedures [111]. Kang and colleagues reported a technology based on a nanofountain probe, based on a cantilever tip placed in proximity of cells and provided with a certain voltage. This technology allowed to delivery molecules to target cells with high selectivity and efficiency, yet keeping a high cell viability [112].

Nanochannel electroporation recently emerged as an advanced method for facilitating cellular uptake of drugs or silencing RNA and DNA strands [113, 114]. By means of this technique, electrical pulses push the therapeutic agent out of a reservoir and direct them through a nanometer-scale channel in the device that penetrates into the cell. By adjusting the number of pulses and the channel width, the drug/oligonucleotide dose can be varied. Arrays of nanochannels embedded in PDMS microridges have been proposed, and their efficacy in rapidly and safely allowing cell transfection by plasmids was demonstrated [115]. Finally, a 3D nanochannel electroporation platform based on magnetic tweezers, recently described by Chang and colleagues, showed unique performances in terms of dosage control, high-throughput features and negligible cell damage [116].

CONTROLLABLE MICRO/NANOROBOTS

The merging of skills and techniques derived from materials science and nanotechnology and those typical of micro/nanorobotics could allow to solve one of the main issues related to targeted therapies, namely how to bring a therapeutic agent close to the required tissue in a finely controlled way. Micro/nanorobotic systems for targeted therapy are essentially unthetered, wirelessly powered and wirelessly controlled devices able to get to the hard-to-reach areas of human body and to perform minimally invasive procedures difficult or unfeasible with traditional tools. Current prototypes show dimensions ranging from some μm to few hundreds of μm, whereas truly nanometric systems are rather far to achieve, yet. The specific action mechanisms of medical microrobots range from those related to targeted therapy (*e.g.* drug delivery, hyperthermia and brachytherapy) to those related to telemetry, material removal and reconfigurable structures deployment [117]. It is worth conceiving these systems as complex platforms, whose features strongly depend on the working environment and on the procedure to be performed. The robot normally acts as a carrier for the therapeutic agent. To enable its action, a sensing/localization system and a control strategy, required both for robot locomotion and therapy triggering, must be considered. In this section, attention will be focused mainly on how to provide micro/nanorobots with locomotion capabilities and on which are the challenges to be faced to bring these systems and other related robot-assisted procedures to the clinical practice.

When we think about locomotion of microrobots inside the human body, we refer to the ability of "swimming" through a dense network of low caliber fluid-filled channels, including but not limited to the cardiovascular system. In such environment, Reynolds number (Re) is lower than one, thus making impossible certain motion strategies, typically performed at the macro-scale. Reciprocal motion, for example, results in a null displacement [118] and surface and capillary forces are more relevant than volumetric ones [119], [120]. This compels to abandon the traditional paradigm sensor-actuator-controller that normally dominates the robotics field and to look for new actuation strategies, efficient at low Re numbers and compatible with the narrow space available. In the recent literature, few medical robots are passive: they simply move as the environment dictates, thus making it difficult to precisely get to the desired district. In most cases, however, micro/nanorobots are provided with the capability to convert energy into motion and thus to swim in a controlled or semi-controlled way towards the target; this can be achieved by exploiting wireless powering sources or self-propelling strategies (on-board motors) (Fig. **4**).

The most exploited wireless powering source is based on magnetic fields that can be easily produced by means of an MRI scanner [121] or a dedicated electromagnetic setup [122]. Magnetic fields enable different locomotion strategies, ranging from the simple pulling to the more bioinspired helical [123] or elastic tail propulsion [124]. The main advantage of magnetic locomotion is the possibility to wirelessly control a robot in a three-dimensional space and with multiple degrees of freedom, thus allowing it to follow really compli-

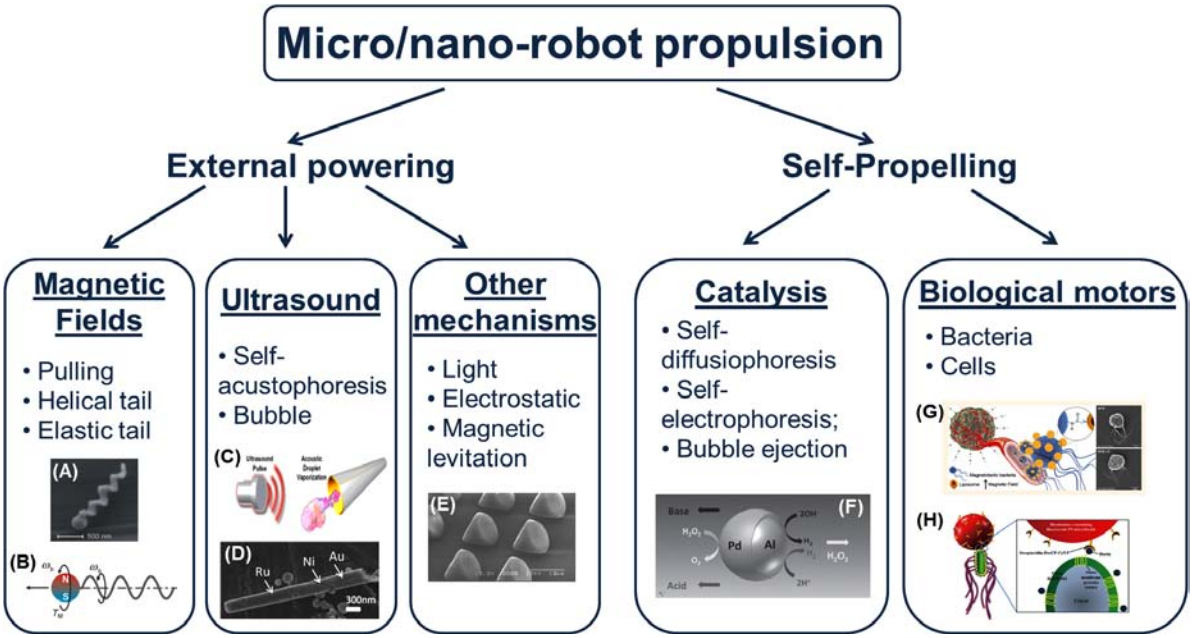

Fig. (4). Overview of different micro/nanorobots locomotion strategies actuated by means of external powering or on board motors. **A**) Artificial bacteria flagella. Reprinted with permission from [146]. Copyright (2009): American Chemical Society. **B**) Helical tail locomotion by means of rotating magnetic fields. Reprinted with permission from [123]. Copyright (2012): John Wiley and Sons. **C**) Expansion and vaporization of perfluorocarbon emulsion droplets produced by ultrasound. Adapted with permission from [130]. Copyright (2012): American Chemical Society. **D**) Self-acoustophoretic robot. Reprinted with permission from [131]. Copyright (2013): American Chemical Society. **E**) Light-driven microrobot. Adapted with permission from [132]. Copyright (2012): AIP Publishing LLC. **F**) Self-diffusiophoresis mechanism. Reprinted with permission from [135]. Copyright (2013): John Wiley and Sons. **G**) Functionalized magnetotactic bacteria. Adapted with permission from [142]. Copyright (2014): American Chemical Society. **H**) Theranostic bacteria-based microrobot. Adapted with permission from [126]. Copyright (2014): Nature Publishing Group.

cated paths, if needed, to reach the target region [125, 127]. However, as already mentioned, magnetic field intensity and gradients rapidly decay with distance. Furthermore, to exploit them, dedicated bulky external magnet setups and complex control algorithms are required. US, and in particular self-acoustophoresis mechanisms based on the formation of local pressure gradients on the concave end of a nanowire [128] or kinetic energy production through the expansion and vaporization of perfluorocarbon emulsion droplets [129], can be exploited to provide wireless powering and to propel medical microrobots [130]. In the case of self-acoustophoresis, however, robots move on a plane, thus making quite difficult to translate this technology into *in vivo* applications. In the case of expansion and vaporization of droplets, the need of a specific fuel (droplets) for the actuation mechanism makes these systems able to achieve only really short strokes. Furthermore, in both cases, magnetic fields are nevertheless required to orient and steer robots [131]. Further actuation strategies, such as light [132], magnetic levitation [133] or electrostatic forces [134], have been investigated so far. However, they provide only 2D locomotion, thus resulting more suitable for *in vitro* and lab-on-chip applications rather than for *in vivo* targeted therapy.

An alternative to wireless powering is represented by self-propulsion mechanisms that can rely on the interaction between the robot and the environment or on the integration of "biological motors" (*e.g.* bacteria). One of the most promising class of self-actuated micromotors is based on catalysis and on the exploitation of different mechanisms such as self-diffusiophoresis [135], self-electrophoresis [136] or bubble ejection [137]. This actuation strategy, differently from many others, is quite efficient also at the nanoscale. However, the need of specific and usually toxic fuels, such as H_2O_2, necessary for the chemical reactions to occur, makes this technology scarcely usable in biological environments. To overcome this limitation, some groups focused their attention on the development of systems able to convert the chemicals available in body fluids, such as glucose into propulsion energy, without the need of any toxic compound [138].

While several groups are trying to develop magnetically actuated artificial bacteria flagella [139], some others investigate strategies to exploit living microorganisms such as bacteria or cells, able to efficiently swim at low Re, as on-board powering sources [140, 141]. Really encouraging results have been reported in this field by controlling magnetotactic bacteria under MRI [142] or by combining polystyrene microbeads and flagellated bacteria for solid tumors treatment [126]. Despite the reduced dimensions, the possibility to overcome fabrication issues and the high propulsion efficiency provided by the microorganisms, this technology still appears controversial due to the pathogenic risks associated with the introduction of such agents in the human body, and due to the swarm behavior that does not allow a fine control of single robots.

It is rather clear that several technological tools are still needed, to make true what some decades ago was science fiction, namely the development of controllable integrated therapeutic micro/nano-robots. First of all, to be able to effi-

ciently get in every district of our body, size reduction is required. The examples previously reported, in fact, refer to robots whose dimensions range from some μm to few hundreds of μm, whereas nanometric vectors, which would represent the optimal solution, are under investigation but still in the modeling phase [143-145]. We can affirm that magnetic powering, employed to wirelessly propel the robots or in combination with other strategies to orient their locomotion, is one of the most efficient and studied strategies. However, there are still debated issues related to the biocompatibility of magnetic microrobots: it will be necessary to find out a trade-off between magnetization maximization, thus to enhance robot controllability, and their biocompatibility [127]. Furthermore, it will be necessary to increase robot efficiency in presence of a payload to be carried. The motion dynamics and the required actuation power are strongly affected by the dimensions, geometry and nature of the therapeutic components (*e.g.* drugs) loaded in the vector. Thus, it will be of key importance to take into account such payload already in early design phases. Usually, robots or robot swarms are provided with the capability to push drug molecules [146] or to temporarily trap them [123], but the risk to lose the load during the path and the low dose they are able to carry are issues that still have to be faced.

To go beyond the limitations of current therapeutic microrobots, a possibility could be the development of a multistage therapeutic system [147, 148], based on a combination of different external and on-board energy sources, and embedding controlled locomotion capabilities, smart release mechanisms and nanomaterials able to carry drugs or to perform physical therapy. Another great challenge will concern the development of a strategy not only for the deployment of the micro/nanorobots in the human body, but also for their retrieval, in order to avoid long-term toxicity and excretion issues.

INTEGRATION OF BIO-COMPONENTS

Bio-components for drug delivery systems constitute an emerging research trend and promising tools to smartly treat cancer and other pathologies. They can confer several advantages, such as enhanced biocompatibility and lack of immunogenicity respect to synthetic and inorganic elements, more efficient targeting of certain body districts and internalization of therapeutic agents within cells. In particular, the recent advances in proteomics and genomics are able to expand the catalogue of the molecular elements of interest for targeted therapies. In this section, we will discuss the potential of deoxyribonucleic acid (DNA), proteins (included antibodies), exosomes and aptamers.

DNA has *per se* rather limited chemical, electrical and optical properties. However, it constitutes an attractive molecule for the design of highly customized nano-devices. The size of DNA strands depends on the number of nucleotides assembled together and can range from sub-nanometric to micrometric lengths. The first approaches to DNA nanotechnology refer to the 1980s [149]; afterwards the discovery of DNA origami [150] and evolutions in nanofabrication and nanoassembly procedures amplified the interest in DNA-related structures [151, 152]. The advantages of using DNA mainly concern the design of assembled structures with a predictable disposition of their building blocks, with sizes ranging from tens of nm to micrometers, and the possibility to control the position of cargo molecules by means of chemical modifications at predefined positions [153-155]. Moreover, DNA possesses molecular recognition properties that enable the development of systems able to identify and bind to a specific cell site [156]. Computer-aided design tools permit to create nanosystems with desired dimensions and characteristics, taking advantages from the information encoded inside DNA. Engineered DNA nanostructures have been employed, for example, as drug-delivery nanovehicles to release and control the activity of cargos inside cells in response to stimuli or signal molecules [157]. DNA origami has been used for the development of three-dimensional containers (Fig. **5A**) to be used as drug delivery systems and to induce apoptosis in cancer cells [158, 159]. This technology has the potential of being integrated with small interfering ribonucleic acids (siRNAs), small molecules able to "silence" specific genes within cells. Preliminary studies showed that self-assembled DNA nanovectors loaded with siRNA, once injected systemically or intratumor, permitted the suppression of the expression of targeted genes, without relevant immune response [160]. This approach looks promising for exploiting the recognized therapeutic potential of siRNAs, overcoming the obstacles related to carrier nanotoxicity, highlighted in previous literature [161].

Proteins are biocompatible and versatile materials showing a size range of 1-100 nm that can be used as carriers for cancer therapy [162]. Protein nanocontainers can play an important role, enhancing biocompatibility and extending the lifetime of circulation carriers, with low toxicity [163, 164]. Their design versatility, achieved through bioengineering techniques, make them promising elements. In particular, protein cages are the most promising vectors. They derive from viruses and virus-like materials and are constituted by repetitive subunits showing self-assembling properties. Protein cages have ideal sizes for endocytosis and a high degree of flexibility, with the possibility to functionalize their sites by means of protein engineering tools. Precise interfacial locations for specific applications are possible by modifying genetically and chemically protein subunits [165]. The main drawback is represented by their small size, which limits their drug load capacity.

Exosomes derive from multivescicular bodies and play an important role in the intercellular communication. They have a diameter of about 40-100 nm and are tailored for the transport and intracellular delivery of proteins and nucleic acids through endogenous mechanisms. Their functions recently raised a great interest in the scientific community and opened the way to an emerging paradigm shift in the field of drug delivery. Exosomes can be used as vehicles for the delivery of therapeutic drugs since, in comparison with synthetic liposomes, they are more tolerated inside the body: they are not subjected to attack by opsonins and antibodies in the circulation, thus showing high stability in blood and a long circulating half-life [166]. In addition, they possess an intrinsic homing ability that can be also enhanced thanks to external membrane modifications. A high-impact study showed for the first time in 2011 the capability of exosomes to deliver siRNA *in vivo*, overcoming the BBB after systemic injection [167]. Other studies highlighted the added

values that exosomes can bring to intracellular drug delivery [168, 169]. Exosome-mediated drug delivery represents a promising research topic that could permit to efficiently face some challenging aspects of drug delivery, such as the crossing of impermeable biological barriers, and the identification of biocompatible drug vehicles from patient-derived tissues [170-172].

Mesenchymal stem cells (MSCs) represent an ideal source of exosomes. MSCs are multipotent fibroblast-like cells that can act as bioreactors able to produce and secrete therapeutic exosomes. These cells possess immunomodulatory properties, exerting suppressive and regulatory effects to enhance the longevity of MSC exosome-derived drug delivery [173]. The advantages of this approach are that the cell source is non immunogenic, easily accessible and inclined to immortalization without compromising exosome production. MSC engineering is a promising process for the production of exosomes and for embedding them with therapeutic molecules (Fig. **5B**) [174]. Obviously, the size of cells (tens of microns) do not allow to use them as nanosystems, but rather as implantable nanosystem "incubators".

Other interesting bio-components are aptamers (typical size: 3-5 nm), oligonucleotides showing flexibility for a wide range of applications at the nanoscale. Aptamers are DNA/RNA strands that can be selected in a large variety of sequences and folding patterns through an automated method to identify aptamers sequences and isolate them *in vitro*, named systematic evolution of ligands by exponential enrichment (SELEX) [175, 176]. Through this selection process, scientists can obtain custom cell-selective aptamers to be coupled with targeted drug delivery systems, in order to reach diseased cells. Recently, aptamers designed for specific cancer cells have been designed [177]. Compared to antibodies, aptamers possess a greater specificity as targeting agents, higher thermal stability, lower costs for the synthesis process and lower immunogenicity.

The conjugation of aptamers with liposomes and nanoparticles has appeared helpful to achieve efficient treatments [178, 179], but aptamers have been also directly loaded with drugs: aptamer-conjugated doxorubicin has been demonstrated to be more effective in comparison with the unconjugated drug for cancer treatment. This added value is brought by a molecular specificity that lacks in many drug delivery strategies, and which inhibits nonspecific uptake [180, 181]. Aptamer-based systems have proven effective also in the intracellular release of siRNA, both *in vitro* and *in vivo* for cancer and also for HIV treatment in animal models [182]. Cancer cells were successfully treated by Dassie *et al.* with aptamer-siRNA systems, achieving a significant regression of the tumor factors in mice after systemic administration [183].

Aptamers can also control the release of cargos in space and time acting as smart gating mechanisms triggered by the aptamer-target interactions [184]. Similar gating systems were also studied for the development of DNA nanorobots: signaling pathways were actuated by an aptamer-based lock mechanism that opened in response to specific antigens, thus behaving like an "AND" logic gate (Fig. **5C**) [185].

Nanoclaws are recently described devices able to perform analyses on cancer cell-surface markers, thus providing diagnostic information [186]. They represent additional functional sensing elements to be integrated on board of future therapeutic micro/nano robots. More complex systems can

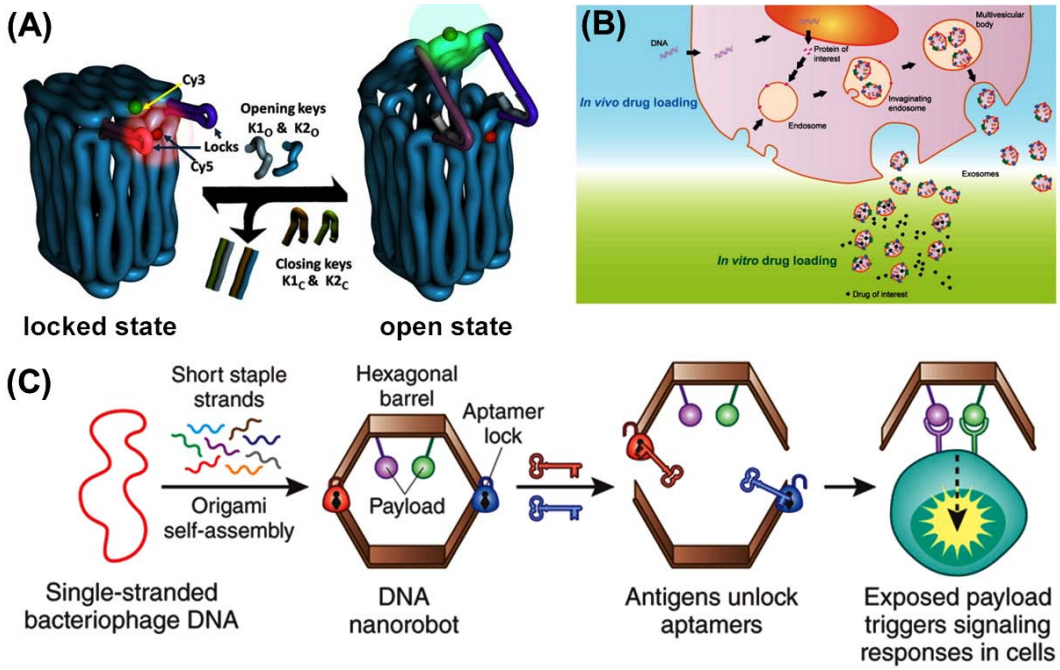

Fig. (5). Examples of nanovehicle systems for drug delivery: **A**) 3D DNA box origami with a switchable lock system based on key factors. Reproduced with permission from [158]. Copyright (2012): American Chemical Society; **B**) Exosomes loading that can be implemented directly *in vitro* or *in vivo* inside cells Reproduced with permission from [174]. Copyright (2013): Macmillan Publishers Ltd; **C**) Smart DNA self-assembled nanorobot characterized by an aptamer-lock based system that unlock in an AND logic configuration for drug delivery. Reproduced with permission from [185]. Copyright (2012): Elsevier.

be obtained by integrating aptamers and quantum dots for synchronous imaging and therapy of cancer, with the possibility to verify drug release at the target site [187].

Although the described bio-components enable interesting functions for advanced targeted therapies, several issues still have to be faced: the stability of DNA and protein nanovehicles in physiological environment (blood) and their scarce penetration ability through biological membranes still strongly limit their applicability. On the other hand, difficulties in the characterization of pharmaceutical exosomes and a little understanding on how they pass through physiological barriers are other important aspects to consider. Possible solutions for future therapeutic paradigms concern the increase of resistance to degradation and the use of cell-penetrating peptides (CPPs) [188]. CPPs could be used as vector tools through cell membranes, to safely deliver small molecule drugs or RNA strands inside cells.

CONCLUSIONS

The fields of nanomedicine and targeted therapy are rapidly growing in terms of both public awareness on the theme and scientific literature production. However, in most cases, current paradigms still remain strictly adherent to a traditional route, simply based on advancements concerning nanocarrier chemical formulation/functionalization and/or novel drug development/encapsulation techniques.

In this article, we highlighted the most used vectors in nanomedicine, reporting their main advantages and drawbacks, and we depicted the main therapeutic strategies they are based on. Passive nanocarriers have been extensively studied in the literature, but they show clear limitations, especially concerning their capability to really reach the target cells. Site-directed systems show greater potential, but they assure only a monotone drug delivery kinetics. Remotely triggerable systems are highly appealing, in particular those based on magnetically triggerable and ultrasound-responsive materials. These paradigms currently represent hot topics with a great potential, but issues related to long-term biocompatibility and controllability/repeatability of the process still need to be faced. Micro and nanorobots have been also described, shading light on the potential that a robotic-oriented approach may bring to the fields of targeted therapy, once technological limitations (mainly related to component miniaturization) will be overcome. Finally, the most important bio-components have been described. They are able to provide advanced sensing capabilities and to constitute smart mechanisms at the nanoscale, thus enabling additional functions for therapeutic nanosystems. However, their low stability in physiological conditions still hampers their pre-clinical and clinical use.

By analyzing the literature of the mentioned fields, it appears highly desirable that future research efforts will orient towards the design of smart integrated nanorobots capable of precisely reaching the targeted areas, of analyzing cell molecular inputs with high sensitivity and of modulating targeted therapy by means of internal mechanisms or remotely triggered ones. To this aim, a real merge of competences between nanotechnology, chemistry, robotics and biotechnology is highly desirable and it shows the best promises. With the help of genomics and proteomics we will be increasingly able to collect information on how to properly design drugs, on when certain proteins or genes are implicated in pathological diseases, etc. Indeed, a predictive and personalized medicine paradigm seems to be the future [189, 190], but it should be associated with important steps ahead in the development of micro-nano-bio systems for a targeted therapy delivery.

ABBREVIATIONS

BBB	=	Blood-brain barrier
CCPs	=	Cell-penetrating peptides
DNA	=	Deoxyribonucleic acid
EPR	=	Enhanced permeation and retention
HPMA	=	N-(2-hydroxy propyl) methacrylamide
MB	=	Microbubbles
MI	=	Mechanical index
MR-HIFU	=	Magnetic resonance-guided high intensity focused ultrasound
MRI	=	Magnetic resonance imaging
MSCs	=	Mesenchymal stem cells
NIR	=	Near infrared radiation
PEG	=	Poly(ethylene glycol)
PEO	=	Poly(ethylene oxide)
PnP	=	Peak negative pressure
PRP	=	Pulse repetition period
Re	=	Reynolds number
RES	=	Reticulo-endothelial system
RGD	=	Arginylglycylaspartic acid
SAR	=	Specific absorption ratio
si-RNA	=	Small interfering ribonucleic acid
ThI	=	Thermal index
TI	=	Therapeutic index
US	=	Ultrasound

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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