Review Article

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Recent developments and applications of smart nanoparticles in biomedicine

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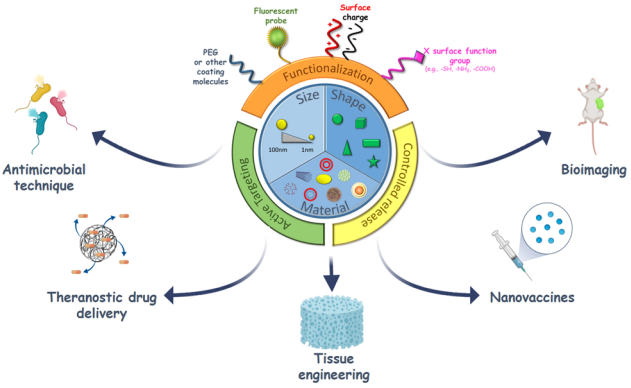
Abstract: Over the last decades, nanotechnology applied in medicine (nanomedicine) has sparked great interest from the scientific community, thanks to the possibility to engineer nanostructured materials, including nanoparticles (NPs), for a specific application. Their small size confers them unique properties because they are subject to physical laws in the middle between classical and quantum physics. This review is proposed to explain better how to design a specific NP and clarify the relationship between the type, size, and shape of NPs and the specific medical applications. NPs are classified into inorganic (metallic NPs, quantum dots, carbon-based nanostructures, mesoporous silica NPs) and organic (liposomes and micelles, dendrimers, and polymer NPs). Here, we report an accurate description of the potential of each NPs type focusing on their multiple areas of application, including theranostics drug delivery, imaging, tissue engineering, antimicrobial techniques, and nanovaccines. All these features make NPs a promise to revolutionize the new era of nanomedicine.

Keywords: organic and inorganic nanoparticles, drug delivery, antitumor therapy, tissue engineering, bioimaging, antimicrobial techniques, nanovaccines



Graphical abstract

1 Introduction

Nanoscience represents one of the most exciting fields of modern science, with a highly interdisciplinary character; indeed, it develops by combining different doctrines like chemistry, biology, physics, and engineering, taking advantage of their principles and processes. It is based on understanding and knowledge of the matter properties on the nanometric scale (between 1 and 100 nm). The realization of materials, systems, and apparatuses on this size scale determines nanotechnology [1].

The term "nanotechnology" was first defined by Norio Taniguchi of Tokyo Science University in 1974 [2]. In the 1980s, the idea of nanotechnology as deterministic, rather than stochastic, handling of individual atoms and molecules was conceptually explored in depth by Dr. K. Eric Drexler, who called it molecular nanotechnology [3]. It is a continually evolving field that finds application in many productive sectors, including cosmetics, coating and paints, nano-hard disks, and memory chips. One of the significant applications concerns the biomedical environment (nanomedicine) focused on tissue engineering [4,5], drug delivery [6], nanovaccines [7], antimicrobial techniques [8], and bioimaging [9].

Nanoparticles (NPs) are dispersion solutions of atomic aggregates or solid particles with a size between 1 and 300 nm and specific properties, like the high surface-to-mass ratio. Furthermore, the small size permits them to circulate more freely in the human body. NPs present unique chemical, magnetic, mechanical, and biological properties that increase biocompatibility and cellular uptake. The possibility to engineer their surface permits multifunctional

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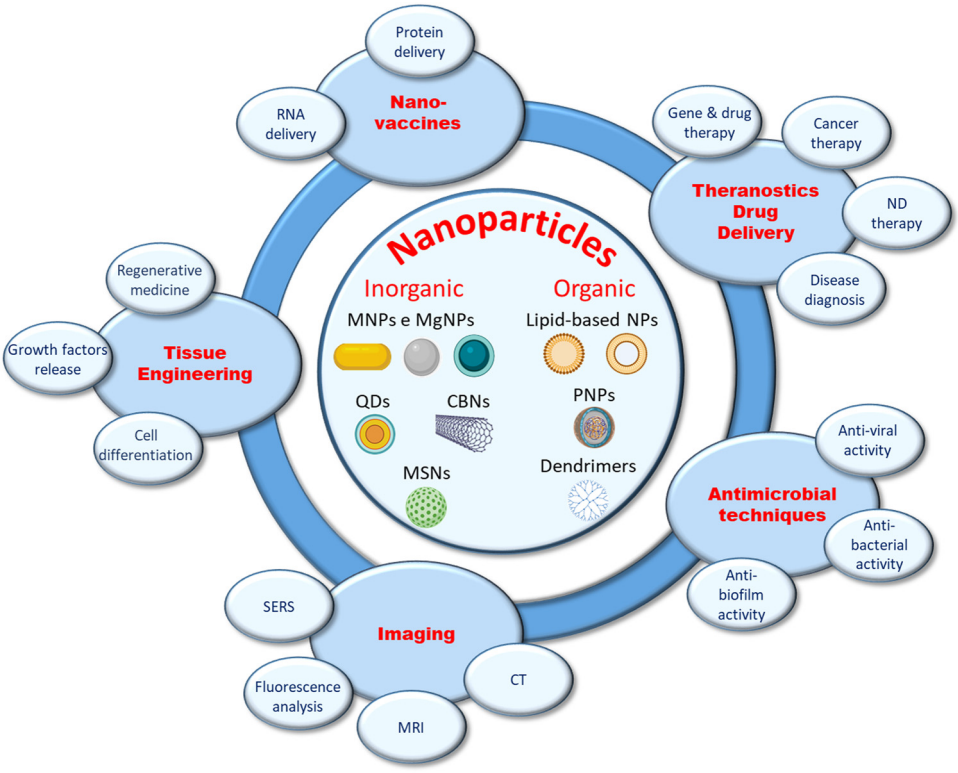


Figure 1: Nanoparticles classification and applications in biomedicine. Inorganic NPs include metal and magnetic NPs (gold, silver, SPIONs), QDs, CBNs, and MSNs; organic NPs collect lipid-based NPs (liposomes and micelles), PNPs and dendrimers. Depending on the material, size, shape, and functionalization, NPs can be employed in nano-vaccines both for protein and RNA delivery; theranostics drug delivery that include treatment and diagnosis of many pathologies like cancer and NDs; antimicrobial (anti-viral, bacteria and biofilms) techniques; imaging for CT, MRI, SERS, and fluorescence analysis; tissue engineering in regenerative medicine for growth factors release or cell differentiation.

applications, especially in the clinical environment for diagnosis and therapies [10]. The classification and applications of NPs in biomedicine are illustrated in Figure 1. The combination of diagnosis and therapy (theranostic) applications has amply been employed for treatment of diseases. Depending on the nature and the functionalization (*e.g.*, with fluorescence probes), NPs have been applied for different bioimaging studies, including magnetic resonance imaging (MRI) contrast agents, Positron emission tomography (PET), and optical, magnetic, and radioactive imaging (Figure 1). In this manner, NPs can be adopted for early disease detection, image-guided disease treatment, and the evaluation of a therapeutic effect.

Conversely, NPs can act like vectors able to carry biological molecules (*e.g.*, drugs) to a specific tissue and release them with a controlled mechanism. These characteristics make them optimal candidates as drug delivery systems (DDS) in many pathologies, including neurodegenerative diseases (NDs) and tumours. Despite many progressions in the last decades, cancer remains one of the most devastating diseases globally, causing 10 million deaths in 2020 (data from OMS). The tumour heterogeneity limits the formulation of standard therapy. Therefore, conventional drug administration systems (CDASs; parenteral, oral, cutaneous, or topical) for diseases and, in particular, cancer chemotherapy can induce side effects because of their nonspecific action as they act both on healthy and malignant cells [11]. Based on the tumour cells' high capability to divide out of control, CDASs are developed to destroy the rapidly dividing cells, unfortunately including the body's other rapidly proliferating cells, such as cells in the hair follicles, myelopoietic bone marrow precursor, and intestinal epithelial cells [12]. Therefore, the knowledge of definite cancer physiology and structure can be the starting point for designing engineered NPs for a specific tumour targeting.

Furthermore, the drug dilution in the bodily fluids limits its absorption in the target tissue, so it is necessary to administer substantial doses to have a high local concentration. Conversely, the use of nanosystems as DDS

permits a controlled release of the conjugated drug, depending on physiological conditions of the targeted site (site-specific targeting) and modulation during the release time (temporal modulation), related to the physical properties of the microenvironment [13].

Disease treatment is only one of the multiple applications in which NPs can be involved. Depending on their nature, NPs can present antibacterial or antiviral properties and also anti-angiogenetic and anti-neoplastic effects [14,15] (Figure 1). Furthermore, they are abundantly employed in tissue engineering to promote tissue differentiation, thanks to the possibility of local delivery of bioactive (growth factors, chemokines, inhibitors, cytokines, genes, *etc.*) and contrast agents in a controlled way [16].

Moreover, in the last decades, the use of different kinds of NPs as delivery systems in vaccines sparked great interest from the scientific community, thanks to their potential to improve vaccine efficacy and reduce the risk of attenuated vaccines. The encapsulation protects the antigens from early proteolytic degradation, permits a controlled antigen release, and helps antigen uptake and processing by antigen-presenting cells. In addition, the possibility of obtaining a specific target can improve vaccine formulation [17].

Therefore, NP systems, including the choice of the size, shape, composition (material), and surface properties, play a pivotal role in optimizing their use in biomedical applications.

2 Project of NPs

NP functionalization plays a crucial role in their activities in a wide range of delivery applications, including treatment of different diseases (tumours, neurodegenerative, and metabolic pathologies), bioimaging, tissue engineering, nanovaccines, and antimicrobial techniques (Figure 1). The engineered nanomaterials can be synthesized by two different approaches, top-down and bottom-up. Top-down is a physical approach that reduces macrostructures, named bulk materials, through incisions, grindings, and cuttings [18]. Conversely, bottom-up is a chemical approach that produces NPs from atoms or molecule aggregates [19]. It is the typical synthesis mechanism adopted in the biomedical field because it permits the formulation of nanostructures with the desired properties through a specific controlled process [20]. Starting from this, the size, shape, composition (material), and surface properties must be considered and analysed to increase the circulating half-life, biocompatibility, drug loading, corresponding site-specific release, and the specific site targeting [14].

2.1 NPs size

Morphological characteristics, like size and shape, play a pivotal role in NP-based drug delivery. Size change in the nanoscale can influence physical properties (like optical absorption or melting points that decrease in a size-dependent way), chemical reactions (like thermodynamic features), and magnetic properties, especially for metal NPs (MNPs) and electrical properties. The size of NPs can influence different aspects like body and tissue distribution, cellular response, and blood extravasation as shown in Figure 2.

In fact, the size of NPs needs to be chosen with particular attention because nanosystems have to be small enough to escape the capture from the cells of the mononuclear phagocyte system (*i.e.*, in the spleen and the liver) and big enough to avoid their rapid leakage into blood vessels following by the renal clearance [21]. Depending on the administration technique, cytotoxicity and adsorption of NPs across the epithelial barrier are related to their size (Figure 2). For example, inhalation enables penetration in the lung parenchyma, showing a different localization in the respiratory tract. Conversely, as reported by Braakhuis *et al.*, the cytotoxic effect in rats of inhaled silver NPs is related to their dimension: NPs of 18 and 34 nm induced cell damage in a concentration-dependent way. Simultaneously, there was no dose-dependent toxicity of 60 and 160 nm NPs [22].

Many studies have evaluated the pharmacokinetics of NPs (*in vivo* distribution) and revealed a size-dependent different organ distribution as assessed by Ibrahim *et al.*: 5 nm gold nanoparticles (AuNPs) preferentially addressed to the liver, while bigger AuNPs of 20 and 50 nm localized on the spleen [23]. De Jong *et al.* also had analysed AuNPs size-dependent tissue distribution reporting an exclusive localization of 10 nm AuNPs in the testis, thymus, heart, and brain [24]. For instance, the size of NPs plays a pivotal role in passive tumour targeting by the enhanced permeability and retention effect due to the leaky vascularization of tumour tissues. It is unlikely that NPs bigger than 200 nm reach tumour mass due to spleen clearance, while NPs with a size smaller than 30 nm can travel back from the cancer mass to the blood vessels. Therefore, the optimal nanosystem size for passive tumour targeting is between 30 and 200 nm [25].

Smaller NPs are generally cleared by drainage organs like the kidney (<5 nm) or the liver (10–20 nm), while the bigger ones (20–200 nm) accumulate in the spleen, bone marrow, and other reticuloendothelial systems (RES) [26]. The clearance of NPs also depends on the inhalation process; for instance, pulmonary clearance by alveolar macrophages concerns big aerosol-based NPs (>100 nm). By comparing the biodistribution of 13 and 105 nm of inhaled AuNPs, Han and

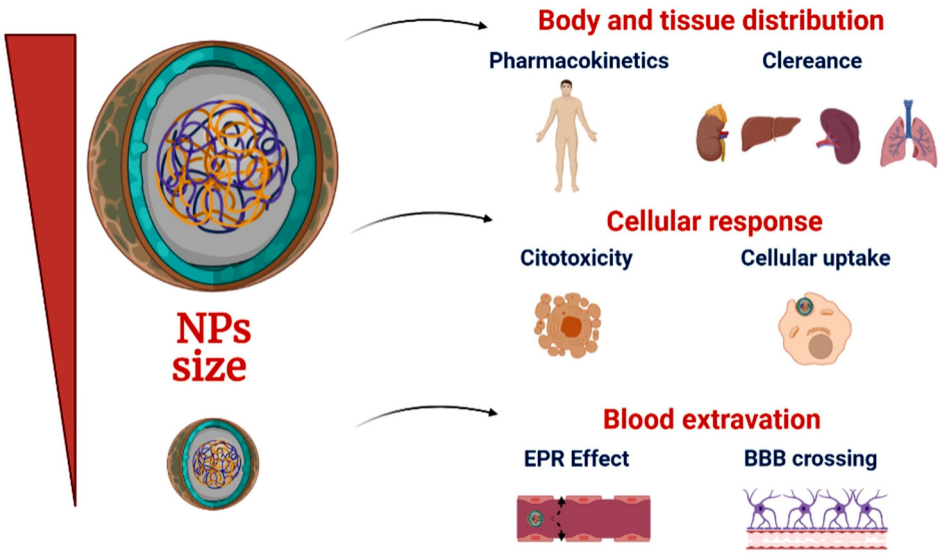


Figure 2: Effect of nanoparticles size on body and tissue distribution, cellular response, and blood extravasation. Depending on the size, NPs can localize differently in body compartments (pharmacokinetics) and can cleaned from different organs: kidney (<5 nm), liver (10–20 nm), spleen (20–200 nm), and lung (inhaled NPs >100 nm). Cellular response exploited cytotoxicity and cellular uptake mediated by micropinocytosis (big NPs), clathrin-mediated endocytosis (100 nm) and caveolae-mediated endocytosis (15–80 nm). NPs size influence also the EPR effect for passive tumour targeting and the cross thought BBB.

colleagues had noticed an initial rat lung deposit of both NPs, but significantly higher biodistribution from the lung to other organs of the smaller AuNPs. In addition, lung clearance was considerably higher for bigger NPs.

The optimal size of NPs must be ranged between 1 and 100 nm, especially to cross the blood–brain barrier (BBB), as suggested by neurodegenerative disease studies, including Alzheimer, Parkinson, or glioma. The biggest problem with treating cerebral pathologies is the impossibility or high limits of drugs to pass through the BBB. Their conjugation with NPs of different natures (*i.e.*, polymeric, inorganic, or liposomes) permits them to cross the BBB by active (receptor-mediated or adsorption-mediated endocytosis or carrier-mediated transport) or passive (diffusion through endothelial cells) transport mechanisms [27]. NPs smaller than 10 nm cross BBB through a transcellular passage, while bigger NPs are involved in the transcytosis mechanism [28]. For example, one of the most used drugs for Alzheimer's disease is the anti-amyloidogenic drug curcumin, which is unable to cross the BBB. Hence, Barbara *et al.* encapsulated it in polylactide-*co*-glycolic-acid (PLGA) NPs modified with a g7 ligand that permits the BBB crossing. An intensive decrease of Aβ aggregates in response to curcumin-loaded NPs was registered, suggesting a possible approach to treating Alzheimer's disease [29].

A decrease in NP dimension corresponds to a higher surface area-to-volume ratio, suggesting that more conjugated drugs could be associated with or near the NP surface [30]. Furthermore, cellular uptake also depends on the size [31] (Figure 2). NPs are internalized faster and 15–250 times more than microparticles of 1–10 μm through many mechanisms: large NPs are generally involved in micropinocytosis; 100 nm NPs in clathrin-mediated endocytosis; and 15–80 nm NPs in caveolae-mediated endocytosis [32,33].

2.2 NP shape

The shape of NPs confers peculiar features that influence blood lifespan, macrophage uptake, and cell membrane interaction (Figure 3). Generally, NPs are injected into the blood vessels and are subjected to Brownian motion and convective forces, inducing rotation and rolling, especially for oblate-shaped spherical NPs [34]. In fact, blood circulation depends on nanosystem shape, as suggested by Geng and coworkers who showed that polymer filomicelles persisted in the circulation of rodents about ten times more than their spherical counterparts (more than 1 week against 2–3 days), probably due to the possibility to align to the blood fluid [35]. Zhao *et al.* also confirmed these data, which reported the more prolonged bloodstream circulation of the long rod mesoporous silica nanoparticles (NLR) compared with the short rod and spherical ones [36]. They also investigated the body biodistribution after rat oral administration; although the liver and kidney took

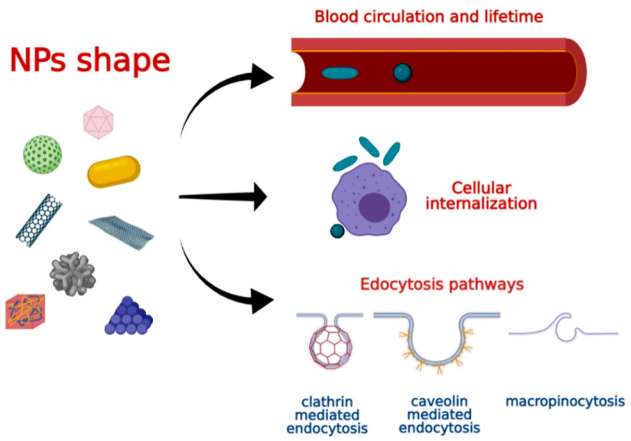


Figure 3: Factors affected by nanoparticles shape: blood circulation and lifetime; cellular internalization (depending on the NPs AR and the contact angle); and endocytosis pathways (clathrin-mediated endocytosis, caveolin-mediated endocytosis, and micropinocytosis).

up all the NPs, NLR had the longest residence time in the gastrointestinal.

The major part of the NPs' nonspecific clearance depends on the spleen and liver's mononuclear phagocytic system (MPS). Their retention can induce an inflammation state [37]. Therefore, their blood lifetime is improved by evoking the macrophages' phagocytosis in the reticuloendothelial system to reach the target tissue. Many strategies have been adopted to avoid the MPS, including the functionalization with polyethylene glycol (PEGylation), which permits the formation of a hydrating layer due to the association with water molecules [38]. This manner prevents the NP aggregation and interaction with blood components like opsonins, prolonging systemic circulation time [39]. PEGylated particles' behaviour is related to PEG molecular weight and surface density, which influences its superficial properties [40]. Another approach consists of the NP's "mimetic effect" by conjugating them with "self" molecules like CD47 peptides [41] or coating them with cell membranes extracted from autologous leukocytes [42] and red blood cells [43].

Cellular uptake and internalization depend on the NPs' aspect ratio (AR) and the contact angle between NP and surface. Elongated rod nanosystems with very high AR attached better to the cell membrane than spheroidal or rod NPs with lower AR, but are phagocytosed less efficiently [44]. This is probably related to the alignment of the longer axis parallel to the cell membrane: in this case, its internalization is more difficult compared to sphere-shaped NPs. Furthermore, the geometry of the initial contact of the NP with the macrophage (tangent angles) determinates the cell response: the cell starts to remodel the actin cytoskeleton to cover and engulf the nanosystem only when the smaller axis of the oblate-shaped NP contacts the cell membrane. On the contrary, depending on the local particle shape, an incorrect interaction fails to correctly organize the actin, inducing a superficial spreading without any internalization [45,46]. Shape-dependent different macrophage uptake is also attributed to the different endocytosis pathways: spherical AuNPs are generally internalized by clathrin- and caveolin-mediated endocytosis. In contrast, the cylindrical ones are clathrin-mediated endocytosis. Moreover, the elongated shape induces a more efficient interleukin 6 inflammatory response than the shorter rod or spherical ones [47]. Shape-related differential uptake grade was also individuated in other cells, such as the tumour cells. For instance, breast cancer cells show a preferential uptake of rod NPs, followed by dishes and spheres [48].

Furthermore, the shape can also influence specific nanosystem features. Xu *et al.* reported a relation between morphology and reaction rate of silver NPs (AgNPs): the reaction rate of nanocubes was 14 times higher than that of triangular ones and four times more than the semi-spherical ones [49]. The morphology of AgNPs also influences their antibacterial effects: naocomplexes with a higher specific surface area resulted in more toxicity for bacteria than smaller ones due to the difference in the Ag ion release depending on the shape [50,51]. Conversely, the shape plays a pivotal role in mechanical properties and adhesion with hydrogel materials, as suggested by Arno *et al.* Analyzing the interaction between polymeric NPs and calcium-alginate hydrogels, they found an increase in the adhesion and the material's mechanical strength concerning spherical or cylindrical counterparts [52].

2.3 NPs material

NPs can be classified into inorganic and organic depending on the material used. As shown in Figure 1, the first one includes MNPs, quantum dots (QDs), carbon-based nanostructures (CBNs), and mesoporous silica NPs (MSNs). At the same time, liposomes and micelles, dendrimers, and polymeric NPs represent the organic ones. A summary of the features of the major NP classes covered in this review is presented in Table 1.

2.3.1 Inorganic NPs

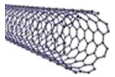
Inorganic NPs play a fundamental role in modern materials science due to their unique physical characteristics

Table 1: Advantages, disadvantages, and biomedical applications of each type of nanoparticle

NPs		Advantages	Disadvantages	Functionalization	Applications in nanomedicine	Ref.
Metallic NPs	AuNPs	• Biocompatibility	• Not biodegradable	• Targeting molecules (antibodies; glycans, *etc.*)	• Bio-imaging (CT/ X-Ray)	[61,64,66–68,72,73]
		• Easy to synthesize and conjugate to biological molecules • High X-ray attenuation • SPR	• Nanoparticles aggregation	• PEG	• Drug delivery • Photothermal therapy • Photodynamic therapy • Tumour therapy • Nano-vaccines	
	AgNPs	• Easy to synthesize • Antibacterial and antiviral activity • Anti-inflammatory and antitumor capacity • Antiangiogenic effects	• Toxic at higher concentrations • Various ecological problems if released into the environment	• PEG	• Drug delivery • Antiviral and antibacterial activity (inhibition of bacterial biofilm formation and EPS production) • Antineoplastic effect	[76,79–82,84,85,90,91]
						
Magnetic NPs	• Possibility to magnetize by the external field		• Limitation to maintain efficacy in the target organ once the magnetic field is removed	• Targeting molecules (folic acid, *etc.*)	• Magnetic biosensing	[98,99,101,104]
		• Well-controlled activation/ deactivation mechanism	• Nanoparticles aggregation	• Coated with silicon, dextran citrate or PEG	• MRI	

(*Continued*)

Table 1: *Continued*

NPs	Advantages	Disadvantages	Functionalization	Applications in nanomedicine	Ref.
		• Toxic effect		• Magnetic separation • Drug and gene delivery • PTT • PDT • RT	
QDs	• Imaging properties	• Toxic effect of metal core	• Biological molecules (peptides, proteins, nucleic acids, *etc.*)	• Drug delivery	[109,112–115,120,121,129]
	• Capability to conjugate different biological molecules	• Nanoparticles aggregation	• Targeting molecules (antibodies, hyaluronic acid, folic acid, *etc.*)	• Bio-imaging	
			• PEG	• Cancer diagnosis and treatment • Theranostic application	
CBNs	• Easy to synthesize and conjugate to biological molecules	• Not biodegradable	• Targeting molecules (antibodies, *etc.*)	• Drug delivery	[142–145,153,159,160]
	• Large surface area	• Potential material toxicity	• Fluorescence probes	• Bio-imaging	
	• Protect entrapped molecules	• Poorly soluble in water	• Biological molecules (proteins, nucleic acids, *etc.*)	• Cancer diagnosis and treatment	
			• Drugs • PEG	• Tissue engineering • Photothermal therapy • Biosensors	

(*Continued*)

Table 1: *Continued*

NPs	Advantages	Disadvantages	Functionalization	Applications in nanomedicine	Ref.
MSNs	• High surface to volume ratio to conjugate with biological molecules	• Not biodegradable	• Targeting molecules (antibodies, *etc.*)	• Bio-imaging	[185–187,189,192,193,196–198, 200,202]
	• Stability	• Potential cell lysis caused by silanol groups interacting with membrane lipids	• Fluorescence probes	• Drug delivery	
	• Easy control of morphology, pore distribution, and size		• Biological molecules (proteins, nucleic acids, *etc.*)	• Cancer treatment	
	• Biocompatibility		• Drugs • PEG	• Tissue engineering • Nano-vaccines	
Lipid-based NPs (LNPs)	• Biocompatibility	• Low solubility and stability	• Targeting molecules (antibodies, *etc.*)	• Drug delivery	[213,215,218,219,221,224,226, 228,230–232,264,268,273,274, 277]
	• Biodegradable	• Tends to agglomerate	• PEG or heparin or albumin or polysaccharides (chitosan)	• Cancer treatment	
	• Amphiphilic	• Some may be allergic	• Hydrophilic and/ or hydrophobic drugs	• Neurodegenerative disease treatment	
	• Longer duration of circulation	• May trigger an immune response	• mRNA	• Trojan Horse Liposome (THL) technology (*e.g.*, to cross BBB)	
			• Drugs	• Nano-vaccines	
Dendrimers	• High drug loading ability	• Immunoreaction	• Targeting molecules (antibodies, RGD, *etc.*)	• Drug delivery	[293,301,304,306,308,309,311, 349]
	• Low polydispersity,	• Haematological toxicity	• Biological molecules (peptides, siRNA, small DNA, *etc.*)	• Cancer treatment	

(*Continued*)

Table 1: *Continued*

NPs	Advantages	Disadvantages	Functionalization	Applications in nanomedicine	Ref.
	• Reproducible pharmacodynamics and pharmacokinetic behaviour	• Toxicity for prokaryotic and eukaryotic cells	• Drugs	• Antiviral and antibacterial activity	
	• High cellular uptake		• PEG	• Tissue engineering	
	• Capability to cross BBB		• Anionic groups (acid or sulfonate residues)		
Polymer NPs	• Biocompatibility	• Inflammatory response	• Targeting molecules (antibodies, folic acid, *etc.*)	• Drug delivery	[32,337,339–341,343–347]
	• Biodegradable	• Nanoparticles aggregation depending on the polymer used	• PEG	• Cancer treatment	
	• Variety for chemical composition		• Biological molecules (GF, peptides, *etc.*)	• Tissue engineering	
	• Stability			• Nano-vaccines	

like size-dependent optical, magnetic, electronic, and catalytic properties. Moreover, they can be quickly and cheaply synthesized and mass-produced, and hence, they can also be more readily used for many applications. These inorganic NPs include metallic ones like gold and silver, QDs, CBNs, and MSNs [53].

2.3.2 Metallic nanoparticles and magnetic nanoparticles

MNPs are amply employed in biomedical applications such as targeted drug delivery, antimicrobial activity, bio-imaging, and diagnosis and disease therapy [54] (Figure 4; Table 1). In addition, they can be used as imaging probes in many techniques like ultrasound (US), X-ray, computed tomography (CT), PET, MRI, optical imaging, and surface-enhanced Raman imaging (SERS) [55].

In this context, AuNPs have recently attracted interest for their use as CT imaging contrast agents, thanks to their high X-ray attenuation, simple synthesis, surface properties, and biocompatibility [56]. Furthermore, they present peculiar absorption and scattering properties like surface plasmon resonance (SPR) that can be tuned by controlling the specific size and shape (sphere, rod, and clusters), depending on the synthesis method [57]. AuNPs can be produced through physical (like the microwave and ultraviolet irradiation or laser ablation), chemical (like the Turkevich method that consists of the reduction of gold chloride with sodium citrate), and biological (plants and microorganisms mediated) ways [58]. The last one is relatively new and attracted great attention because of its eco-friendly character because microorganisms can adsorb gold atoms and collect AuNPs by secreting enzymes involved in the enzymatic reduction of gold ions [59].

AuNPs are widely used in academic research for the tumour treatment [60]. Some studies revealed their potential in limiting angiogenesis and tumour progression; Li *et al.* suggested and demonstrated the AuNPs inhibition effects on epithelial–mesenchymal transition and tumour vasculature normalization [61,62].

Furthermore, their functionalization with targeting molecules permits their specific cancer mass penetration and the release of the associated anticancer drug [63]. Pedrosa *et al.* had designed multifunctional AuNPs carrying a novel chemotherapeutic candidate (ZnD) and monoclonal antibody cetuximab to recognize the epidermal growth factor receptor (EGFR) overexpressed in cancer cells. Data suggested a specific tumour targeting in a colorectal DOX-resistant model, leading to a reduction in the tumour growth without systemic toxicity [64].

Furthermore, AuNPs show an optical scattering that can be exploited for the nanophotolysis technique using a short-pulse laser. Laser photothermal therapy (PTT) is based on the capability of cancer-targeted AuNPs to absorb light and convert it into heat, which leads to thermal

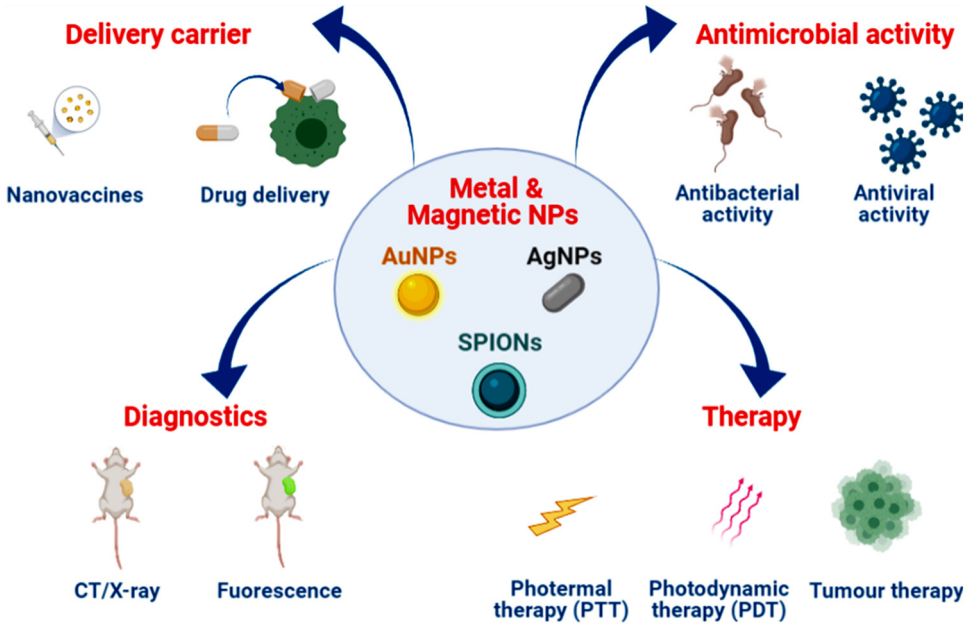


Figure 4: Metal and magnetic nanoparticles (MNPs and MgNPs) including AuNPs, AgNPs, and SPIONs. They present a natural antimicrobial activity (antibacterial and antiviral); can act as delivery carriers both in drug delivery and nanovaccines; can be employed in diagnostics (CT/X-ray and fluorescence analysis) and in disease therapy (*e.g.*, tumour therapy, photothermal, and photodynamic therapy).

explosion when the threshold laser flow is 25–40 mJ/cm^2 [65]. Many studies suggest this approach for cancer therapy because it is an effective way to selectively kill tumour cells without affecting healthy ones. In this context, Zheng *et al.* have synthesized a hybrid nanoplatform made of photothermal gold nanostars and a glycopolymer containing both galactose groups (to target the tumour asialoglycoprotein receptor) and glucose groups (to recognize concanavalin A [ConA], which is cytotoxic to hepatoma cells). A decrease in the tumour size was observed under infrared laser irradiation due to the synergic effect of photothermal and drug therapy [66]. Another form of light therapy adopted against cancer cells and pathogenic bacteria is photodynamic therapy (PDT) based on AuNPs as a photosensitizer (PS). When excited by near-infrared (NIR) light, AuNPs transfer energy to the surrounding O_2 to generate reactive oxygen species (ROS), inducing cell death [67]. PTT and PDT can be combined with radiation therapy (RT) to produce a multifunctional nanotheranostic gold nanocage, as reported by Xy and colleagues. They had synthesized hyaluronic acid-modified Au nanocages (AuNCs-HA) acting as a contrast agent for enhanced photoacoustic (PA) imaging to provide contour, size, and location information of the tumour. By combining radiotherapy and phototherapy, AuNCs-HA could inhibit the tumour growth compared to each therapy alone [68].

All the AuNP features make them an optimal candidate for vaccine formulations acting both as delivery systems and adjuvants [69]. Peptide–AuNP conjugates can be internalized by macrophages, resulting in their activation, and AuNPs of 8–17 nm size bring a strong antibody response with low cytotoxicity [70,71]. The NPs' efficiency as adjuvants could depend on their shape as observed by Tazaki *et al.*: only gold nanorods, but not spherical ones, were able to enhance the intranasal inactivated influenza vaccine adjuvanticity [72]. Conversely, Gulla *et al.* have proposed AuNPs as vectors for *in vivo* mannose receptor-mediated targeting of DNA vaccines to mouse dendritic cells (DCs). The formulated nanovaccines delivered the melanoma antigen (pCMV-MART1) encoded DNA to DCs, inducing a long-lasting immune response against murine melanoma and significant inhibition of the melanoma growth [73].

AuNPs can be recognized from the plasma proteins (opsonization) and processed by the RES in the bloodstream. To prevent this process, AuNPs can be functionalized by adding PEG (PEGylation), which prolongs their blood circulation [74].

PEGylation is also adopted for AgNPs because it increases human cell biocompatibility and inhibits platelet aggregation under flow conditions [75]. Furthermore, due to their low-cost production and low toxicity and immunological response [76], AgNPs are amply used in biomedical applications for drug delivery, bioimaging, and molecular diagnostics, and some of them are FDA (Food and Drug Administration) approved and available in the market (Table 2) [77].

Moreover, AgNPs present excellent antimicrobial and antiviral properties. AgNPs interact with microorganisms and release Ag^+ ions that can bind the negatively charged cell walls (due to carboxyl and phosphate groups) and alter the cell permeability [78]. Consequently, ion uptake inhibits the mitochondria respiratory enzymes leading to ROS production, resulting in oxidative stress, ATP production inhibition, and DNA replication [79]. Gram-negative bacteria are more susceptible to AgNPs than Gram-positive ones: in fact, the thick peptidoglycan layer of Gram-positive microorganisms acts as a protective barrier, limiting AgNP internalization [80].

The antibacterial effect of AgNPs is correlated with their size and shape. Hong *et al.* reported a higher antibacterial effect to *E. coli* of silver nanocubes to spheres and wires [81]. At the same time, their antimicrobial activity decreases with the increasing particle size, as reported by Raza *et al.* [50,82]. Therefore smaller spherical AgNPs are more inclined to release silver ions due to their large surface area, bringing a high antimicrobial effect [83]. Many studies suggest the role of AgNPs in inhibiting bacterial biofilm formation and extracellular polymeric substance (EPS) production, mainly when associated with the plant-derived drug-like quercetin [84,85].

The antiviral activity is due to the interference of AgNPs with (1) viral proteins involved in cellular interaction and consequently in cell internalization inhibition or (2) viral DNA/RNA by blocking virus replication and propagation [86]. For instance, AgNPs (especially in NPs size ranging from 1 to 10 nm) are involved in HIV-1 virus infection by interacting with glycoprotein (*e.g.*, gp 120) and preventing CD4-dependent virion binding [87–89]. On the other hand, Morris *et al.* demonstrated the effect of AgNPs on reducing respiratory syncytial virus (RDV) replication and pro-inflammatory cytokine production both *in vitro* and *in vivo* experiments [90]. Recently, their potential inhibition effect on SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2) was also observed. By comparing AgNPs of different sizes, Jeremiah *et al.* had recently demonstrated that 10 nm AgNPs could disrupt viral integrity *in vitro* [91]. Meanwhile, Balagna *et al.* applied the antiviral effect of silver to confer an antimicrobial/virucidal effect on individual protection equipment (*e.g.*, facial masks) by a silver nanocluster/silica composite sputtered coating, directly applied on the FFP3 (filtering facepiece 3) masks [92].

Table 2: Approved marketed nanoparticles and their application in biomedicine

Name	NPs type/drug	Application	Approval date	Ref.
LNPs				
Diprivan	Liposome/propofol	Anaesthesia	1989	[203,352]
Doxil	Liposome/doxorubicin	Kaposi sarcoma, ovarian cancer	1995	[203,352–354]
DaunoXome	Liposome/daunorubicin	Kaposi sarcoma	1996	[203,352–354]
AmBisome	Liposome/amphotericin B	Fungal infection	1997	[352–354]
DepoCyt	Liposome/cytarabine	Lymphoma	1999	[352,353]
Visudyne	Liposome/verteporfin	Myopia, ocular histoplasmosis	2000	[203,352–354]
Mepact	Liposome/mifamurtide	Myosarcoma	2009	[203,352]
Marquibo	Liposome/vincristine	Acute lymphoblastic, leukaemia	2012	[203,352–354]
Onivyde	Liposome/irinotecan	Pancreatic cancer	2015	[203,352–354]
Vyxeos	Liposome/cytarabine/ daunorubicin	Acute myeloid leukaemia	2017	[203,352–354]
Onpattro	Liposome/patisiran sodium	Transthyretin-mediated amyloidosis	2018	[203,352,354]
Comirnaty [BNT162b2] [Pfizer-BioNTech]	Lipid-based NPs/mRNA	COVID-19	2020	[352,354]
Spikevax [mRNA-1273] [Moderna]	Lipid- based NPs/mRNA	COVID-19	2020	[352,354]
Polymer-based nanoparticles				
Oncaspar	Polymer-protein/pegaspargase	Acute lymphoblastic leukaemia	1994	[352–354]
Copaxone	Polymer-protein/glatiramer acetate	Multiple sclerosis	1996	[352–354]
Verelan	PLGA NPs/verapamil HCl	Hypertension, angina, rhythm disorders	1998	[352]
Renagel	polyallylamine hydrochloride/ epichlorohydrin	Renal disease, hyperphosphatemia	2000	[353]
PegIntron	PEGylated IFN alpha 2B	Hepatitis C	2001	[352,354]
Neulasta	PEGylated GCSFprotein	Neutropenia, chemotherapy induced	2002	[352–354]
Pegasys	PEGylated IFN alpha 2A	Hepatitis Band C	2002	[352,353]
Eligard	PLGH/leuprolide acetate	Prostate cancer	2002	[352–354]
Somavert	PEGylated/visomant	Acromegaly	2003	[352,353]
Estrasorb	Micellar/estradiol	Vasomotor symptoms of menopause	2003	[353]
Cimzia	PEGylated/certolizumab	Crohn's disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis	2008	[352–354]
Krystexxa	Polymer-protein/pegloticase	Chronic gout	2010	[352,353]
Plegridy	Polymer-protein/peginterferon beta-1A	Multiple sclerosis	2014	[352–354]
Adynovate	Polymer-protein/pegylated factor VIII	Haemophilia	2015	[352–354]
Zilretta	PLGA hydrogel/trimconolone acetonide	Osteoarthritis	2017	[352,353]
Inorganic nanoparticles				
INFeD	Iron dextran colloid	Iron-deficient anaemia	1992	[203,354]
DexFerrum	Iron dextran colloid	Iron-deficient anaemia	1996	[203,354]
Ferrlecit	Iron gluconate colloid	Iron deficiency in chronic kidney disease	1999	[203,354]
Venofer	Iron sucrose colloid	Iron deficiency in chronic kidney disease	2000	[203,354]
Feraheme	Iron polyglucose sorbitol carboxymethylether colloid	Iron deficiency in chronic kidney disease	2009	[203,353,354]
Injectafer	Iron carboxymaltose colloid	Iron-deficient anaemia	2013	[203,354]
Ferumoxytol	Magnetic nanopartilces	MRI	2009	[103]
Nanoparticles for imaging applications				
Optison	Human serum albumin-stabilized perflutren microspheres	Ultrasound contrast agent	1997	[203]
Definity	Perflutren lipid microspheres	Ultrasound contrast agent	2001	[203]
SonoVue	Phospholipid stabilized microbubble	Ultrasound contrast agent	2001	[203]

AgNPs also act on eukaryotic cells as antineoplastic drugs by inducing apoptosis. They increase ROS levels by reducing mitochondrial membrane potential, releasing cytochrome C into the cytosol, activating JNK, and translocating Bax to mitochondria [93]. This occurs, for example, in Dalton's lymphoma ascites cell lines both *in vitro* and *in vivo* as reported by Sriram *et al.*: AgNP treatment reduces the volume of the ascitic fluid in tumour-bearing mice by 65%, increasing their survival time by about 50% in comparison with tumour controls [94]. The cytotoxic effect of NPs was also evaluated on healthy human tissues. Many studies have suggested a correlation between toxicology and NP size and morphology: smaller particles can affect cytoplasm and cellular organelles more than bigger ones [95].

In recent decades, great interest was also given to MgNPs, thanks to their unique properties. MgNPs are typically produced from pure metals (such as Fe, Co, Ni) or alloys (such as FeCo, FePt, PtCo; FePd), iron carbides (such as Fe_5C_2, Fe_3C, Fe_2C), or ferrites (such as $CoFe_2O_4$) [96]. There are different MgNP synthesis methods including chemical and physical (like the ball milling method, coprecipitation, thermal decomposition, hydrothermal, microemulsion, sol-gel method) or biological processes starting from bacteria, plants, fungi, or algae [97].

The possibility to magnetize MgNPs by an external field opens a wide opportunity to be used in a huge range of biomedical applications including magnetic biosensing (diagnostics), magnetic particle resonance (MRI), magnetic separation, drug and gene delivery, and PTT, PDT, RT, *etc*. In addition, once the external magnetic fields are removed, the magnetization of MgNPs is extinguished, permitting a well-controlled activation/deactivation mechanism.

Magnetic hyperthermia is based on the possibility to generate heat when an external alternating magnetic field is applied to MgNPs. This technique is amply adopted for tumour therapy because the lower pH of the tumour microenvironment makes cancer cells less thermotolerant and, therefore, more susceptible to hyperthermia [98]. The specific cancer effect can be increased by conjugating specific cancer-targeting molecules like folic acid (FA) to obtain a synergistic anti-tumour effect of chemotherapy and heat treatment as suggested by Wang *et al.* [99]. Superparamagnetic iron oxide nanoparticles (SPIONs) are the most commonly used MgNPs for tumour therapy because of their high biocompatibility, low toxicity for healthy cells, and the capability to induce ROS-mediated cancer cell death by Fenton reaction: Fe^{2+} ions released by SPIONs can destabilize the hydrogen peroxide (H_2O_2) generating $^{\cdot}OH$ radical groups [100]. Fenton metals, like iron, are amply employed in MRI as contrast agents able to deeply penetrate the tumour mass and provide multimodal imaging modalities [101]: the acid tumour microenvironment induces NP clustering limiting their escape into the bloodstream and increasing the imaging resolution [102]. Therefore, some SPIONs like Ferumoxytol and Endorem were approved by FDA as contrast agents for MRI [103] (Table 2).

ROS generation is involved also in the antibacterial mechanism adopted by MgNPs: in fact, ROS can interfere with the major of bacteria components. This permits the use of magnetic NPs against multi-drug-resistant bacteria and biofilm [104]. In the same manner, NIR-triggered PTT can destroy bacterial cell membranes and cause protein denaturation, selectively killing microorganisms [105]. Under physiological conditions, iron oxide NPs present low solubility and can aggregate in the bloodstream. To reduce these limitations, magnetic NPs can be coated with silicon, dextran citrate, or PEG especially when they are used as contrast agents in target organs [106].

2.3.3 QDs

QDs are very small (2–10 nm) NPs or nanocrystals with an inorganic core of semi-conductor of group II/IV (*e.g.*, cadmium/selenium, cadmium/technetium) and an aqueous organic coated shell (*e.g.*, zinc sulphide, cadmium sulphide). Typically, their semiconducting nature confers unique optical and electronic properties. Depending on the core structure and composition, QDs can emit different colours over a wide spectral range if excited by the same light source (Figure 5). Therefore, they are amply employed as fluorescent probes in cellular and *in vivo* molecular imaging [107]. In this context, Zhou *et al.* have developed the faster method point of care testing quantum dot fluorescence immunoassay to detect the high-sensitivity cardiac troponin (a significant biomarker of myocardial injury and necrosis) in whole blood samples [108].

On the other hand, the outer shell can be functionalized by conjugating different molecules like peptides, proteins, or DNA acting as diagnostic and therapeutic agents for cancer diagnosis, PDT cell labelling, and biosensors [109,110] (Table 1). For instance, conjugation with specific antibodies permits a specific targeting of the tumour so that Ab-modified QDs can be used for the detection of primary tumours (such as ovarian, breast, prostate, and pancreatic cancer), as well as local lymph nodes and detached metastases [111,112] (Figure 5). Karakoçak *et al.* formulated hyaluronan-conjugated carbon QDs for bioimaging of tumour cells. *In vivo* studies

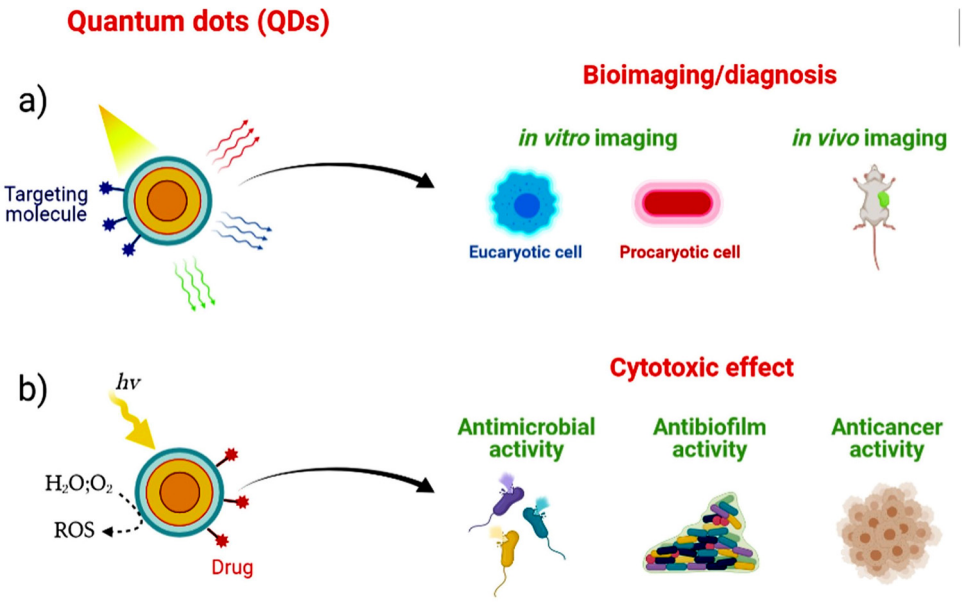


Figure 5: QDs properties and biomedical applications. (a) If excited by a light source, QDs can emit different colours over a wide spectral range. This feature makes them optimal candidate for cellular (on both eukaryotic and prokaryotic cells) and *in vivo* bioimaging and diagnosis (if conjugated to targeting molecules). (b) QDs present also a cytotoxic effect that can be exploited as antimicrobial, antibiofilm, and anticancer activity. When are photoexcited, QDs generate ROS, bringing cell death. The same effect can be obtained by conjugating killing drugs that can be released in a specific tissue.

demonstrated the ability of conjugated hyaluronic acid to recognize CD44 receptors overexpressed by breast cancer tissue and, therefore, selectively detect tumour mass compared to the surrounding tissue [113].

Many studies reported the use of QDs for cellular and targeted drug delivery in cancer treatment as proposed by Ruzycka-Ayoush *et al.* who synthesized FA-conjugated Ag–In–Zn–S QDs modified with 11-mercaptoundecanoic acid (MUA), L-cysteine, and lipoic acid encapsulated with the anticancer drug doxorubicin. Their studies had demonstrated specific targeting, controlled drug delivery, and imaging on adenocarcinoma human alveolar basal epithelial cells overexpressing folate receptors [114]. Conversely, Qin *et al.* demonstrated the double effect of carbon quantum dots (CQDs) for imaging and photocatalytic inactivation of cancer cells. After cell internalization, CQDs emitted a yellow fluorescence signal when excited under blue light and simultaneously started to produce ROS, bringing a 40% decrease in relative cell viability [115].

Despite their extraordinary potential as fluorescence probes, QDs present some biomedical application limitations because of their high toxicity for eukaryotic cells: cadmium could cause interferences in DNA repair or stimulate free radical synthesis [116,117]. In this context, recently, cadmium-free QDs (Cd-free QDs) made of indium/palladium were amply used because of their higher biocompatibility [118,119]. For example, fluorescent Ag–In–S/ZnS quantum dots (AIS/ZnS QDs) are Cd-free QDs amply adopted in bioimaging, including tumour draining lymph node imaging [120] and visualization of transplanted adipose tissue-derived stem cells [121].

Overall, the QD application had given many results *in vitro* systems, especially cellular pathways' understanding. Anyway, even if QDs were removed from heavy metals, the translation to *in vivo* systems still presents some limitations due to the RES system action and side effects. QDs' potential toxicity can be limited by surface modification, like adding PEG [122] or carbohydrates to QDs [123,124]. PEGylation can reduce the liver and spleen uptake decreasing their clearance [125,126]. Therefore, the length and the molecular weight of the PEG and the degree of substitution can modulate the circulation half-life. For example, mPEG-5000-coated QDs circulate longer in mice than mPEG-750-coated QDs that were completely cleared from the bloodstream after 1 h of injection [127].

PEGylation (just as, for instance, the adding of polyethyleneimine and poly-L-lysine) can be also employed to enhance the intrinsic QDs antimicrobial activity. Many studies suggested them as an efficient alternative to traditional antibiotic drugs because they can (1) destroy cell walls/cell membranes, (2) act as oxidizing agents producing ROS, and (3) inhibit cell proliferation by binding with nucleic material (DNA/RNA) [128]. These phenomena

are more evident in positively charged carbon quantum dots (PC-CQDs) than in negatively charged CDs, thanks to the more robust interaction with the bacterial cells. The PC-CQDs showed a stronger antibacterial effect on Gram-positive bacteria than on Gram-negative ones, as suggested by Hao *et al.* who tested its efficiency on different microorganisms such as *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, drug-resistant *E. coli*, and drug-resistant *P. aeruginosa* isolated from the clinic [129]. *In vivo* experiments demonstrated the PC-CQDs ability to support the antibacterial treatment of mixed *S. aureus* and *E. coli* infected wounds in rats with low *in vivo* toxicity. Conversely, Ristic *et al.* synthesized graphene quantum dots (GQD) NPs that present higher biocompatibility for eukaryotic cells and antibacterial activity in infectious diseases. If photoexcited, GQDs generate ROS due to increased propidium iodide cellular uptake, resulting in the killing of the pathogenic bacteria strains such as methicillin-resistant *S. aureus* and *E. coli* as illustrated in Figure 5 [130]. Moreover, QDs optical properties also permit their use for sensing microorganisms, including their detection, Gram type identification, biofilm imaging, and microbial viability assessment [131–134].

2.3.4 CBN

CBNs are amply employed in many biological applications like bioimaging, drug delivery, tissue engineering, diagnosis, and cancer therapy due to their unique features, including thermal, mechanical, electrical, optical, and structural properties [135–137] (Table 1). CBNs include graphene oxide (GO), carbon nanotubes (CNTs), fullerenes, carbon nanohorns, carbon nanodots (CDs), and nanodiamonds (Figure 6). Graphene is a two-dimensional sheet of hexagonally arranged carbon atoms isolated from its three-dimensional parent material, graphite [138]. The oxide form consists of single-atom-thick carbon sheets with carboxylate groups on the periphery, providing pH-dependent negative surface charge and colloidal stability [139]. The basal surfaces contain functional groups of hydroxyl (–OH) that permit the conjugation with many molecules such as antibodies, fluorescence probes, proteins, drugs, or nucleic acids permitting different biomedical applications including tumour therapy, tissue engineering, PTT, and bioimaging (Figure 6) [140]. The basal planes also include unmodified graphene domains that are hydrophobic and capable of steaking (π–π) interactions employed on biological molecules' adsorption like nucleic acid, including small interfering RNA (siRNA) for drug delivery applications [141]. siRNA gene therapy can also be combined with PTT, as proposed by Yin *et al.* for pancreatic cancer. They developed PEGylated GO nanosheets conjugated with the tumour targeting molecule FA to co-deliver two siRNA causing apoptosis, proliferation, inhibition, and cell cycle arrest. The synergistic combination of gene silencing and NIR light thermotherapy *in vivo* mouse model showed tumour volume growth inhibition by >80% [142].

Another significant feature of graphene NPs is the capability to promote the growth, proliferation, and

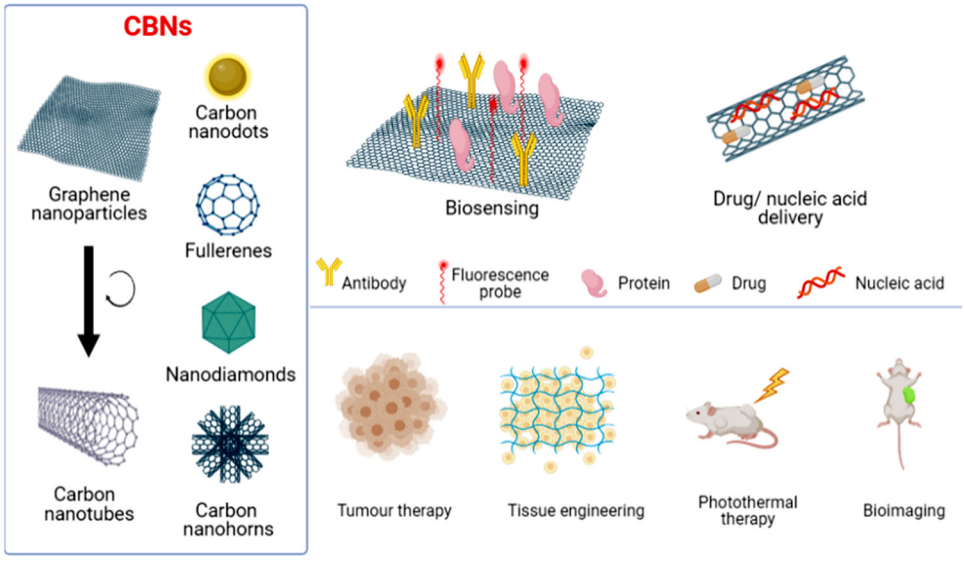


Figure 6: CBNs including graphene NPs and its wrapping structure carbon nanotubes, carbon nanodots, fullerenes, nanodiamonds, and carbon nanohorns. Thanks to the possibility to conjugate fluorescence molecules, antibodies, proteins, drugs, or nucleic acids, CBNs can be employed in tumour therapy, tissue engineering, photothermal therapy, and bioimaging.

differentiation of mesenchymal stem cells (MSCs) [143], neural stem cells [144], and induced pluripotent stem cells [145] into tissues of various lineages [146–148]. Therefore, its possible employment in tissue engineering and regenerative medicine has generated significant interests, thanks to the further possibility of combining it with other materials like poly-L-lactide (PLLA) [149]. For example, 3 wt% of graphene added to PLLA scaffolds facilitates the differentiation of bone marrow-derived MSCs/stromal cells (BMSCs) and increases the calcium deposition and formation of collagen type I [150].

Furthermore, graphene-specific features, including the electrical conductivity, high electron transfer rate, and capability to immobilize different molecules, make them optimal candidates as biosensors for the electrochemical detection of nucleic acids (through dsDNA), ions and molecules (through enzymes), or cells and microorganisms (through antibodies) [151]. In fact, graphene-based materials are amply used in diagnostics, thanks to their ability to recognize specific molecular biomarkers (*i.e.*, circulating tumour cells, exosomes, circulating nucleic acid, *etc.*) in liquid biopsies [152]. Recently, the use of graphene for SARS-CoV-2 detection has sparked great interest in the scientific community. For instance, Seo *et al.* developed a graphene field-effect transistor (FET)-based biosensing device conjugated to anti-SARS-CoV-2 spike protein antibody to detect the virus in human nasopharyngeal swab specimens [153].

CNTs are also amply used in biosensing applications, thanks to their unique features like high AR, stability and thermal and electrical conductivity, strong mechanical strengths, and fast electron transfer rate [154,155]. They originate by wrapping graphene into a cylinder structure forming a tubular structure of 1–2 nm in diameter: the rolled sheets can be single (single-walled CNTs- SWCNTs), double (double-walled CNTs DWNTs), or more than two (multi-walled CNTs – MWCNTs) [136,156]. Their limited solubility on all solvents generates toxicity problems that can be solved by chemical modification with peptides, proteins, nucleic acid, and therapeutic molecules that can increase the cellular uptake and drug release when used as DDS [136,157,158]. Su *et al.* conjugated iRGD-polyethyleneimine (PEI) and candesartan (CD) to develop MWCNTs targeting the tumour endothelium and lung cancer cells (by recognition of αvβ3-integrin and AT1R). In addition, plasmid AT2 (pAT2) was assembled to form iRGD-PEI-MWNT-SS-CD/pAT2 complexes. Co-delivery of CD and pAT2 synergistically inhibited angiogenesis by downregulating vascular endothelial growth factor and inducing tumour growth suppression in A549 xenograft nude mice [159]. Moreover, the nature of CNTs makes them ideal elements for tissue engineering; for instance, Vaithilingam *et al.* introduced multi-walled CNTs to 3D scaffolds to stimulate human pluripotent stem cells to differentiate into cardiomyocytes and modulate their behaviour [160].

Similar to SWCNTs, carbon nanohorns (CNHs) present a conical hollow configuration constituted by sp^2-hybridized carbon atoms highly resistant to oxidation. Although easily synthesised and functionalised, CNHs present some limitations during biomedical applications because of their aggregation into spherical clusters [161]. Some strategies were developed to increase CHHs polydispersity such as the addition of potassium naphthalenide [162,163]. On the other hand, CDs present water-dispersible properties in addiction to fluorescent ones. In fact, they are fluorescent carbon nanostructures with a size less than 10 nm amply employed in biological imaging. The modification with nitrogen, sulphur, and phosphorus can improve the bioluminescence features, while the conjugation with drugs or targeting molecules increases their interest in biomedical applications, especially in cancer theranostics [164]. For instance, when CDs are irradiated by NIR light, ROS that selectively kills tumour cells is generated (NIR light-irradiated PDT) [165,166]. Notwithstanding the interesting properties, *in vivo* CD applications present some limits due to the concentration-related toxicity for the blood compound: a concentration higher than 0.1 mg/mL induces red blood cells lysis, complement activation, and platelet-mediated coagulation stimulation [167].

Fullerens consist of hexagonal and pentagonal rings than confer the typical curvature to generate hollow spheres (named buckyballs), ellipsoids, or tubes. Buckminsterfullerene (C_{60}) is the most common fulleren amply adopted in biological applications, thanks to its electrochemical, physical, and photographic features [168]. Although fullerens are soluble only in organic solvents, their structure permits the addition of hydroxyl groups (fullerenols) that increase their solubility in water and also the conjugation with a huge range of biological molecules for biomedical applicants in tissue engineering and photothermal and cancer therapy and also antibacterial and antiviral activities [169,170]. Therefore, the first water-soluble fullerene form was developed in 1993 by Wudl and coworkers who studied its action as an inhibitor of the HIV-1 protease (HIV-1 PR) [171,172].

Conversely, the capabilities to bind a high amount of drugs and to easily enter into the cells make nanodiamonds (NDs optimal candidates for drug delivery in biological systems. They present a truncated octahedral structure, faceted or with a rounded shape, often modified by additional functional groups (like hydroxyl, carbonyl, carboxyl, anhydrides, and lactones) that limit aggregation, improving

biocompatibility [173]. Their good stability, biocompatibility, and good optical properties permit them to be used as biological markers for bioimaging investigation, as DDS for cancer or metabolic diseases and as fillers of biocomposite scaffolds for tissue engineering. NDs present a low osteogenic differentiation capability that can be increased by conjugating chemical compounds like icariin, as proposed by Choi *et al.* [174,175].

2.3.5 MSNs

MSNs are porous spherical particles of 50–300 nm constituted by a polymeric structure of siloxane (–Si–O–Si–O–) rich in silanol (Si–OH) groups on their surface that can be modified by conjugation with biological molecules to obtain multifunctional nanoconjugates [176]. The porous structure permits conjugating molecules (*e.g.*, drugs, fluorescence probes, targeting molecules, nucleic acid) in the inner part and surface (Figure 7). The controlled chemical synthesis permits to regulate their morphology, pore distribution, size, and biodegradability [177]. Parameters like pH, surfactant, silica precursor, and temperature can modulate NP size and shape that play a crucial role in cellular uptake, immune escape, and elimination rate [178]. For example, long-rod MSNs are captured by the spleen with a low elimination rate, while the short-rod ones are localized in the liver [179].

Generally, MSNs are highly biocompatible because they degrade to silicic acid naturally present in body fluids and connective tissue, such as hair, nails, bone, skin, and tendons, and are rapidly eliminated through urine [180–182] (Table 1). In this context, MSN circulating time can be highly regulated: surface modification like the PEGylation can prolong their permanence in the bloodstream. At the same time, the increase of their pore size or the addition of metal ions can accelerate their elimination from the body [183,184]. By analysing the MSNs biodistribution and clearance kinetics in healthy rats, Dogra *et al.* showed that big NPs (142 nm) presented lower accumulation in the liver and spleen compared to the smaller ones (32 nm) [185].

Furthermore, the significant amount of pores and channels that confers a high surface-to-volume ratio permits to accommodate a large number of biological molecules, including therapeutic agents or drugs that make them optimal candidates as drug delivery carriers for tumour therapy as suggested by Duo *et al.* [186,187] (Table 1, Figure 7). They synthesized doxorubicin-loaded MSNs coated with polydopamine (PDA) to obtain a pH-sensitive drug release. Furthermore, NPs were functionalized with PEG to increase the stability and biocompatibility of nanosystems. *In vitro* and *in vivo* analysess in the breast cancer model had suggested a higher cellular uptake and a controlled drug release with an improved anticancer activity than a free drug [187].

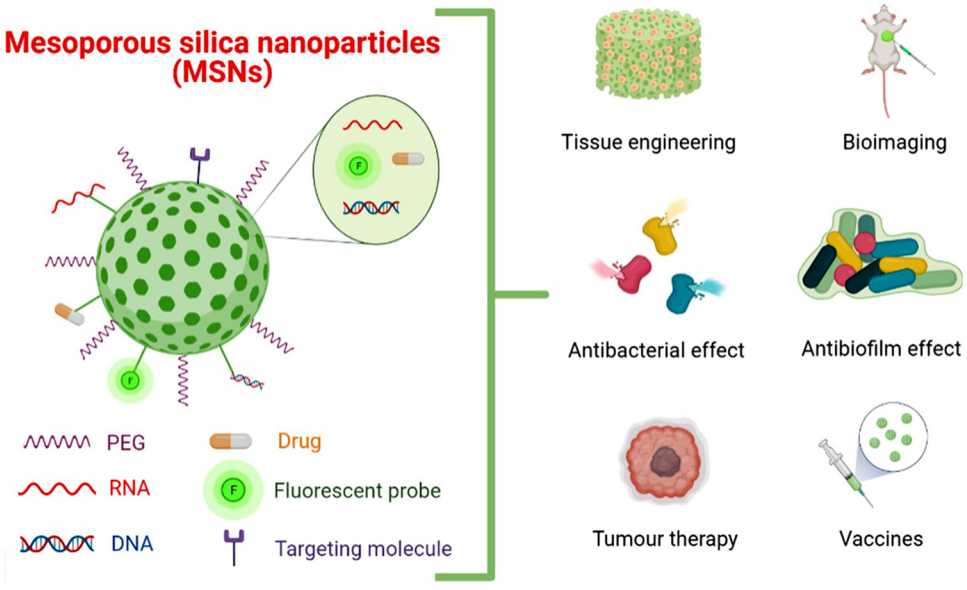


Figure 7: Mesoporous silica nanoparticles (MSNs) functionalization and biomedical applications. Biological molecules (*e.g.*, fluorescent probes, drugs, DNA, and RNA) can be encapsulated in the pores or conjugated to the surface of MSNs just as targeting molecules and PEG. Fictionalized MSNs are employed not only in tissue engineering, bioimaging, tumour therapy, and vaccines but also in antibacterial and antibiofilm treatment.

Silica can also reduce the toxicity of other NPs containing, for example, heavy metals like gadolinium (Gd), which is typically used as a contrast agent in MRI [188]. In the same manner, their functionalization can occur for other bio-imaging applications such as optical imaging, PET, and CT [189–191]. Recently, MSNs are also employed in PA imaging, thanks to the possibility to encapsulate the PA contrast agent ICG that produces heat under NIR irradiation [192]. Due to the heat-induced thermoelastic change, the tissue generates US waves detected by US transducers [193].

Zhang *et al*., to limit the aggregation and increase the drug loading to AuNPs, synthesized a mesoporous silica shell-coated AuNPs conjugated with doxorubicin as an anticancer agent [194,195]. At the same time, Ramasamy *et al*. developed AuNPs coated with silica to deliver the antibiofilm agent cinnamaldehyde to eradicate bacteria [196]. The MSN structure is ideal for loading, protecting, and transporting antimicrobial molecules to the target bacteria and/or biofilm and releasing them in a stimuli-responsive way, as reported by Yang *et al*. [197,198]. They described the *in vitro* and *in vivo* antibacterial efficiency of MSNs carrying gentamicin and bacteria-targeting peptide ubiquicidin coated by bacterial toxin responsive lipid bilayer surface shell. Their data demonstrated the *S. aureus* growth inhibition and the downregulation of inflammation-related gene expression in infected preosteoblast or macrophage [198].

Moreover, MSNs could be incorporated on scaffolds for tissue engineering, especially bone tissue engineering: MSNs loaded with bioactive factors can be combined with scaffolds to improve repair efficacy [199] (Figure 7). MSNs can release Si ions that can influence stem cell behaviour, especially in gene expression in differentiation and osteogenesis [200].

Moreover, mesoporous silica NPs present an interesting potential as a vaccine adjuvant, as suggested by Oliveira *et al*. who had investigated the MSNs vector efficiency against the parasite *Schistosoma mansoni*. In particular, they had developed MSNs associated with SWAP (soluble worm antigenic preparation) to test their higher immunization activity compared to a conventional immunization system (SWAP-associated aluminium salt) [201]. In this scenario, MSNs pore sizes play a key role in the presentation of peptide-major histocompatibility complex (MHC I) complexes to $CD8^+$ T cells, as suggested by Hong *et al*. They had shown that the association of ovalbumin (OVA) tumour antigen with MSNs enhanced both antibody and T cell responses and, in particular, the large-pore MSNs had established the strongest antitumor effects and immune response. Nanosystems, indeed, facilitated OVA escape from lysosomal degradation for MHC I restricted [202].

2.3.6 Organic NPs

In the last years, many researchers have focused their studies on the possible use of organic (ONPs) in different sectors, especially biomedical ones. Indeed, the organic nature of these systems highly reduces their toxicity and, therefore, side effects. There are different types of ONPs depending on their composition and structure like liposomes and micelles, dendrimers, polymeric NPs, and nanogels.

2.3.7 Lipid-based nanoparticles

Lipid-based nanoparticles (LNPs) are the most common FDA-approved NP type due to their features like the easy synthesis process, biocompatibility, and capability to deliver molecules of different nature (Table 2, Figure 8) [203]. It is a vast family of different types of nanosystems, including liposomes, micelles, exosomes, niosomes, solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), lipid hybrid nanoparticles (LPHNPs), and lipid calcium phosphate nanoparticles (LCPNPs) that differ for the lipid composition and are employed in many applications like the "Trojan horse liposome" (THL) technology, tumour therapy, tissue engineering, and vaccines (Figure 8).

Liposomes, an early version of LNPs, are vesicles constituted by a self-assembled phospholipid bilayer that assumes a spherical shape delimiting an aqueous core of 50–1,000 nm diameter [204]. Depending on the bilayer's number, it is possibly classified into small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), giant unilamellar vesicles (GUVs), multivesicular vesicles (MVs), and multilamellar vesicles (MLVs), in which the layers are separated by aqueous spaces [205]. This unique structure permits to load of both hydrophilic and hydrophobic molecules: the hydrophilic ones are localized in the inner core or between the bilayers; in contrast, the hydrophobic molecules are associated with the phospholipid membranes [206]. This leads to multidrug loading and, consequently, a sequential drug release from the two different compartments. They are primarily employed in the DDS because they can fuse with the plasmatic membrane and release the drug inside the cell [207]. Furthermore, PS inclusion in the liposomes permits a light-induced cargo release (light-induced liposome technology) [208]. Instead, micelles are characterized by a single lipid layer that defines a spherical structure with a hydrophobic core, fundamental for transporting lipophilic molecules like many antitumor drugs [209].

Thanks to their nature, liposomes and micelles are non-toxic, biocompatible, biodegradable, and non-immunogenic (Table 1). Moreover, their chemical–physical properties can

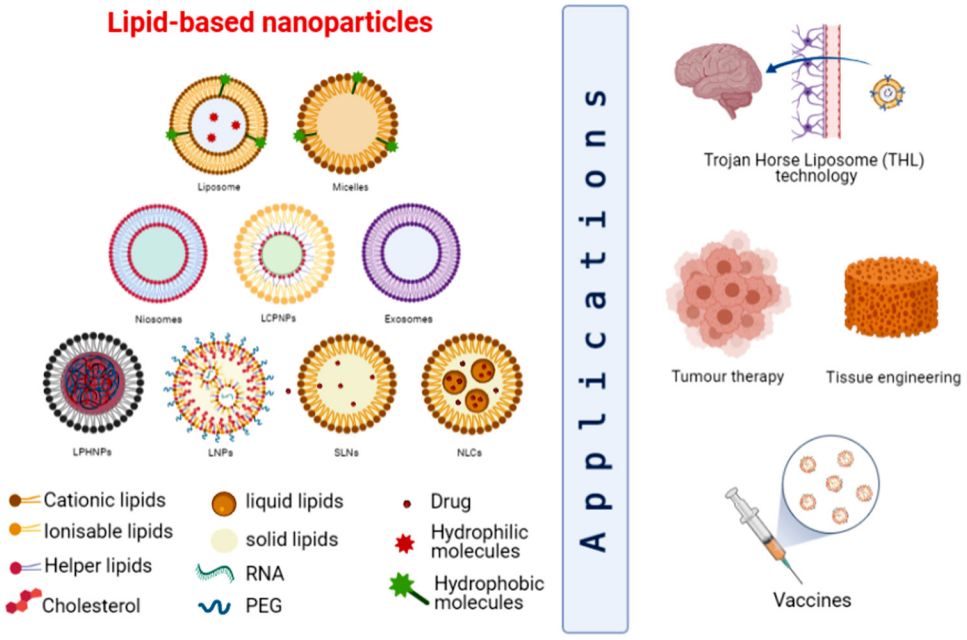


Figure 8: LNPs including liposomes, micelles, exosomes, niosomes, solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), lipid hybrid nanoparticles (LPHNPs), and LCPNPs and their application in biomedical research. Liposomes can encapsulate hydrophilic molecules in the inner core, while the hydrophobic ones are associated with the phospholipid membranes; micelles can link lipophilic molecules in the hydrophobic core; LNPs can encapsulate nucleic acid like RNA. SLNs are composed by solid lipids, while NLCs by both solid and liquids. Thanks to their characteristics, LNPs are amply studied in Trojan Horse Liposome (THL) technology for NDs, cancer therapy, tissue engineering, and in vaccine formulations.

be accurately modified by mixing different lipids molecules and changing the superficial charge, size, and functionalization [210].

Even if they have a good distribution in the organism, they present some disadvantages like low solubility and half-life, the possibility to leak the loaded drugs, especially when they occasionally change lipid components with high- or low-density lipoprotein (HDL and LDL respectively) and thus modify their size and composition, and also their accumulation into the tissues [211]. In addition, surface modification like PEGylation can elongate the circulating time in the bloodstream, while the addition of targeting molecules can improve targeted delivery [212]. In this context, PEGylated liposomes are amply studied in the THL technology for transvascular non-viral gene therapy of the brain. Cationic liposomes of THL carrying non-viral gene expression plasmids are functionalized with specific antibodies able to recognize antigens on the BBB and, in this way, permit its crossing [213,214]. Cationic liposomes' encapsulation of genetic material limits their degradation from ubiquitous nucleases. The exogenous gene is expressed within 1 day of a single intravenous administration, as Jiang *et al.* had demonstrated; indeed, THL treatment reduced tissue inclusion bodies in the brain and peripheral organs [215,216]. This technique is also applied in NDs like Parkinson's disease [217,218] and tumour therapy, as described by Zhang *et al.* They reported that monoclonal antibody-targeted THLs carrying a siRNA knocking down the EGFR were capable of increasing the survival time of mice with intracranial brain cancer [219,220]. Liposomes and micelles are the most primarily studied vector for drug targeting macrophages in treating diseases like salmonellosis, leishmaniosis, tuberculosis, rheumatoid arthritis, and cancer [221–226]. Furthermore, liposomes can mimic pathogens' features, inducing humoral and cellular immune responses, thanks to their capability to present antigens to antigen-presenting cells. Therefore, they can be optimal candidates as vaccines. Depending on the saturation grade of the lipids, they could induce a Th2 (if they are unsaturated) or Th1 (if they are saturated) response [227]. For example, Huang *et al.* reported the improved efficiency of the Pfs230 malaria transmission-blocking antigen candidate when it was loaded on liposomes containing cobalt–porphyrin–phospholipid and the synthetic monophosphoryl lipid A (PHAD). They had shown an increase in anti-Pfs230C1 IgG response in mice after 250 days, the inhibition of parasite transmission, and the immunization of rabbits [228].

Cationic lipid NPs are amply employed as nucleic acid-based vaccines (mRNA-carrying LPNs). They differ

from liposomes because, inside the core of the particles, they present micellar structures in which nucleic acid, including mRNA, can be protected from enzymatic degradation. Generally, they consist of cationic or ionisable lipids that interact with negatively charged nucleic acids and helper lipids (like phospholipids, cholesterol, and/or PEGylated lipids) that increase their stability and promote cellular uptake and consequently the nucleic acid delivery [229]. Espeseth *et al.* demonstrated a higher cellular immune response of mRNA/lipid NPs than to the protein-based vaccine. They tested lipid NP-encapsulated mRNA vaccine encoding respiratory syncytial virus F protein on rodent animals, highlighting the total absence of vaccine-enhanced respiratory disease that generally occurs after protein immunization [230]. Furthermore, two of the most used COVID-19 vaccines (mRNA 1273 and BNT162b2) were developed using LPNs as a vector to deliver mRNA encoding for a full-length, perfusion stabilized spike (S) protein of SARS-CoV-2 [231,232]. After intramuscular injection, LNPs are internalized by host cells in which the mRNA is release, translated into the S protein that is exposed in the cell membrane to immune system cells [233].

Exosomes are small extracellular vesicles constituted by a phospholipid-bilayer membrane that enclose various molecules depending on the type of mammalian cell that secreted them [234]. Based on this, they can be used as potential biomarkers for early detection and monitoring of the progression of cancer or other pathologies like neurodegenerative ones [235,236]. In the same manner, exosomes can deliver drugs or other biomolecules acting as DDS [237]. Generally, they are isolated by ultracentrifugation and filtration, even if these techniques limit the purity and can partially destroy the NPs. Other methodologies were optimized like size-exclusion chromatography (SEC) or polymer precipitation, but the isolation protocol could be optimized to obtain more pure exosomes [238]. Because of their nature, exosomes are highly biocompatible, present innate stability and low immunogenicity, and can easily enter cells and tissues. Conversely, they present some disadvantages like the limited drug loading, the rapid clearance by the reticuloendothelial system, and the difficulty in specific targeting that depends on the homing sources. For example, tumour cell-derived exosomes can target metastatic and cancer cells, while macrophage-derived exosomes can cross the BBB [239]. Despite their great potential, further improvements need to be carried out for their use in clinical.

Niosomes present also a bilayer structure that, in this case, is formed by self-association of non-ionic surfactants and lipids such as cholesterol in an aqueous phase. Depending on the type of components, the size, the surface charge, and the number of lamellae, niosomes can be classified into three groups: SUV (10–100 nm), LUV (100–3,000 nm), and MLV, where more than one bilayer is present [240]. As liposomes, niosomes are able to load both hydrophobic and hydrophilic drugs. However, they present some advantages compared to liposomes [241]. The uncharged single-chain surfactants confer higher stability in contrast with neutral or changed double-chain phospholipids of the liposomes. Furthermore, the lower cholesterol concentration in niosomes bring to higher drug loading and longer self-life [242,243]. Thanks to their nature, different administration methods can be adopted, like parental and oral. It has been amply reported that niosomes can enhance the oral bioavailability of different drugs, including paclitaxel, griseofulvin, carvedilol, and nateglinide [244,245]. Conversely, the nasal administration permits brain uptake through the direct nose-to-brain pathway for the neurodegenerative or glioma therapies [246], while the possibility to use with the stratum corneum permitting drug penetration in dermal tissue (dermal administration) [247]. Finally, niosomes could prolong residence time in the cornea and could be incorporated in foaming gels, creams, and ointments for ocular administration [248].

In 1991, SLNs were developed by combining features of the classical NPs (metal or polymeric NPs at that time) and the lipid-based liposomes [249,250]. SLNs are generally spherical with a diameter between 50 and 1,000 nm and are constituted by lipids (including fatty acids, triglycerides, steroids, and waxes) and surfactants as stabilizing agents [251]. Surfactants, like lecithin phospholipids, Tween, Span, sodium cholate, polysorbates, poloxamers, and bile acids, increase the SLN stability by limiting the interfacial tension and the aggregation of the NPs, especially if used as a mixture [252]. SLNs present not only good biocompatibility and biodegradability as well as other lipid NPs but also easy sterilization and increased stability due to the rigid core lipid matrix, permitting the encapsulation of both lipophilic and hydrophilic drugs [253,254]. Differently from liposomes, they are synthesized by organic solvent-free methods (through high-pressure homogenization technique or micro-emulsification processes), allowing large-scale manufacture at a low cost. Another significant advantage of SLNs is the wide range of administration routes, including oral [255], intravenous [256], pulmonary [257], ocular [258], and transdermal [259].

In contrast to other NPs, the lipid nature of SLNs makes them an optimal candidate for oral administration that is feasible as aqueous dispersions or in the dosage forms of capsules, tablets, and pellets. Indeed, the solid

lipid matrix of SLNs can enhance their bioavailability by protecting encapsulated drugs from chemical and enzymatic activity in the gastrointestinal tract (GIT) [260]. SLNs exploit the oral drug delivery *via* intestinal lymphatic transport, reaching the lymphatic system through microfold cells (M cells) [261,262]. Moreover, SLNs can be surface modified by adding heparin, albumin, PEG, and polysaccharides like chitosan to prevent the high drug release due to the low pH of the stomach [263]. The bioavailability could also be increased by conjugating P-gp inhibitors as proposed by Shah *et al*. They synthetized SLNs as carriers for the anti-diabetic drug linagliptin (LGP) and functionalized them with Tween 80 as a P-gp inhibitor. Data reported that this formulation increased the oral bioavailability from 29.5% (LGP alone) to 300%, carrying to a reduction in glucose levels compared to the drug alone [264]. Oral administration of NPs can also be adopted for the treatment of other pathologies including, cardiovascular diseases [265], cancer [266], and neuropathologies, thanks to their ability to cross BBB [267]. For example, Shen *et al*. developed doxorubicin and SPION-loaded SLNs for a chemo/magnetothermal colon tumour therapy. The active targeting was obtained by adding folate (FA), and the entire complex was covered and protected by a dextran shell. Once arrived in the small intestine, the shell is degraded by dextranase, bringing the FA exposure and the NPs colon tumour cellular uptake. In this manner, the drug stability was increased not only by the SLNs themselves but also by the possibility to functionalize them (*e.g*., dexatran). Compared with the drug alone, the anticancer efficiency of the nanocomplex was significantly higher, bringing to the effective inhibition of primary colon tumour and metastasis [268].

Despite several advantages, SLNs have drawbacks including poor drug-loading capacity and drug expulsion during long-term storage because of lipid crystallization [252]. Therefore, NLCs were developed to overcome these limits. NLCs consist of both solid and liquid lipids that reduce the crystallinity of the lipid core, increasing the drug loading and long-term stability. Based on this, NLCs can be classified into imperfect, amorphous, and multiple types [269]. Imperfect type is constituted by different lipids, like glycerides carrying fatty acids of different lengths and saturation, engendering imperfections in the crystal order. The amount of loaded drug depends on the imperfections grade (a higher imperfection grade corresponds to a higher drug loading). In the amorphous NLCs, the solid and liquid lipids are mixed to form an amorphous unstructured non-crystalline matrix. Multiple NLSs are oil-in-fat-water-carriers (O/F/W) in which oil nano-compartments, hosting drugs, are surrounded by solid lipids acting as a barrier to protect drugs and control their release [270].

As reported earlier for SLPs, NLCs can also be administered in different ways like oral, ocular, transdermal, intranasal, intravenous, and pulmonary routes [271]. The intranasal (IN) route is a non-invasive approach amply adopted for nose-to-brain delivery using NLCs as vectors to increase the drug bioavailability. Three pathways can be involved in this process: the systemic ones in which, after reaching the systemic circulation, drugs can cross the BBB; the olfactory pathway, where drugs go into the brain tissue via the olfactory bulb; and the trigeminal way where the drugs arrive in the nervous system through trigeminal nerve [272]. Since the drug absorption can be limited by the mucociliary clearance in the nasal cavity, NLCs can be superficially modified with cationic polysaccharides, such as chitosan, which were able to interact with the negative charge of the mucosa. For instance, Singh *et al*. developed chitosan-coated NLC loaded with the antipsychotic drug asenapine for intranasal delivery [273]. Their data showed that NLCs as carriers increased the systemic and brain bioavailability by 2.3- and 4-folds compared to drugs alone. Pai and Vavia also adopted the chitosan coating to make mucoadhesive on the surface of NLCs [274]. They used NLCs as a vector for the antineoplastic eye drug etoposide for ocular administration, showing NP localization around the ocular surface confirmed by the high drug concentration. Furthermore, many assays have demonstrated non-toxicity and the absence of ocular irritancy.

On the other hand, all the mentioned SLNs and NLCs properties make them optimal also for cosmetic and pharmaceutics applications [249]. In particular, many studies reported the NLCs' ability to improve wound healing by enhancing drug penetration and maintaining skin hydration and moisture [275,276]. Starting from this, Chato-Astrain *et al*. combined the NLCs properties to bioartificial human dermis substitute for tissue engineering application [277]. In particular, they functionalized fibrin-agarose biomaterials with antimicrobial-loaded NLCs to treat infected wounds, typical of severe burns. Their data showed that the antibacterial effect was directly proportional to the NLC amount and that these NPs were able to improve some important biomechanical properties of the artificial construct.

The special properties of LNPs can be combined with those of polymeric nanoparticles (PNPs) to develop the LPHNPs. Lipid component increases the biocompatibility, stability, and permeation of drug across the cell membrane, while the nature of PNPs permits to regulate the drug loading and release [278]. Taken together, these characteristics make LPHNPs an optimal candidate for DDS with excellent drug

release kinetics, high encapsulation efficiency, and low toxicity. LPHNPs are constituted by (1) a hydrophobic polymeric core, in which the therapeutic drugs are encapsulated, surrounded by (2) a phospholipid monolayer that makes more biocompatible the polymeric core, and (3) an outer lipid-PEG layer that confers stability increasing the *in vivo* blood circulation time of the LPHNPs. Based on their structure, LPHNPs can be classified into [279,280]:

- Monolithic hybrid NPs (random dispersion of lipids in a polymeric matrix);
- Polymer-core lipid-shell NPs (polymeric core surrounding by lipid shell);
- Hollow core lipid–polymer–lipid NPs (hollow aqueous core surrounding by cationic lipids followed by polymer layer and a neutral lipids layer);
- Erythrocyte membrane-coated LPHNPs (polymeric core surrounding by a cell-derived membrane);
- Polymer-caged liposomes (liposomes coated by an external polymer layer).

By integrating two different kinds of NPs, LPHNPs can be employed in a huge range of biomedical applications including cancer therapy because the polymeric part permits the conjugation with hydrophobic chemotherapeutic drugs and the lipid part protects them from early degradation and permit the association with targeting molecules, like FA [281]. For instance, Khan *et al.* developed FA-conjugated chitosan LPNPs and showed an increase in their breast cancer cellular uptake and efficiency compared to non-targeted NPs [282]. Many studies also reported the use of LPHNPs for gene therapy, thanks to their large-DNA incorporation ability [283,284]. Cationic LPHNPs are employed also for siRNA delivery as proposed by D'Angelo *et al.* who investigated the use of PLGA and dipalmitoylphosphatidylcholine-based LPHNPs for siRNA pulmonary delivery for cystic fibrosis (CF) therapy. Their data highlighted the efficiency of these nanosystems to be internalized inside the airway epithelial barrier and are able to transport siRNA and release it reducing the relative protein expression [285]. Despite good *in vitro* results amply reported in the literature, few data of *in vivo* data can be found, suggesting that more investigation into the use of LPHNPs for gene therapy needs to be carried out.

Conversely, it is amply reported in the literature on the use of LCPNPs for gene delivery. Calcium ions are able to complex with nucleic acids protecting them from the serum nucleases, and calcium phosphate (CaP) is dissolvable at a pH of 4–5 [286]. This pH is typical of the lysosomes in which CaPs can go after cellular internalization, permitting the drug release. The coating of CaP with cationic lipid compound form LCPNPs, and this increases their stability and the siRNA delivery, as suggested by Tang *et al.* [287]. The NP's features, including size and drug loading, depend on the Ca^{2+}/PO_4^{3-} ratio. Due to the pivotal role of the calcium and phosphate ions in bone resorption and deposition regulation (healthy bone cells communicate through Ca^{2+} and PO_4^{3-} channels), LCPNPs are amply involved in bone tissue regeneration [288]. In fact, many studies demonstrated the use of this type of NPs as a delivery system of factors for bone application, including antibiotics, anti-inflammatory agents, and growth factors (GFs) [289]. Among these, bone morphogenetic factors (BMPs) are the most used GFs. Some studies reported also the delivery of small plasmid DNA carrying genes like BMP, microRNA, or siRNA [290].

2.3.8 Dendrimers

Dendrimers are nanovectors with a spherical shape constituted by polymeric macromolecules capable of self-assembling. They present three different parts: a central hydrophobic core available for the encapsulation of drug molecules; ramification repeated units named "dendrons" that determinate the generation of the dendrimer and its globular structure; hydrophilic functional groups at the outer side that can be conjugated with specific molecules for the complex formation or other functionalizations, as shown in Figure 9 [291]. Their synthesis is based on the polymerization process of the ramification units from the surface to the core (convergent synthesis) or *vice versa* (divergent synthesis), and it can be patterned to control the drug release [292]. Thanks to this globular shape, they present a high drug loading ability through both covalent and noncovalent bonds, low polydispersity, reproducible pharmacodynamics, and pharmacokinetics (Table 1). Moreover, the positive charge on their surface due to amino groups' presence permits the interaction with nucleic acids, like siRNA or small DNA and the association with cell membranes with cellular uptake [293,294] (Figure 9).

On the contrary, the cationic charge makes them toxic for both prokaryotic and eukaryotic cells so that they are rapidly eliminated from the bloodstream by the mononuclear phagocyte system [295,296]. Therefore, generally, they are modified with molecules like PEG that are able to shield the positive charge, improving circulation time and making them more biocompatible even if it depends on PEG molecular weight, degree of PEGylation, and tested cell lines [297]. The antibacterial activity is related to the ratio of surface cationic charge to hydrophobicity. It is probably mediated by disrupting the bacterial outer and inner membranes due to positive charges

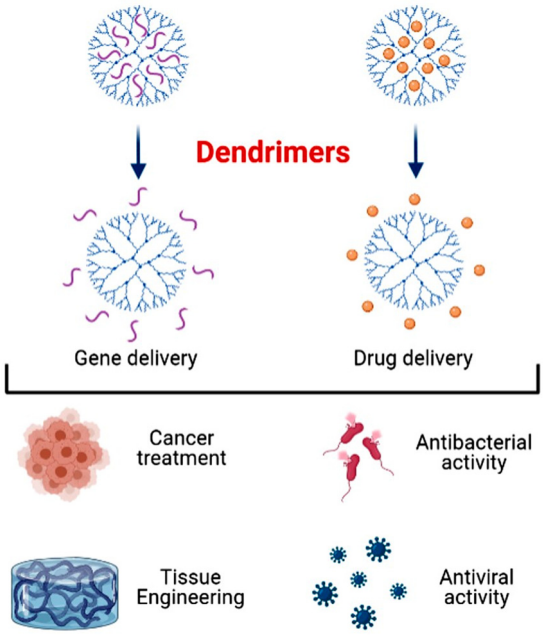


Figure 9: Dendrimers features and biomedical applications. Thanks to their ability as gene and drug delivery systems, dendrimers are amply employed in cancer treatment, tissue engineering, and antimicrobial (bacterial and viral) activity.

of terminal amino groups, as Kannan *et al.* have suggested [298]. Furthermore, Valikala *et al.* demonstrated this effect against both Gram-positive and Gram-negative bacteria even after PEGylation [299]. Conversely, their functionalization with anionic groups, such as acid or sulfonate residues, permits limiting the eukaryotic cell toxicity and determinates artificial mimics of the anionic cell surfaces to exploit an antiviral function. Based on the virus–cell interaction depending on the binding to the cell membrane's sulphated residues, dendrimers can compete with cells for binding of virus and, therefore, stop the infection [300,301].

There are over 100 families of dendrimers depending on their functionalization moieties and on the initiator cores (carbon, nitrogen, and phosphorus). They are classified as polyamidoamine (PAMAM), polypropylenimine (PPI), carbosilane (CBS), poly-L-lysine (PLL), and phosphorus dendrimers [302]. PAMAM and PPI are amply employed in pharmaceutical sciences and biomedical engineering, thanks to the possibility to work as a delivery system and overcome drug resistance. In this context, they are amply used to treat infectious diseases like malaria, leishmania, schistosomiasis, toxoplasmosis, HIV, meningitis, hepatitis, and herpes and especially in tumour therapy [303–308] (Table 1 and Figure 9). Easy surface and core modifications with DNA, siRNA, plasmids, peptides, antibodies, or drugs make them optimal candidates for drug delivery, especially for brain tumours like glioma, because they can cross the BBB and deliver biological molecules in a controlled way like that proposed by Lu *et al.* They had formulated PAMAM dendrimers conjugated with the PEG, the Arg-Gly-Asp (RGD) tripeptide for tumour targeting, and the anticancer drug arsenic trioxide (ATO): the use of the dendrimer vector permits the drug crossing of the BBB, enhancing its antitumor effect [309,310]. The capability to incorporate many biological molecules and the ramified structure are optimal tissue engineering applications [311]. In this context, dendrimers can act as a polymerizing agent in hydrogel scaffolds and simultaneously release growth factors in a controlled manner. In particular, they are amply used in bone tissue engineering, as reported by Oliveira *et al.* who showed an increase in the ectopic early osteogenic differentiation of rat bone marrow stromal cells in osteoblasts in hydroxyapatite (HA) and SPCL (starch–polycaprolactone) scaffolds in the presence of Dex-loaded CMCht/PAMAM dendrimer NPs (dexamethasone-loaded carboxymethylchitosan/poly(amidoamine) dendrimer) [16]. Furthermore, the presence of PMAM dendrimers in hydrogel scaffolds can be related to its specific mechanical properties, as reported by Pistone *et al.* They developed a chitosan–dendrimer–HA hydrogel doped with an anti-inflammatory drug (ketoprofen). They identified a correlation between dendrimer molecular weight and chitosan–HA matrix's rheological properties, investigating its drug release kinetic [312].

2.3.9 Polymer nanoparticles

PNPs present a size ranging from 1 to 1,000 nm and are classified into nanocapsules and nanospheres [313]. The first one is constituted by an oily core in which the drug or the biological molecule is retained, surrounded by a polymeric shell that controls the drug release mechanism. Conversely, nanospheres present a polymeric matrix able to encapsulate the drug: in this way, the drug is carried both in the inner and outer parts. Based on the polymer source of origin, it is possible to individuate natural and synthetic polymers [314]. Natural polymers include sodium alginate [315], albumin [316], chitosan [317], polypeptides [318], cellulose [319], inulin [320], and gelatin [321]. Conversely, some examples of the synthetic ones are PLGAs [322,323], polyglycolides [324], poly(malic acid) [325], poly(methyl methacrylate) [326], polyacrylamide [327], poly(*N*-vinyl pyrrolidone) (PVP) [328], polyorthoesters [329], poly(methacrylic acid) [330], and poly-L-lactide [331,332] that can be modified with particles like HA for bone tissue engineering applications [333]. Polymers can also cross-link to form nanogels

that are able to encapsulate theranostic cargos and conjugate targeting molecules to obtain a specific drug release profile [334].

Generally, PNPs can be formulated by direct monomeric polymerization or dispersion of pre-existing polymers. The polymerization process can be obtained from monomers by different preparation techniques like emulsion, miniemulsion or microemulsion, interfacial, controlled/living radical (C/LRP). At the same time, polymer dispersion can be developed by solvent evaporation, nanoprecipitation, salting out, dialysis, and supercritical fluid technology [335]. Conversely, amphiphilic copolymers with distinct hydrophobic and hydrophilic segments can self-assemble to form micelles in an aqueous solution, wherein water-insoluble elements form the core hydrophilic components form the corona [336]. These features permit a huge biomedical application range, so that many PNPs are currently FDA approved and used as therapeutic agents (Table 2).

Polymers present many functional groups permitting the conjugation with biological molecules involved in specific targeting or a controlled drug release mechanism as shown in Figure 10. Adamo *et al.*, for example, designed PVP nanogels bringing both FA for a specific tumour targeting and the pro-apoptotic Bcl-2 siRNA through a redox-sensitive linker. Their data suggested a selective death induction only on cancer cells [337,338]. Their biodegradability and biocompatibility make them optimum candidates for the treatment of cancer and neurodegenerative disorders and cardiovascular diseases[339] (Table 1, Figure 10). By investigating the use of NPs for Alzheimer's disease (AD), Carradori *et al.* had demonstrated the therapeutic efficacy of PNPs conjugated with the antibody against Aβ1–42 peptide to reduce soluble forms of Aβ and rescue memory in AD mice [340]. Conversely, Tan *et al.* had developed PNPs to encapsulate apomorphine (AMP) drug commonly used in Parkinson's disease. In this manner, AMP was able to cross the BBB and was protected by oxidation preventing toxic form formation [341]. Moreover, many studies suggest their application for ischemic protection since passive targeting may be doable because the blood–brain barrier's permeability increases upon ischemia [342]. Zamanlu *et al.*, for example, had formulated PEGylated PLGA NPs conjugated with the tissue plasminogen activator for the treatment of ischemic stroke: circulating time and thrombolytic activity were increased by association with the nanocomplex [343]. PLGA and the other PNPs can be functionalized to obtain

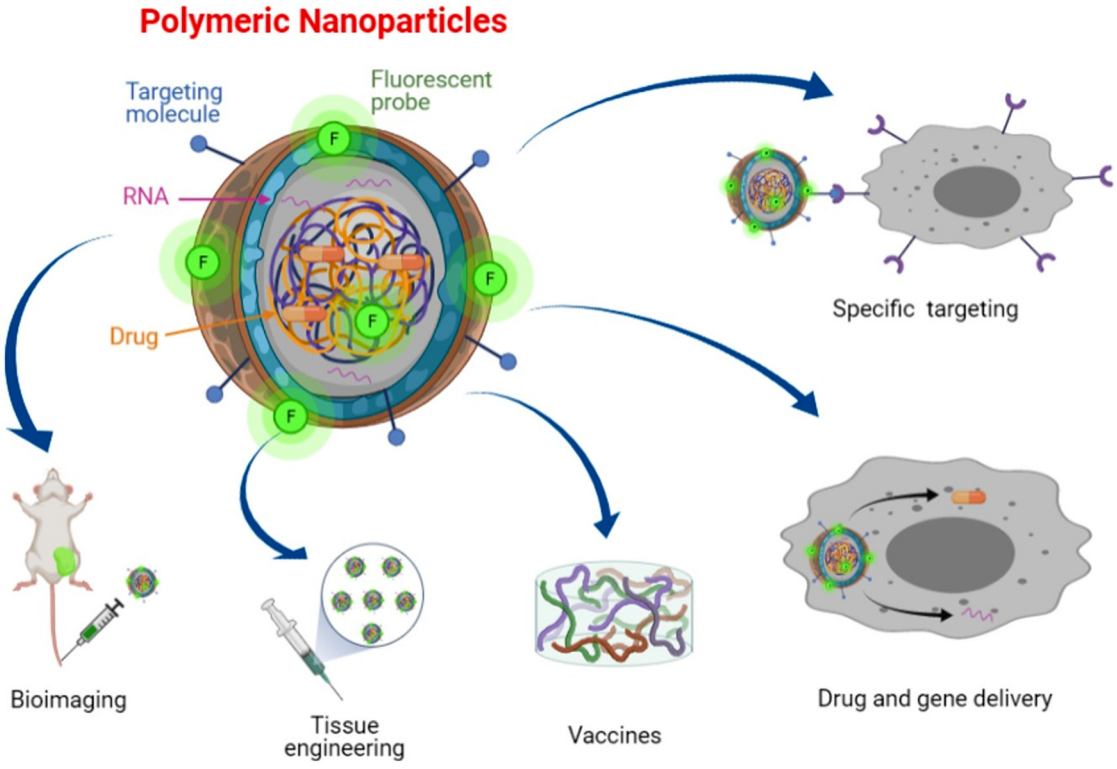


Figure 10: PNPs and their application in biomedical research. PNPs can be conjugated with targeting molecules (specific targeting); with biological molecules including drugs and RNA (drug and gene delivery) and fluorescence probes (bioimaging). Furthermore, PNPs are amply used also in tissue engineering and vaccines.

a sustained, spatial, and temporally controlled delivery of growth factors (GFs) involved in cell growth and differentiation. GFs can be loaded, together with cells, into solid scaffolds or hydrogels to elaborate 3D structures for tissue engineering, as proposed by Sokolova *et al.* [344]. They encapsulated DNA-loaded calcium phosphate NPs into PLGA/hydroxylapatite (nHAP) composite scaffolds for bone tissue engineering. HeLa cells' test suggested a higher cell attachment, higher cell viability, and good gene transfection activity. In this context, Park *et al.* developed a co-delivery system of a gene and a protein into cells [345]. They synthesized Runt-related transcription factor 2 (RUNX2) protein-loaded PLGA NPs conjugated with bone morphogenetic protein 2 (BMP-2) plasmid DNA to enhance osteogenesis. *In vitro* analysis of human mesenchymal stem cells (hMSCs) treated with these NPs showed increased differentiation grade. Furthermore, *in vivo* experiments demonstrated NPs' ability to enhance the osteogenesis of the hMSCs.

Furthermore, many studies have been dedicated to PNPs application as a controlled-release vaccine delivery system (Table 1 and Figure 10). Some parameters like surface charge and antigen loading can influence the immune responses, as suggested by Gu *et al.* who had tested PLGA NPs positively or negatively charged conjugated to ovalbumin (OVA) by adsorption or encapsulation. They reported that the negative charge facilitated the cytoplasmic antigen delivery by inducing the activation of DCs in lymph nodes 5 days after the primary vaccination. Conversely, when the antigen was encapsulated, more potent and long-term antigen-specific antibody responses were registered, compared to those of antigen-adsorbed NPs [346]. Moreover, NP immunogenicity can be improved by adding adjuvants like chitosan or glycol chitosan. *In vivo* studies suggested that glycol chitosan induces significantly higher systemic and mucosal immune responses than only chitosan or NPs alone [347].

3 Conclusions

With a particular focus on nanomedicine, nanotechnology provides an entirely new concept and new approaches in vast fields of modern science and medicine. The small size of NPs confers them unique properties because they are subject to physical laws in the middle between classical and quantum physics. In this context, the NPs' project plays a key role because the material, size, shape, and functionalization need to be chosen and optimized to reach the desiderated aim, as suggested by this review. The NP size-dependent cytotoxic effect was amply analysed as well as the influence of size on cellular uptake and cytotoxicity. On the other hand, the shape is deeply related to body distribution, blood lifespan, macrophage uptake, and membrane internalization.

Moreover, the synthesis processes and the material adopted can modulate other features like biocompatibility, aggregation, and stability. Indeed, each type of NPs presents specific advantages and disadvantages (Table 1), which confer unique properties for specific biomedical applications. Furthermore, the possibility of conjugating them with a large number of different molecules permits obtaining a controlled release mechanism (*i.e.*, mediated by pH, temperature, redox state) and specific targeting, as adopted in cancer treatment to overcome chemotherapy limitations [348]. In this manner, NPs can be employed as DDS not only to act on malignant cells selectively but also for diseases diagnosis, thanks to the capability to detect, for example, primary tumours, lymph nodes, and metastasis or to act as contrast agents in medical imaging techniques. In addition, nanosystems are amply employed in tissue engineering and regenerative medicine to promote tissue differentiation, thanks to the possibility of local delivery of bioactive molecules (*i.e.*, growth factors). Finally, it is recently an object of interest by the scientific community to recruit nanotechnology in vaccine delivery (Table 1), as adopted for BNT162b2 mRNA Covid-19 vaccine, consisting of lipid NPs to deliver mRNA vaccine [231].

Taken together, these features make NPs the starting point for the future of nanomedicine, having a considerable impact on human health. The potential application sectors in which they can be involved are more than those reported in this review. For example, these molecules could permit personalized DDS by improving the patient's life quality or could be involved as nano-robots to make repairs at the cellular level. Undoubtedly, intelligent multifunctional nanosystems will be the most promising candidates as vectors of biological molecules for a vast range of applications in nanomedicine. Furthermore, different features of each kind of NPs can be integrated to generate inorganic/organic hybrid NPs like lipid-polymer hybrids NPs or mesoporous silica shell-coated AuNPs, cell membrane-coated NPs, or dendrimer-conjugated MNPs [349–351]. Therefore, the use of nanotechnology in biomedical application continues to be an evolving field.

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