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Functional nanoparticles and their interactions with mesenchymal stem cells

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Abstract

Mesenchymal stem cells (MSCs) have become one of the most important cell sources for regenerative medicine. However, some mechanisms of MSC-based therapy are still not fully understood. The clinical outcome may be restricted by some MSC-related obstacles such as the low survival rate, differentiation into undesired lineages and malignant transformation. In recent years, with the emergence of nanotechnology, various types of multifunctional nanoparticles (NPs) have been designed, prepared and explored for bio-related applications. There is high potential of NPs in biomedical applications, attributed to the high capacity of cellular internalization in MSCs and their multiple functionalities. They can be used either as labeling agent to track MSCs for mechanism study or as gene/drug delivery carriers to regulate the cellular behavior and functions of MSCs. However, the application of NPs may be accompanied by some undesirable effects, as some NPs can induce cell death, inhibit cell proliferation or influence the differentiation of MSCs. Aiming to provide a comprehensive understanding of the interaction between NPs and MSCs, recent progress in the design and

preparation of multifunctional NPs is summarized in this review, mechanisms of cellular internalization of the NPs are discussed, the main applications of multifunctional NPs in MSCs are highlighted and overview about cellular response of MSCs to different NPs is given. Future studies aiming on design and development of NPs with multifunctionality may open a new field of applying nanotechnology in stem cell-based therapy.

Keywords: mesenchymal stem cells, polymer, nanoparticles, microparticles, multifunctionality, interaction

1. Introduction

Stem cells represent an opportunity for regenerative medicine to initiate, support and control the regeneration of damaged tissues, due to their capacities of differentiation and release of bioactive substances [1, 2]. MSCs, an important type of adult stem cells, have drawn a lot of attention in regenerative therapies. They are multipotent stromal cells that can differentiate into a variety of cell types, such as adipocytes, osteocytes and chondrocytes [3, 4]. They can be isolated from many sources including the peripheral blood [5], periosteum [6, 7], umbilical cord blood [8], synovial membrane [9], pericytes [10], trabecular bone [11, 12], adipose tissue [13, 14], limbal stroma [15], amniotic fluid [16], lung [17], dermis [18] and muscle [19]. They can be expanded to a large scale through the standard *in vitro* or *ex vivo* culture without major loss of their potency. After transplantation to the site of injury, MSCs contribute to tissue regeneration by not only the differentiation to tissue-specific cells but also the secretion of soluble factors [20]. MSCs also present a high potential to deliver anti-tumor agent to target tumors, since they own the ability to migrate to cancer tissues [21-24]. Importantly, MSCs are immunoprivileged and display the immunosuppressive effect, which largely facilitate the allogeneic transplantation of MSCs [25, 26]. Owing to these intrinsic advantages, MSCs have been utilized to treat diverse human diseases, such as myocardial infarction, Parkinson’s disease, cancer, hurler syndrome, spinal cord injury and acute graft-versus-host disease [27].

In spite of the clinical benefit obtained from MSC-based therapy, the therapeutic efficacy is still limited by some obstacles. For example, the phenotypes and differentiation potential of MSCs may be altered by the culture conditions and the prolonged cell passaging [28, 29]. MSCs may undergo transformation during culture and lose their therapeutic effect [30]. The beneficial impact of MSCs can be limited by their poor survival rate after transplantation and the age-related functional decline [31-33]. And the safety concerns can be generated by the transplanted MSCs which may undergo the differentiation into the undesired lineages in the tissue [34].

Therefore, many efforts have been made to pursue the improved clinical benefits of MSC-based therapy [35]. In recent years, with the emergence of nanotechnology, various types of functional NPs, such as polymer-based and organic-inorganic hybrid NPs, have been developed and utilized in biomedical applications. NPs are nanoscale constructs with unique physical and chemical properties. Their small size, large surface-to-volume ratio, multiple functionalities and high capacity of cellular entry endow them a high potential in stem cell study and other biological applications [36-39]. For example, NPs could serve as cell tracking agents to track and monitor MSCs after transplantation. They also could deliver genes or drugs to MSCs to regulate the cellular behavior and functions. Initially, NPs for biomedical applications were prepared with a single function. For instance, the micelles were only used as delivery carriers and quantum dots were applied for imaging. To improve the performance of NPs and address the emerging challenges in biomedical applications, over the past years, multifunctional NPs have been designed and engineered with more complex structure and/or composition. The integration of multiple functionalities in one NP provides the ability to carry out multiple tasks. For example, a NP, which serves as delivery vehicle, could be conjugated with targeting ligands to actively deliver cargoes to the target cells, or with cell penetrating peptides to enhance the cellular internalization, or with imaging agents to monitor the delivery process [40]. To date, multifunctional NPs have become an effective tool to improve the outcome of MSC-based therapy and help us to understand the regeneration processes and mechanisms. However, NPs may also induce undesirable effects to the cells, such as leading to cell death, slowing down cell proliferation and even influencing the differentiation pathway of stem cells, for which care must be taken.

Here, an overview about multifunctional NPs and their applications in MSCs is given, and principles of the interaction between NPs and MSCs are discussed. The strategies adopted currently to prepare the multifunctional NPs are summarized and the mechanisms of cellular internalization of the NPs are discussed. In particular, the main applications of multifunctional NPs for studying and modulating MSCs are highlighted. The influence of different NPs on the cellular behavior of MSCs is described whereby biosafety issues are taken in view. Besides nanoscale particles, some microscale ones are also discussed here.

2. Design and engineering of multifunctional NPs

To date, diverse NPs with different compositions, structures, properties and functionalities have been developed and utilized in nanomedicine, playing important roles in imaging, diagnosis and delivery of therapeutic agents [41, 42]. They could be classified as inorganic, organic and

hybrid NPs according to their composition, or as nanotube, capsule, sphere, liposome and so on, according to their structural morphology (Figure 1).

The properties of NPs such as the size, shape and surface characteristics directly affect their applications in cell biology by dictating their bioavailability, biodistribution, endocytic pathway, intracellular trafficking and cytotoxicity [43, 44]. To meet the increasing demands of modern biomedical applications that the NPs and their cargoes should be efficiently delivered to the target sites to exert their desired efficacy without inducing unwanted side effects, preparing NPs with multifunctionality is of great importance, which involves the adoption of many strategies (Figure 2). Here, the rationales for design and engineering of NPs with tailored physical, chemical and biological properties are outlined.

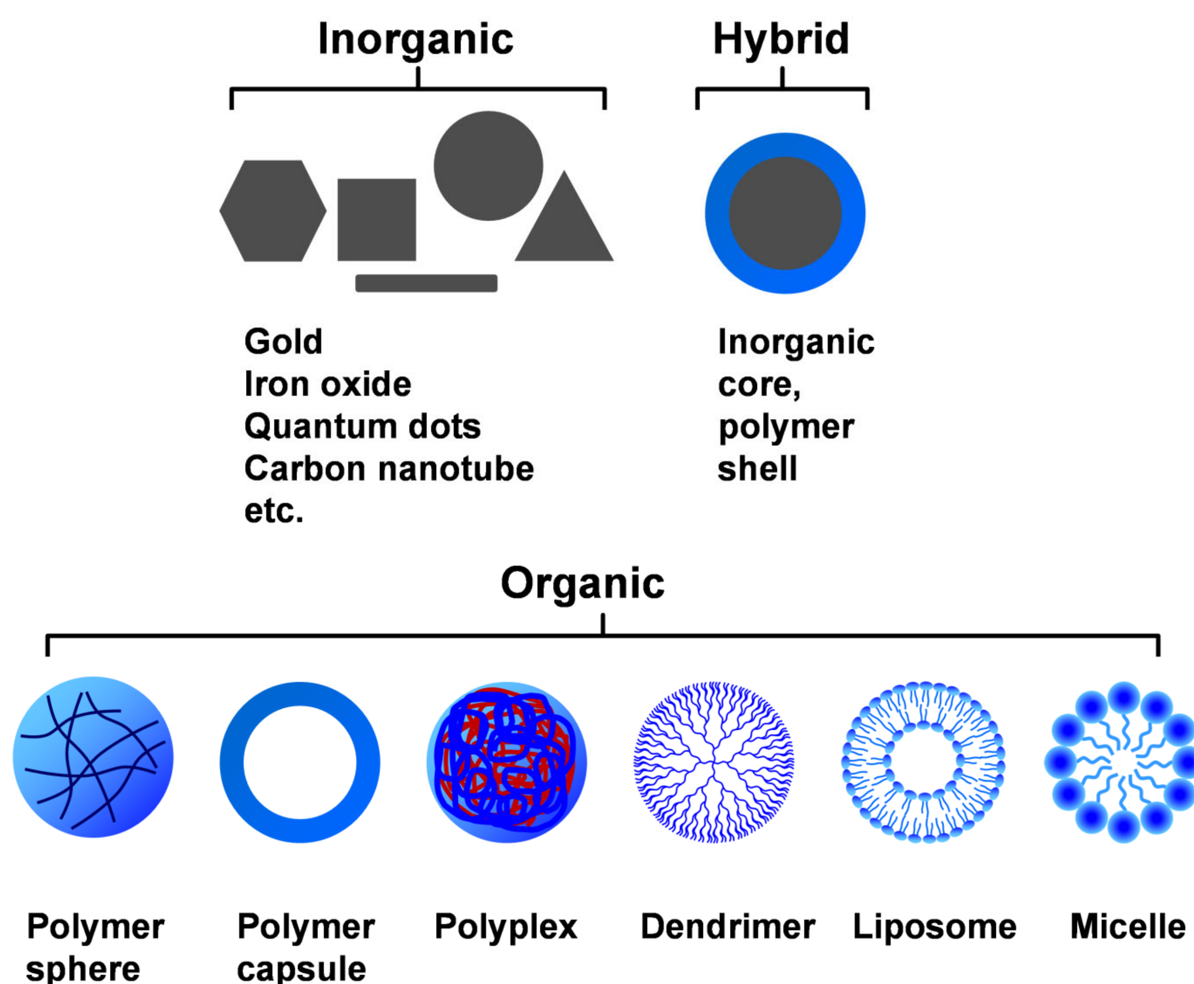

Figure 1. Classification of NPs used in biomedical applications.

2.1 Cell biocompatibility

Cell biocompatibility is the most important feature to ensure an effective and safe application of NPs, and thus must be most carefully considered in design and preparation of multifunctional NPs. Biocompatibility was re-defined by Williams as follows: "biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimising the clinically relevant performance of that therapy [45]". Therefore, the NPs with high cell biocompatibility should be capable to exert their desired functions with low toxicity as well as only limited immunogenic, thrombogenic and carcinogenic effects. Among diverse biomaterials, members of the polyester family, such as polylactide (PLA), polyglycolide (PGA), poly(ε-caprolactone) (PCL) and poly(lactide-co-glycolide) (PLGA), have been most widely employed and investigated. The PLA and PLGA have been approved by the US Food and Drug Administration as delivery carriers for human use [46, 47]. One thing should be noticed that the biocompatibility of the NPs is related to a number of relevant factors besides the material itself, such as the particle size, dose as well as the cell types [48-50].

The cellular behaviour of MSCs could be strongly influenced by the biomaterials [51, 52]. Therefore, the functional particles should be utilized cautiously since they might induce the undesired effects on cells. The influence of selected nanoscale and microscale particles on MSCs has been summarized in Table 1. It should be noted that the results from different studies might be controversial, which could be explained by the influence of other factors such as the features of the particles and the experimental conditions. The influence of the particles on MSCs is strongly dependent on multiple factors, including the particle type and size [48, 53-57], crystallinity [58], dose and concentration [48, 53, 59-62], incubation time [53, 59, 63] as well as the serum in the culture medium [62]. Besides the items listed here, some other cellular responses induced by the particles have also been observed, including the cell adhesion [64, 65], cytoskeletal architecture [60, 66], cell migration [67] and phenotypical appearance following implantation [68]. Huang et al. found that Ferucarbotran, the ionic superparamagnetic iron oxide (SPIO) nanoparticles, could promote proliferation of human MSCs. The mechanism study revealed that the internalized SPIO nanoparticles could diminish intracellular H_2O_2 through the intrinsic peroxidase-like activity. Also, SPIO could accelerate cell cycle progression, which might be mediated by the Fe irons released from lysosomal degradation [69]. The particles can also influence the MSCs in an indirect manner. For example, the indirect

exposure to calcium-deficient apatite particles severely impaired the osteogenic maturation of human MSCs due to the uptake of Ca^{2+} by the particles from the culture media [70]. The conditioned medium collected from titanium particles treated MSCs showed the cytotoxicity to MSCs and induced cell apoptosis [59].

Table 1. The influence of particles on MSCs

Particles	Approximate size (nm)	V	P	AP	Differentiation			CF	E/S	S	Reference
					O	A	C				
Dextran coated SPIO	120-150				●			●	●		[71]
Dextran coated SPIO	80-150	●	●		●	●	▼				[72]
Dextran coated SPIO	80-150	●	●		●	●					[73]
Dextran coated SPIO	80-150				●	●	●				[68]
Carboxydextran coated SPIO	60-80	▲	▲		▼						[67, 69]
Carboxydextran coated SPIO	60-80		●		●	●	●		■		[74]
N-alkyl-PEI2k stabilized SPIO	50-70	●	●	●	●		●		■		[75]
Starch coated SPIO	200	●	●	●	●	●					[76]
Starch coated SPIO	2000								■		[77]
Protamine modified SPIO	20-30				●	●				●	[78]
PLMA coated and FITC conjugated SPIO	10-20	●			●	●					[79]
Polystyrene coated SPIO	900				●	●			■	●	[80]
SPIO (coated with dextran and complexed with protamine sulfate)	80-150				●	●	●				[81, 82]
Magnetic particles with single iron oxide core (dextran shell) and multi- iron oxide cores (starch shell)	80-150	●	▲		▼	▼	▼				[83]
Iron oxide as core within microgel	600		●		●	●	●		■		[74]
Iron oxide (core) within lipid mixture (shell)	10 (core size)	●	●		●	●					[84]
Iron oxide-pullulan	70-160	●			●						[85]
Superparamagnetic divinyl benzene inert polymer (iron oxide and fluorescein-5-isothiocyanate trapped in polymer matrix)	900		▼		●	●					[63]
Glycol–chitosan coated barium titanate	150	●	●		▲	●					[66]

Hydroxyapatite complexed with PLGA, peptide or both	36				▲						[65]
Hydroxyapatite (embedded in PLLA fibers)	$<$ 200	▲	▲				▲				[86]
Hydroxyapatite (embedded within peptide amphiphile nanofibers)	100	●	▼		▲						[87]
Hydroxyapatite (on or within poly(3-hydroxybutyrate) nanofibers)	$<$ 200		●								[88]
Calcium phosphate incorporated in chitosan	Varied size	▲	▲		▲						[61]
Bone morphology protein-2 encapsulated chitosan/chondrotin sulfate NPs (immobilized on biphasic calcium phosphate scaffolds)	400-700		▲		▲						[89]
FITC labeled silica	110	●	●		●	●	●			●	[90]
Hyaluronic acid/poly-L-lysine bilayered silica NPs	560	▲	●		▲						[91]
Silica conjugated with antibody and loaded with doxorubicin	100-150	▼									[92]
Vinyl phosphonic acid –styrene copolymer conjugated with fluorescent dye	210	●			●	●	●				[93]
Poly lactic acid	136		▼		●	●					[94]
PLGA-chitosan/PLGA-alginate (in the form of colloidal gels)	180-250	●									[95]
PLGA NPs (loaded with 17β-estradiol, on Chitosan-hydroxyapatite scaffold)	240				▲						[96]
Polyethylenimine modified polysaccharide/DNA polyplex	$<$ 100	●									[97]
Polyethylenimine conjugated (α-NaYbF4:Tm^{3+})/CaF_2 upconversion NPs	90	▼	▼		▼						[98]

L-lactic acid oligomer-grafted gelatin micelles entrapped dexamethasone	350				▲						[99]
Lipid nanocapsules	88		▼		●	●					[94]
Silk fibroin in corporated in silk scaffolds	5,000				▲						[100]
Bovine endosteum-derived	40,000-230,000				●	●	●				[101]

Marks: ● no effect or minimal influence, ■ with effect, ▲ promotion, ▼ inhibition. Abbreviations: V: viability, P: proliferation, AP: apoptosis, O: osteogenic differentiation, A: adipogenic differentiation, C: chondrogenic differentiation, CF: colony formation, E/S: expression of genes/proteins and secretion of cytokines/growth factors, S: surface markers.

2.2 Degradability and stability

Compared to non-biodegradable NPs, the biodegradable ones normally offer a higher biocompatibility as well as a better controllable release of the delivered cargoes. Most importantly, biodegradable NPs can be internally digested and subsequently cleared from the body, which avoids the removal procedures and reduces the safety concerns caused by the retention of the NPs in the body.

Biodegradable NPs can be prepared from a variety of natural and synthetic biodegradable materials, among which the most comprehensively employed ones include chitosan, gelatin, PCL, PLGA, PLA, depsipeptide, and poly(alkyl cyanoacrylate) (PAC) [102, 103]. The selection of the polymers as a basis to engineering NPs is based on the design principles and the end application criteria, such as the particle size, the surface characteristics and functionality, the degree of degradability, the properties of the cargoes (aqueous solubility, stability, etc.) to be encapsulated in the polymer and the release profile of the cargoes. Depending on the desired criteria, different methods can be adopted to prepare the NPs including the dispersion of preformed polymers, the polymerization of monomers and the ionic gelation for hydrophilic polymers [104].

The physical stability of the NPs is critical to ensure that the loaded cargoes are well protected and efficiently delivered to the target sites, especially for those dynamically structured NPs such as the micelles. Core-shell structured micelles generated from amphiphilic block copolymers are desirable as delivery vehicles as their hydrophobic core can be loaded with hydrophobic cargoes, while their hydrophilic shell is able to provide colloidal stability to the particles and protect the payload. However, one of the major obstacles for micellar delivery

systems is its relatively poor kinetic stability. Several approaches could be applied to improve the stability of the micelles: (i) covalently crosslinking the core or shell of premixed micelles [105, 106], (ii) utilizing non-covalent interactions (such as hydrogen bonding) [107] or (iii) using stereocomplexation [108]. In a PEGylated starch micellar delivery system, the benefits achieved from disulfide crosslinking included not only the stability of the starch-g-PEG copolymer micelle, but also the improved drug loading capacity [109]. The effect of the enhanced stability of the NPs has also been observed in other delivery systems. For instance, the thiolated crosslinked chitosan nanocomplexes have been used for DNA delivery, which exhibited the physical stability and the effective protection against DNase I digestion, resulting in a sustained DNA release and the significantly higher gene expression than unmodified chitosan [110]. Nevertheless, the biodegradability of some polymers might be impaired by the crosslinking. Introducing biodegradable crosslinkers is an effective method to address this issue [111, 112].

2.3 Delivery efficiency and sustained release

For those NPs used as gene/drug carriers, the efficient delivery of the agents to target sites and the long-term sustained release of the agents are of great importance for the therapeutic efficacy, and therefore should be regarded as principal aims during the design and engineering of such NPs. For example, the efficient gene delivery needs to conquer several barriers including the cellular internalization, the endosomal escape, the cytosolic transport of DNA and the nuclear localization of DNA [113]. Cationic polymers and cationic lipids are two main categories of carriers that were intensively studied and showed high transfection efficiency. Both cationic polymers and cationic lipids can condense negatively charged DNA to form nano-sized complexes termed “polyplex” and “lipoplex” respectively. The complexes bind to the cell surface through non-specific electrostatic interactions between the positively charged complexes and the negatively charged cell surface, followed by the cellular internalization of the complexes through endocytosis or endocytosis-like mechanisms. After internalization, the endosomes containing DNA will transform into lysosomes, which means DNA would eventually be digested by lysosomal hydrolytic enzymes only if it could escape from those endosomes. The mechanisms of endosomal escape for polyplexes and lipoplexes are fundamentally different. The cationic lipids mediated endosomal release is thought to involve the lipid mixing between the endosomal and liposomal membranes, subsequently resulting in the membrane disruption and DNA escape [114]. The mechanism of cationic polymers mediated endosomal release is currently explained by two hypotheses: the physical disruption

of the negatively charged endosomal membrane induced by direct interaction with the cationic polymers [115] and the "proton sponge" effect [116]. After the release from endosomes into cytoplasm, the DNA in free form or in the complexes will be transported to the nucleus where the transcription takes place.

The release of the delivered agents from the NPs is a multiplex process. Soppimath et al. summarized the key aspects, which determine the drug release rate from the NPs: (i) desorption of the surface-bound /adsorbed drug; (ii) diffusion through the NP matrix; (iii) diffusion (in case of nanocapsules) through the polymer wall; (iv) NP matrix erosion; and (v) a combined erosion / diffusion process [117]. Based on this principle, many factors affecting drug release from the NPs have been observed including the properties of the NPs (size, biodegradability and surface characteristics), the polymers to form the NPs (molecular weight, composition and polymer network), the drugs (solubility, dose and loading method) and the drug-NP interactions [118-125]. These findings provided the valuable information to design and prepare the NPs for the controllable drug release.

2.4 Structure

Engineering NPs with desired physical structures may endow the NPs with enhanced biomedical functionalities. For instance, the polystyrene hollow particles with controllable holes in their surfaces presented high potential to carry and deliver the payload [126]. Recently, the porous nanostructures have drawn a lot of attention due to their superiority as drug delivery carriers [127, 128]. The drugs could be loaded into the porous NPs by adsorption or capillary filling, and the release profile of the drugs could be adjusted by varying the pore size and pore surface chemistry [129].

The NPs can be prepared with multilayer structure via layer-by-layer (LbL) assembly, which is a simple and highly versatile approach. Polymeric multilayer NPs have shown promise to be used as advanced drug delivery vehicles as they can be readily engineered and functionalized with specific properties and can encapsulate the large payload of drugs. Usually, such multilayer NPs are generated by sequential deposition of polymer layers from aqueous solutions onto a template, and almost any type of interaction (e.g. electrostatics, hydrogen bonding, covalent bonding, specific recognition) can be used as driving force for the assembly of the multilayer shell [130]. Once the desired multilayer has been assembled, the particles can be used directly in the core-shell form, or the template can be dissolved to obtain the hollow polymeric capsules. A large range of polymers are applicable to form the wall of the multilayer

capsules, which provides the feasibility to control the composition, permeability, stability and surface functionality of the NPs. In addition, the size and shape of the capsules can be adjusted by simply altering the template, and the capsule thickness can be controlled by varying the number of deposited layers [131].

Recently, significant attention has been paid to nano-sized hydrogels, which are named nanogels [132]. A hydrogel is described as a three dimensional network of hydrophilic polymers that is able to swell in aqueous solution and take up large amounts of fluid, while still maintain their internal network structure. The network can be generated through the crosslinking of polymer chains by covalent bonds, hydrogen bonds, van der Waals interactions or physical entanglements [133]. In the past years, hydrogels have attracted a great deal of attention and different types of environmentally-sensitive hydrogels have been developed, showing high advantages for controlled drug release [134]. Since nanogels are nano-sized particles that are able to be readily taken up by cells, they have become attractive vehicles for intracellular drug delivery [135, 136].

2.5 Stimuli sensitivity

Over the last decades, stimuli-responsivity has been implemented in NPs (e.g. polymeric nanocarriers, liposomes, micelles, dendrimers, nanogel and hybrid NPs), as summarized by many excellent review articles [137-143]. These active delivery systems are able to respond to single, dual, or multiple stimuli including endogenous ones (e.g. pH, glucose, redox potential and lysosomal enzymes) and exogeneous ones (e.g. temperature, magnetic field, ultrasound and light). Stimuli-responsivity as a function can accomplish an enhanced release of the loaded agents at the target sites (spatial control) and/or at the right time (temporal control), and consequently improve the therapeutic efficacy and minimize undesired side effects.

2.6 Active targeting and cell penetrating

Targeted delivery of NPs at tissue, cell, or subcellular levels can increase the therapeutic effectiveness and reduce undesired side effects by reducing or eliminating the presence of potentially toxic payload in healthy cells or tissues [144]. The active targeting of the NPs can be realized through the surface modification of the NPs with targeting ligands [145-147]. Targeting ligands can bind to the target molecules as a result of biorecognition, and thereby lead to the highly specific interactions between the NPs and certain cells or tissues. Depending on the disease type and the objectives of the treatment, different ligands can be conjugated onto

the NPs, such as the antibodies, engineered antibody fragments, aptamers, proteins, peptides and some small molecules [44, 148-150].

If the cargoes of NPs exert their functions inside the cells, the delivery of NPs to specific cells or tissues should be followed by an efficient cellular uptake of these NPs. To conjugate cell penetrating peptides (CPPs) onto NPs can enhance the cellular internalization of NPs [151, 152]. The CPPs are short peptides that can be associated with NPs either by covalent binding or by non-covalent binding. It has become more evident recently that CPPs may enter cells through two major cellular uptake mechanisms: direct penetration involving translocation through the lipid bilayer and endocytosis [153]. In gene delivery, the efficient nuclear entry of DNA is a critical step determining the transgene expression. However, the small diameter (~9 nm) of nuclear pore complexes (NPCs) allow only the passive diffusion of ions and small molecules (less than ~40 kDa in mass or ~5 nm in diameter), but restrict the free entry of larger molecules into the nucleus [154, 155]. Nuclear uptake of large proteins in an active manner is mediated by nuclear localization signal peptide (NLS) through a sequence-specific recognition [156]. It has been shown that the modification of DNA with NLS could enhance the transfection efficiency, which might be attributed to the improved nuclear entry of DNA [157, 158].

Targeted delivery of NPs has been applied not only to cells, who overexpress recognition receptors, but also to subcellular organelles [159], including cytosol [160], endosomes/lysosomes [160], nucleus [161] and mitochondria [162]. However, it must be noted that the targeting ligands on the surface of NPs may generate negative effects. For example, the proteins adsorbed on the NPs may accelerate the clearance of the NPs from the bloodstream, and antibodies can potentially induce an immunogenic response [150, 163].

2.7 Stealth coating

The short circulation time of NPs *in vivo* due to the clearance by the mononuclear phagocyte system may strongly restrict their therapeutic and diagnostic applications. Endowing NPs with the feature of stealthiness can inhibit the plasma protein adsorption onto the NPs as well as the recognition by the immune system, and consequently prolong their circulation time [164]. Currently, one of the most effective approaches to achieve this stealth property is conjugating hydrophilic polymers, such as polyethylene glycol (PEG) [165, 166]. In this way the aggregation of NPs can be avoided and the non-specific interactions between NPs and cells can be impeded. In addition, loaded cargoes could be protected by steric hindrance. This is especially relevant if cargoes are sensitive to the physiological environment [44]. At the same

time a hydrophilic NP surface could decrease the cellular uptake efficiency as well as reduce endosomal escape capability after cellular internalization. To overcome these problems, some strategies have been adopted, including the surface modification of NPs by substituting the polymers, conditional removal of hydrophilic shell and biomimetic surface modifications [167].

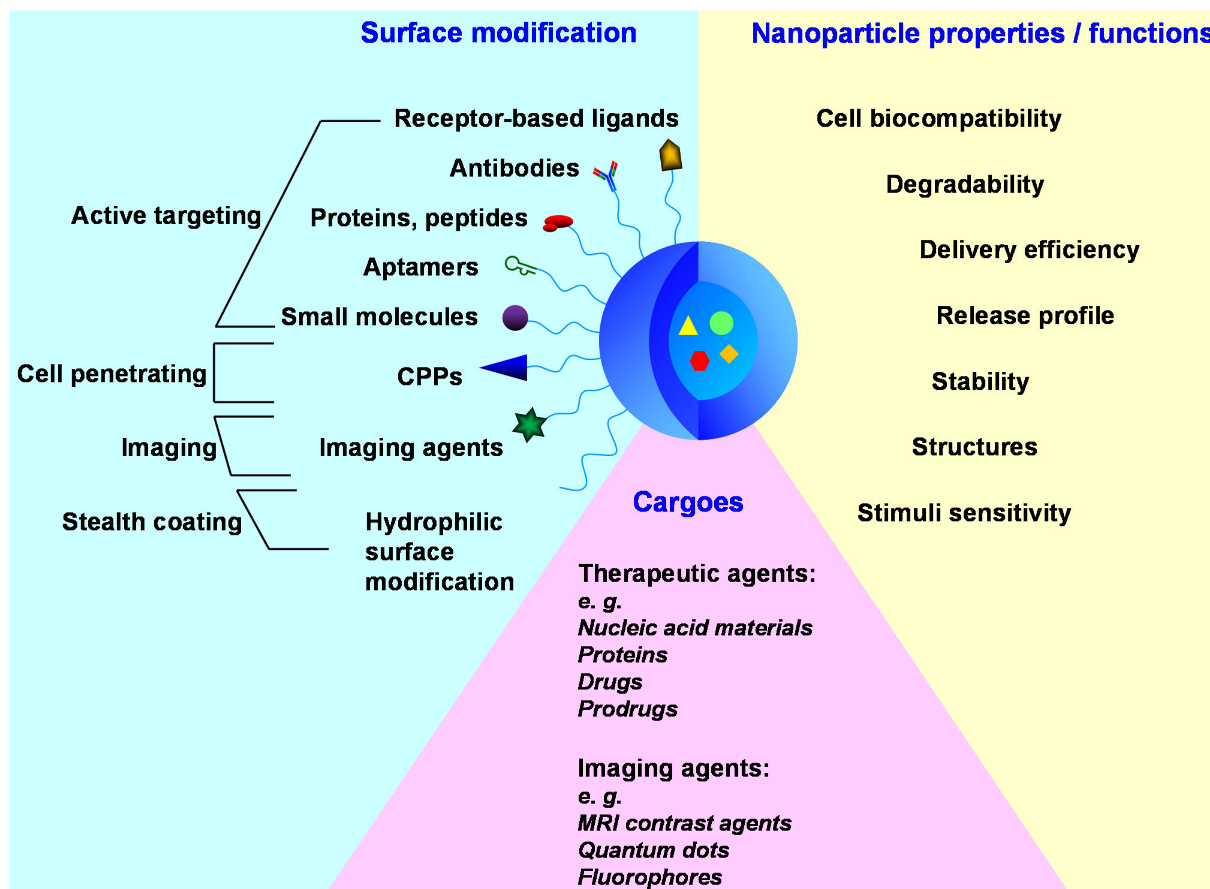

Figure 2. Schematic illustration of engineered multifunctional NPs. The NPs can be prepared from different materials with different structures, properties and functions. Further, the NPs can be functionalized through surface modifications to achieve/enhance the capacities of active targeting, cell penetrating and stealthing. These multifunctional NPs can be used in biomedical applications as imaging agents, delivery vehicles or therapeutics.

3. Cellular internalization of NPs

In most applications, the NPs need to efficiently pass through the cell membrane and subsequently arrive in the appropriate cellular compartment, where they exert their functions. The cell membrane is an elastic lipid bilayer embedded with domains of lipids, carbohydrates and membrane proteins, which segregate the chemically distinct intracellular milieu and extracellular environment. The cellular entry of NPs could be realized via two pathways: active uptake (endocytosis) or passive internalization (physical delivery).

3.1 Endocytic pathways

The active cellular entry of NPs is regulated by the process termed endocytosis, in which the cells take up the NPs by enclosing them in the vesicles or vacuoles pinched off from the plasma membrane. The different endocytic pathways have been described in some excellent reviews [149, 168-172] and are summarized here in Figure 3.

The multiple mechanisms involved in endocytosis can be divided into two broad categories – the phagocytosis ("cell eating", uptake of large particles) and the pinocytosis ("cell drinking", uptake of fluid and solutes). Phagocytosis in mammals is conducted primarily by specialized cells including macrophages, monocytes, and neutrophils. The particles with large size such as bacteria or yeast are engulfed through the assembly of actin and the formation of cell surface extensions that zipper up around the particles [168]. The phagosomes containing the particles bypass the early endosomes and directly fuse with the lysosomes to accelerate the degradation of the internalized particles, since the phagocytosis involves the large volume of cell membrane and therefore requires a prompt and effective processing [173, 174].

In contrast, pinocytosis occurs in almost all types of cells. In this way smaller particles ranging from a few to several hundred nanometers can be taken up. Pinocytosis can be generally classified as the macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis. Macropinocytosis is an important manner of fluid-phase endocytosis, involving the actin-driven formation of the membrane protrusions which can pinch off a large volume of extracellular fluid [175]. However, unlike phagocytosis, the protrusions in macropinocytosis do not zipper up along the particles, but instead they close and fuse with the plasma membrane to form large organelles called macropinosomes. Clathrin-mediated endocytosis is by far the most understood pathway of all of the endocytic mechanisms, and is one of the most important routes in eukaryotic cells for nutrient uptake and receptor signalling [176, 177]. In clathrin-mediated endocytosis, the cell membrane receptors and their associated ligands interact with the adapter proteins and a swarm of accessory factors. Through these interactions, the receptors are bunched into the specialized membrane areas called "coated pits". The coated pits are formed via the assembly of several cytosolic proteins, with clathrin as the main unit. The invagination was induced by calthrin networks with the help of specialized BAR domain proteins such as SNX9 and amphiphysin that can sense and promote membrane curvature, followed by the pinching off of a clathrin-coated vesicle through a small GTPase, dynamin [178-180]. Inside the cells, the clathrin coat is shed off under the influence of a set of proteins and then the vesicle fuses with the early

endosome. Caveolae-mediated endocytosis is the most studied clathrin-independent pathway. It has many biological functions such as lipid regulation, cell signalling and vesicular transport. Caveolae are the flask-shaped invaginations of the plasma membrane, in which cholesterol, sphingolipid and many functional molecules are enriched [181]. Caveolae have a protein coat composed primarily of caveolin-1 (or caveolin-3 in muscle cells), which is a 21 kDa integral membrane protein [182]. Caveolins have an unusual topology, in that the cytosolic N- and C-termini are connected by a hydrophobic hairpin domain, which is buried in the membrane but does not span the bilayer [183, 184]. With this protein caveolae can form the flask-shaped structure and engulf the cargo molecules binding to them. However, in general, the size of caveolae structure (< 100 nm) is smaller than the other endocytic vesicles, whose size may reach several hundred nanometers in diameter. Recently, a number of clathrin- and caveolae-independent pathways have been reported including the Arf6-mediated, flotillin-mediated, Cdc42-mediated and RhoA-mediated endocytosis, that are defined in terms of either the morphologies of the vesicles or the types of the cargos, which are preferentially taken up [171, 178]. Although these endocytic pathways have been intensively investigated, some of the mechanisms at the molecular level are still poorly understood. And there are probably unknown pathways remaining to be discovered.

3.2 Effect of NPs on endocytosis

The cellular uptake of NPs can be strongly affected by the features of the NPs including the size, shape, composition as well as surface properties [43, 185, 186].

The size of the entocytic vesicles might determine their preference and capability for endocytosis. For example, in the caveolae-mediated pathway, the internalization of the NPs with large size could be restricted by the small size of the caveolae [187]. In a study by Chithrani et al., the uptake of gold NPs with various size ranging from 14 nm to 100 nm by Hela cells was investigated, and the result showed that the maximum uptake occurred at a NP size of 50 nm [188]. However, Cho et al. reported the different result, that the SK-BR-3 breast cancer cells had a higher uptake for the smaller gold nanostructures than the larger ones for both nanospheres (15 and 45 nm) and nanocages (33 and 55nm) [189], indicating that the size-dependent uptake might also relate to the other factors such as the cell type or the experimental condition. The particle shape is another important factor affecting the uptake efficiency. The rod-shaped gold NPs showed a lower uptake efficiency than their spherical counterpart, which might be attributed to the longer wrapping time of the cell membrane for the engulfment of the rod-shaped particles [188, 190, 191].

Surface charge of NPs is directly related to endocytosis. Positively charged NPs are more efficient, compared to the neutral and negatively charged NPs, for cellular entry since they are more effective to bind onto the negatively charged groups on the cell membrane [192-194]. Therefore, they are the primary choice as synthetic carriers for gene and drug delivery [154, 195]. However, the uptake of negatively charged NPs has also been described by many groups, despite the repelling force between the negatively charged cells and the negatively charged particles [196-198]. This process was believed to be regulated through the cationic sites distributed on the plasma membrane, which could facilitate the binding of negatively charged NPs and the subsequent endocytosis [191].

The surface charge of NPs can affect not only the internalization efficiency but also the endocytic and transcytotic pathways. A high uptake of negatively charged quantum dots mediated by lipid raft has been observed, which was different from the uptake of positively charged NPs, which was mainly clathrin-dependent [199]. Yue et al. studied the uptake of chitosan-based NPs using eight cell lines and found that the rate and amount of cellular uptake were both positively correlated with the surface charge of NPs in all cell lines. They also demonstrated that some of the positively charged NPs could escape from lysosomes after internalization and exhibited perinuclear localization, but the neutral and negatively charged NPs preferred to localize in the lysosomes [200]. Harush-Frenkel et al. found that the positively charged NPs entered into Hela cells via the clathrin-mediated pathway but the negatively charged NPs did not [193]. Later they studied the influence of surface charge of polymeric NPs on their endocytic and transcytotic pathways in MDCK cells. The result showed that the positive charge apparently increased the internalization of the NPs compared to the negative charge. Both cationic and anionic NPs were targeted mainly to the clathrin endocytic machinery. However, a fraction of both NP formulations was suspected to be internalized through macropinocytosis. Very interestingly, a large fraction of the anionic NPs underwent the degradative lysosomal pathway while the cationic NPs avoided this route [201]. Jiang et al. compared the cellular internalization of two types of cationic polystyrene NPs with similar size and surface charge, one charged by stabilizing surfactant and another functionalized by amino groups. They found that the amine functionalized NPs were rapidly internalized and accumulated to a much higher level in human MSCs, predominantly via the clathrin-mediated pathway. But the NPs charged by the stabilizing cationic surfactant were taken up mainly via clathrin-independent endocytosis [202]. Later they compared two types of anionic polystyrene NPs with identical sizes and surface charge, the carboxyl functionalized NPs and the plain NPs, both coated with anionic detergent. The carboxyl functionalized NPs were internalized into

MSCs more rapidly and accumulate to a higher level than the plain NPs. The uptake mechanism was found to be mainly the clathrin-mediated pathway for the carboxyl functionalized NPs but the macropinocytosis for the plain NPs [203]. Their results indicated that the interaction of the functional groups of the NPs with the receptors on cell membrane might play a crucial role to regulate endocytic pathways, even though the NPs have the similar size and surface charge.

The charge of NPs also contributes to the adsorption of the specific proteins in the cell culture medium onto the NPs through electrostatic interactions as a driving force. And the protein adsorption might lead to an enhanced or a reduced cellular entry of the NPs. In a study by Patil et al., they investigated the effect of surface charge of cerium oxide NPs on the adsorption of bovine serum albumin (BSA) and the particle uptake by A549 cells. They found that the NPs having positive charge adsorbed more BSA while the NPs with negative charge showed little or no protein adsorption. Accordingly, a more efficient uptake of the negatively charged NPs was observed, as compared to the positively charged NPs [204].

The endocytic process can be affected by the physical properties of the NPs. Schrade et al. studied the influence of surface roughness of the NPs on their cellular entry in Hela cells. The result suggested that the rough NPs were internalized by the cells more slowly and through an unidentified uptake pathway, while the uptake of smooth NPs was strongly related to the dynamin, F-actin and lipid-raft. Interestingly, the negatively charged NPs were taken up to a higher extent than the positively charged ones, regardless of the surface roughness [205]. Other features of the NPs influencing the endocytosis include the surface-ligand arrangement [206], conjugation with cell penetrating motifs [207] and mechanical properties [206].

3.3 Direct delivery of NPs

The delivery of NPs into the cells could be performed using physical methods such as physical delivery: microinjection and electroporation to bypass the endocytic pathways (Figure 3). Microinjection is a mechanical process using a micropipette to penetrate the cell membrane and/or the nuclear envelope and inject the materials directly at a microscopic level. It owns some advantages such as high precision and efficiency, high reproducibility and low cytotoxicity, and therefore has been widely used in the studies of transduction-challenged cells, transgenic animal production and *in vitro* fertilization [208]. Microinjection can be used to deliver a set of NPs such as the quantum dots [209-213], polymeric NPs [214], silver NPs and metal oxide magnetite NPs [215] to different types of cells. Electroporation applies the high voltage electrical currents onto the target cells, to make the transient hydrophobic pores on the

cell membrane and thus to allow the free cellular entry of the NPs. It is applicable to a large variety of cell types and is generally safe, efficient and highly reproducible [216]. A variety of NPs could be delivered via this approach, including the quantum dots [217, 218], gold [219], silver [220, 221], silica [222], liposome [223] as well as polymeric NPs [224].

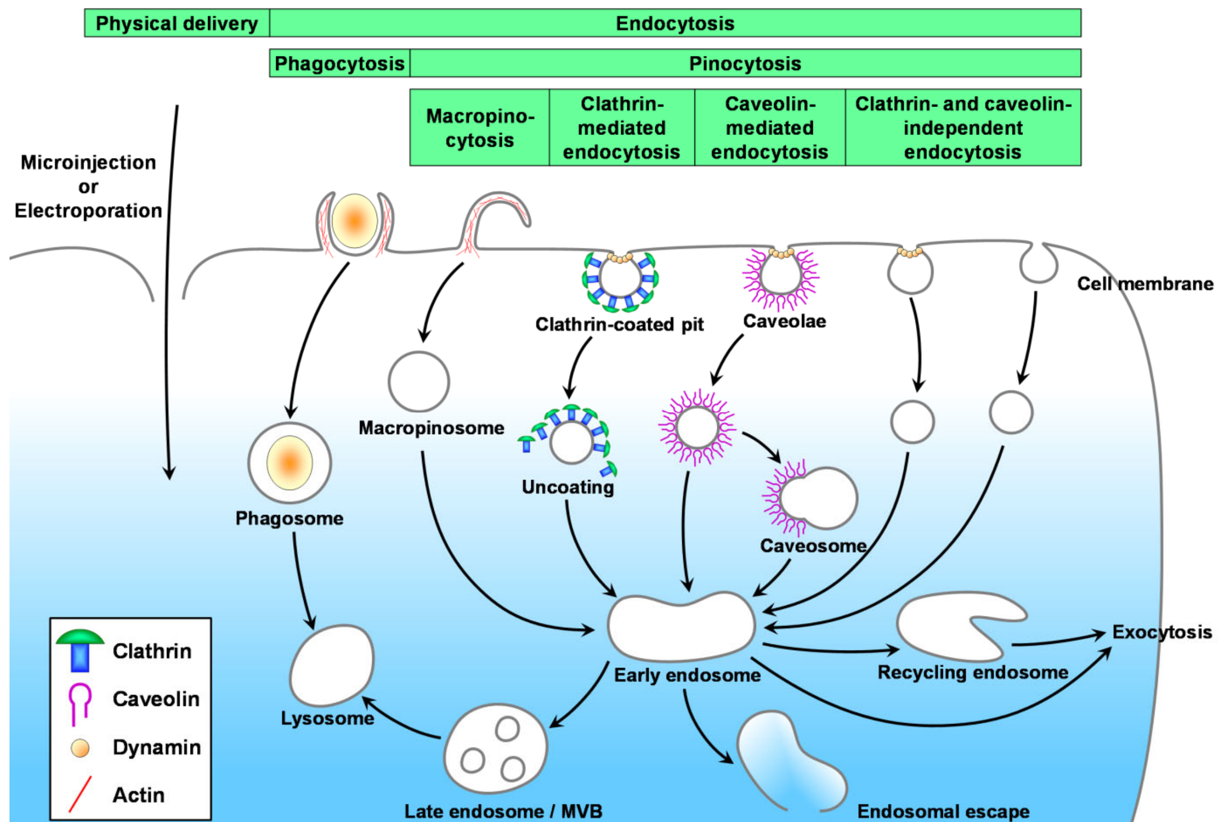

Figure 3. Internalization pathways of NPs in cells. NPs can be internalized through endocytosis, or be delivered through physical methods, in which NPs are directly injected into the cells (microinjection) or freely enter into cytoplasm through physically created pores on the cell membrane (electroporation). The endocytic pathways differ with regard to the mechanism of the vesicle formation, the size of the endocytic vesicle and the nature of the cargo. The phagosomes directly fuse with lysosomes, but the other endocytic vesicles fuse with early endosomes where the internalized particles have three possible fates: 1) to be delivered into lysosomes; 2) to be transported back to cell membrane and subsequently released to the extracellular space via exocytosis; 3) to escape from the endosomes and travel to other intracellular locations.

4. Application of functional NPs on MSCs

4.1 Labeling and tracking

One of the most important applications of NPs in cell biology is to label and track the cells. After cell transplantation, the location, distribution and migration of the labeled MSCs can be

monitored through non-invasive imaging technics. The magnetic resonance imaging (MRI) is a widely used technic for tracking MSCs labeled with magnetic NPs as it offers many advantages such as the high spatial resolution and no exposure to ionizing radiation [74-76, 225-227]. The MSCs labeled with SPIO particles could even be detected by MRI after differentiation, showing the lineage specific signals probably due to the cell-type-specific intracellular iron compartmentalization [68]. An application of MRI to detect the MSCs at the single cell resolution has been described, in which the cells were labeled with the microscale iron oxide magnetic particles [63]. The other types of NPs that can be used for MSC tracking include gold NPs [228], fluorescent dye conjugated NPs [90], and quantum dots [229, 230].

Tautzenberger et al. reported the novel phosphonate-functionalised polystyrene NPs (VPA-based NPs), which were synthesised by copolymerisation of styrene with vinyl phosphonic acid [93]. The phosphonate groups at the outer surface of the particles allowed for their attachment to metal surfaces, showing high potential to be used for coating implants. The density of the phosphonate groups and the size of the particles can be tailored by varying the parameters during polymerization process. Importantly, the particles could be further functionalized with various molecules for different biological applications. The particles labelled using a fluorescent dye with a particle size of 210 nm could be efficiently internalized by MSCs without using any transfection agents, although the exact uptake mechanism has still to be investigated. The NPs did not significantly influence the cell viability after 16 days of particle treatment. The particle treated MSCs maintained their potential for osteogenic, adipogenic and chondrogenic differentiation. In addition, the particles could stay inside the undifferentiated or differentiated MSCs for more than 3 weeks of cultivation period (Figure 4). These results demonstrated the high potential of these NPs for MSC labeling and tracking, and suggested that surface functionalization of NPs with phosphonate groups might be a promising approach for preparing NPs with high intracellular uptake rate.

Recently, the conjugated polymer nanodots (Tat-PFBD) have been reported by Jin et al., as noninvasive fluorescent trackers for tracking MSCs [231]. These nanodots were prepared by using 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] to encapsulate poly[(9,9-dihexylfluorene)-*alt*-(2,1,3-benzoxadiazole)], with a further conjugation of cell-penetrating peptide derived from HIV-1 Tat protein. Such nanodots showed advantages of high brightness, long-term tracking ability as well as negligible influence on MSC properties including proliferation, migration, differentiation and secretion. The internalized Tat-PFBD nanodots allowed detection of MSCs at single cell level after 21 days

upon transplantation, indicating their high potential for investigating in vivo behavior of MSCs. The application of conventional fluorophores is often limited by the so-called concentration quenching or aggregation caused quenching effect, which means the light emission of the fluorophores is quenched when they are at high concentration or in aggregate state. The recently discovered class of nanoparticles with aggregation-induced emission (AIE) characteristics could offer a straightforward solution to address this problem [232, 233]. The AIE fluorogens typically own a propeller-shaped rotorlike structure, which undergo low-frequency torsional motions in dilute solution and emit very weakly. However, they show strong fluorescence in aggregate stage, which is mainly attributed to the restriction of the motions by intermolecular steric interaction [234]. In MSC labeling and tracking, the organic AIE nanodots have shown high potential for both in vitro and in vivo applications [235, 236]. Taking the advantages such as ultrahigh stability and brightness, low toxicity and superb retention with the living MSCs, the AIE nanodots could precisely and quantitatively report the fate and the regenerative capacity of transplanted MSCs for 6 weeks in an ischemic hind limb bearing mouse model [236].

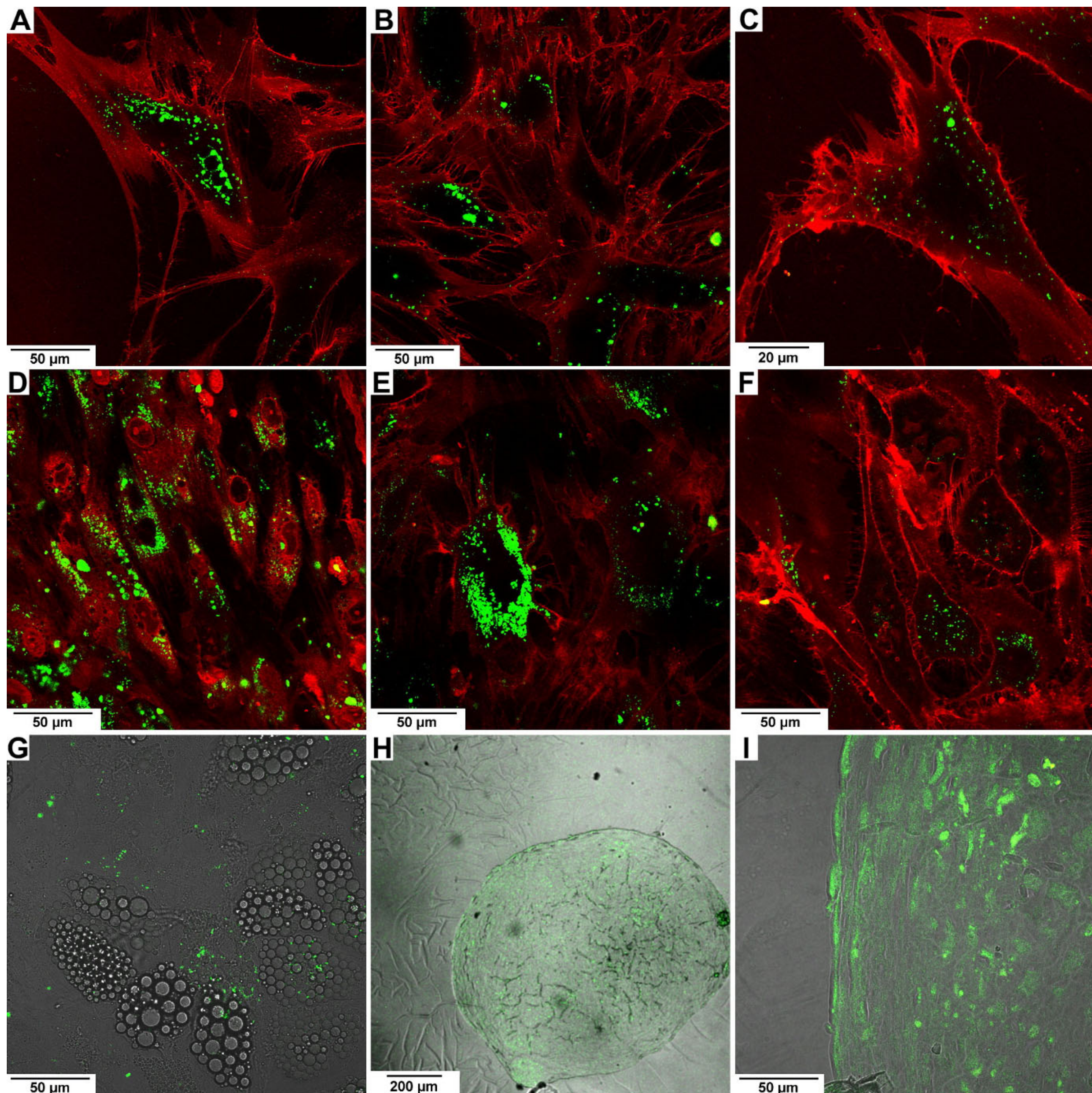

Figure 4. The intracellular localization of fluorescence-labelled VPA-based NPs (green) in human MSCs. At day 5, the particles were observed, mostly in clusters, inside the undifferentiated MSCs (A) and the MSCs cultured under osteogenic (B) or adipogenic (C) conditions. After 23 days, the particles were still visible intracellularly in most undifferentiated MSCs (D) and osteogenically (E) or adipogenically (F, G) differentiated cells. At day 36, the particles could be found inside the entire cell pellets cultured in chondrogenic differentiation medium (H, I). Cell membrane was stained red in A-F, and phase contrast images were combined with particle fluorescence in G-I. Reprinted from Ref. [93], Copyright 2009 with permission from Elsevier Ltd.

4.2 Serving as delivery carriers

NPs can serve as the carriers to deliver the bioactive molecules such as DNA, siRNA or mRNA into MSCs. Genetic modification has been perceived as a promising approach to improve the

efficacy of MSC based therapy, since a series of benefits might be generated such as regulating MSC differentiation, improving their angiogenic effect and enhancing cell survival [237]. Among various types of gene delivery vectors, cationic polymers are the most deeply studied non-viral carriers. Cationic polymers typically contain a high density of amino groups that are protonatable at the neutral pH value. They are dissolvable in solution, and they can form the polymer-DNA complex (polyplex) through the electrostatic interaction when mixed with negatively charged DNA. Polyplexes are normally positively charged particles, and their size varied from nanoscale to microscale depending on the polymer type, DNA size and polymer/DNA ratio. The positive charge facilitates the charge-charge interaction between the polyplexes and the anionic sites on cell surface, and subsequently enhances the cellular internalization of the polyplexes through endocytosis. Inside cytoplasm, the cationic polymers can protect DNA from the degradation by cytoplasm nucleases. Some cationic polymers, e.g. polyethylenimine (PEI), could induce the so called "proton sponge" effect to facilitate the DNA escape from endosomes [116]. We have reported the transfection of human bone marrow derived MSCs from different donors using branched PEI with a molecular weight of 25K Dalton (PEI25) [238]. The gene delivery efficiency was found to be influenced by the polymer/DNA ratio as well as the DNA dose. The highest transfection efficiency was observed at N/P (PEI nitrogen in primary amine/DNA phosphate) ratio 2, in which condition PEI25 could efficiently condense plasmid DNA to form nano-sized polyplexes (particle size around 150 nm) with positive charge (zeta potential around 20 mV). Notably, MSC samples isolated from different donors presented different levels of transgene expression. The transfection efficiency was not dependent on the donors' age and gender, but showed a high relationship with cell cycle (Figure 5). These results indicated that the key factors for MSC transfection include polyplex size, charge as well as cell division, which might affect the polyplex uptake, intracellular transport, endosomal escape and DNA nuclear entry. PEI could also be applied to modify NPs, serving as efficiency carriers to delivery genes into MSCs. For example, polysaccharide conjugated with branched low-molecular-weight PEI (1200 Da) showed higher transfection efficiency as well as relatively lower cytotoxicity compared to PEI25 [97, 239]. Kim et al prepared PEI-modified PLGA NPs to deliver SOX9 gene into human MSCs [240]. The PLGA NPs (40 nm in diameter) with negative surface charge (zeta potential around -14 mV) could be modified with PEI via electrostatic interaction, forming positively charged PLGA-PEI NPs and further loaded with plasmid DNA to form PLGA-PEI-DNA nanocomposite (particle size 80 nm, zeta potential around 13 mV). Plasmid DNA complexed to the PLGA NPs was in a condensed form, which could protect it from denaturation and allow for the efficient cellular uptake by stem cells.

Compared to PEI, PLGA NPs and the commercial gene carrier lipofectamine, such composite NPs exhibited significantly enhanced transfection efficiency and led to an improved chondrogenic differentiation of the MSCs following transplantation into nude mice (Figure 6). These results suggested such PEI-modified NPs might be a good candidate to deliver genes or other therapeutics to stem cells.

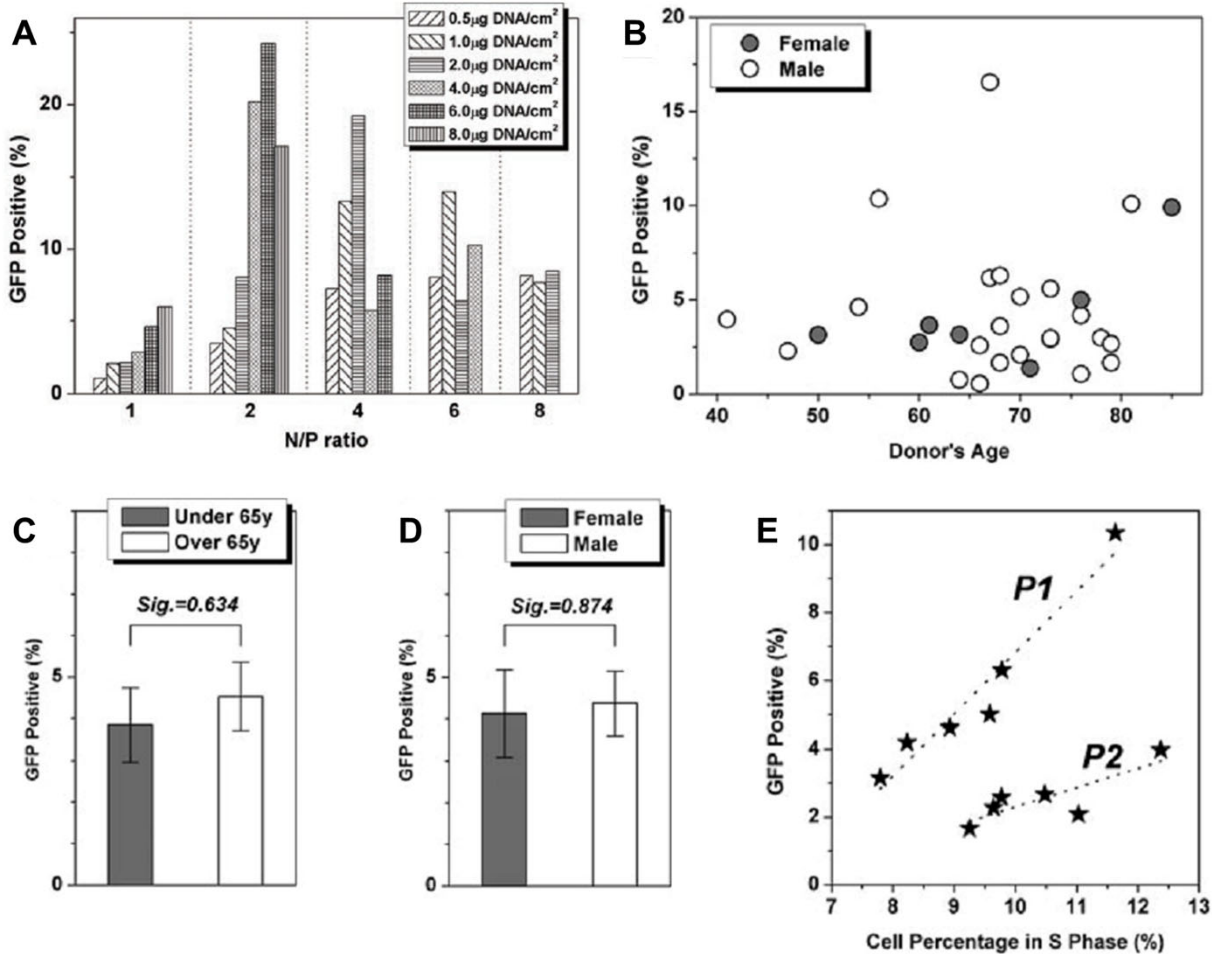

Figure 5. Human bone marrow derived MSCs transfected with PEI-DNA complexes. The highest transfection efficiency was achieved at N/P ratio 2 and DNA dose of 6 μg/cm^2 (A). The large variation of transfection efficiency of different MSC samples was observed (B). The transfection efficiency was influenced by neither age (C) nor gender (D) of the donors, but it was related to the cell cycle and two subpopulations of the MSC samples were observed (E). Adapted with permission from Ref. [238], Copyright 2011 Foundation for Cellular and Molecular Medicine/Blackwell Publishing Ltd.

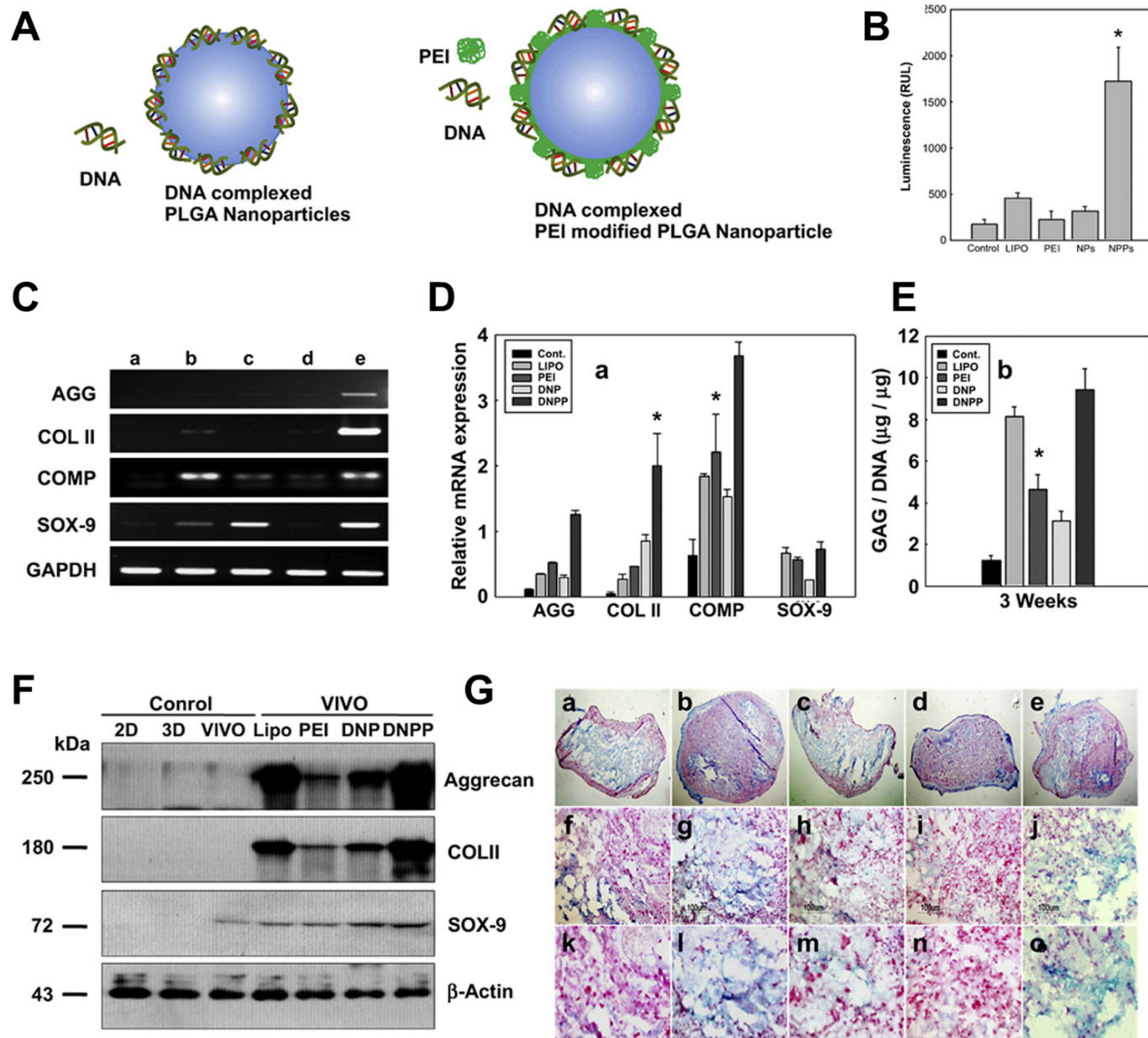

Figure 6. (A) Schematic illustration of the structure of DNA complexed PLGA NPs and DNA complexed PEI-modified PLGA NPs. (B) Quantification of luciferase gene expression of hMSCs transfected with luciferase plasmid *in vitro* using different types of carriers: control, lipofectamine, PEI, PLGA NPs or PEI-modified PLGA NPs. (C-G) Analysis of hMSCs transfected with SOX9 gene following transplantation into nude mice after 3 weeks. C: RT-PCR analysis of expression of cartilage-associated genes (collagen II, cartilage oligomeric matrix protein, aggrecan and SOX9) in control cells (a) or after transfection with lipofectamine (b), PEI (c), PLGA NPs (d) or PEI-modified PLGA NPs (e). D: Real time-PCR analysis of hMSCs transfected using different carriers. E: Glycosaminoglycan (GAG)/DNA levels of hMSCs transfected using different carriers. F: Immunoblot analysis of hMSCs transfected using different carriers. G: Alcian blue staining of hMSCs in control (a, f, k) or after transfection with lipofectamine (b, g, l), PEI (c, h, m), PLGA NPs (d, i, n) or PEI-modified PLGA NPs (e, j, o). Reprinted from Ref. [240], Copyright 2010, with permission from Elsevier Ltd.

Nano- or micro-particles could also deliver drugs or proteins into MSCs, to regulate the differentiation of MSCs such as osteogenic [241-243], chondrogenic [244] and neuronal-like

differentiation [245]. Sarkar et al. reported the utilization of biodegradable PLGA microparticles (1-2 μm in diameter) to deliver dexamethasone (DEX) to regulate MSC differentiation [246]. As an osteogenic differentiation agent acting on cytoplasmic receptors, dexamethasone could be encapsulated in the PLGA particles using a single emulsion encapsulation technique. Their result showed that the particles could be readily internalized by MSCs, likely via phagocytosis, and stably remained inside the cells for at least 7 days. Upon particle modification the MSCs were not impacted with respect to their phenotype, viability, adhesion, proliferation and differentiation potential. A sustained targeted release of the delivered agent could be achieved via this approach, and the release kinetics to the extracellular environment could easily be controlled by tuning the number of internalized particles. Importantly, they found that the intracellular and extracellular release of dexamethasone could promote osteogenic differentiation of the particle-carrying cells, as well as of neighboring cells and of distant cells, which do not contain particles, through intracrine-, paracrine- and endocrine-like mechanisms (Figure 7). Therefore, this study demonstrated a non-genetic model to engineer MSCs to modulate their stem cell fate.

Moreover, efficient drug delivery into MSCs presents the high potential in anti-tumor applications. MSCs have inherent tumortropic and migratory properties, which allow them to serve as efficient vehicles for targeted drug delivery into tumor tissue [21, 22, 247]. Therefore, when MSCs were treated with NPs containing anti-tumor agents, they could be utilized for treatment of localized and metastatic tumors [24, 94, 248].

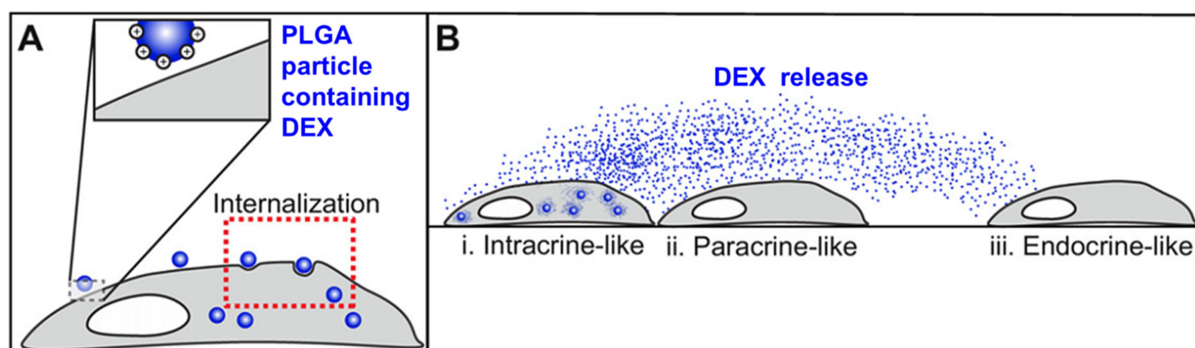

Figure 7. Cellular and extracellular programming of cell fate through internalized biodegradable PLGA particles carrying dexamethasone. (A) Functionalizing MSCs with PLGA particles which were internalized by the cells. (B) The release of the encapsulated dexamethasone could promote osteogenic differentiation of the (i) particle-carrying cell through intracrine-like signaling, (ii) neighboring cells, through paracrine-like signaling and (iii) distant cells through endocrine-like signaling. Reprinted from Ref. [246], Copyright 2011, with permission from Elsevier Ltd.

4.3 Isolation, purification and targeted delivery to MSCs

Labeling cells with magnetic particles offers the feasibility to control the labeled cells by an external magnetic force. Magnetic microbeads conjugated with antibodies have been widely adopted to purify MSCs or isolate the specific sub-populations from MSCs [249, 250]. We have isolated the CD105+ sub-population from human cord blood derived MSCs using antibody-conjugated magnetic microbeads. After being transplanted into the myocardium of the infarcted heart of mice, these CD105+ MSCs led to a more robust preservation of cardiac function, as compared to the normal MSCs [251]. The lack of enough stem cells in the key areas of specific organs is one of the barriers limiting the efficacy of stem cell-based therapy. Magnetically labeled cells could be guided and gathered by magnetic force to the desired sites to present locally significant number and density. *In vitro*, magnetically labeled MSCs accumulated by magnetic force showed the higher cell density and the enhanced cell proliferation rate, as compared to the MSCs in ordinary culture, suggesting a novel culture method to efficiently expand MSCs for clinical treatments [84]. *Ex vivo*, magnetically labeled MSCs have been delivered to the desired place in the knee joint of rabbit and swine with the guidance of magnetic force, indicating a novel and less invasive approach with high potential to repair the cartilage defect [252]. Similarly, *in vivo*, the localization of magnetically labeled MSCs in the retina after intravitreal injection or intravenous injection has been realized by placing a magnet within the orbit of rat [253].

5. Current limitations and future directions

So far, different types of NPs with multifunctionality have been prepared and applied in stem cells, which could fulfill some of the biomedical requirements. Although the combination of several key functions on a single particle becoming increasingly realistic, to prepare NPs that possess all of the above discussed properties and functions is still a challenge due to the limitation of the NP surface chemistry. To control the physicochemical properties and multiple functions of NPs could not be realized via non-covalent modification approaches (ionic and biospecific interactions, physical adsorptions) [41]. Therefore, new chemical strategies are needed to integrate most of the key properties into a single multifunctional NP. Further, it is noteworthy that the improvement of NP function in one aspect might be associated with the decrease of function in another aspect. For example, the high gene delivery efficiency of cationic polymer might be combined with high toxicity [238]. The enhanced polyplex stability

through PEGylation might result in the decrease of transfection activity [166]. The antibody-based targeting of NPs could potentially induce an immunogenic response [163]. Therefore, the first consideration for design of multifunctional NPs should be the specificity of the demand in NP functions. In addition, to use novel strategies to create functional nanoparticles might be an effective approach to realize the multifunctionality of NPs. For instance, the NPs can be produced by miniaturization of bulk polymer materials, if the underlying structural principles are not severely affected [254]. The NPs created via such an approach could be further modified to endow them with multifunctionality.

The cellular internalization of NPs is regulated by different endocytic pathways, and is related to multiple factors including the particle size, shape as well as surface features. Current knowledge on endocytosis could be instructive for design and preparation of NPs with certain physicochemical features, through which their main cellular uptake pathway could be controlled. However, some of the details involved in cellular uptake are still unclear so far. Further studies at the molecular level are still necessary to improve our understanding in endocytosis, with the aim to realize the efficient and targeted delivery of NPs into stem cells through a controllable internalization. Further, after the cellular internalization of the NPs, their intracellular trafficking and their fate are important topics that need to be clearly clarified. More knowledge should be gained about how NPs interact with intracellular molecules and organelles, where they finally locate, and how these processes are affected by the features of the NPs.

NPs might be toxic to the cells and induce the undesired response of MSCs, which could strongly limit their potential clinical effectiveness. The cytotoxicity and influence of NPs are dependent on many factors, such as particle composition, size, dose, concentration and experimental conditions as we discussed. Thus, more studies should be continued to provide more valuable information about the impact of NPs, to understand how the NPs affect the stem cells, and finally to instruct the design and development of NPs with desired properties. For biodegradable NPs, the breakdown of the NPs might induce unpredictable responses of stem cells to the degradation products. For those non-degradable ones, the retention of these NPs in the body may occur following the transplantation of NP-loaded MSCs. All of these subsequences should be carefully considered.

The long-term, sustained and controllable effect of NPs on the cells is critical for the success of many biomedical applications. With the divisions of cells and the elapse of time, the loaded NPs may be diluted out and lose their functionality. For example, the signals of cell-labeling NPs could become weaker with the cellular division. In the applications of gene/drug delivery,

the sudden release of the payload from the NPs could diminish the therapeutic efficacy. Therefore, more studies should be performed to address this issue, although some positive results have been achieved. The future efforts should still focus on the improvement of the "functionality" of the NPs, such as the specific targeting, penetrating and stimuli-responsive properties.

In addition, since the final aim to study the interaction of NPs and MSCs is to serve clinical therapies, the knowledge gained from *in vitro* studies should be well related to clinical applications. What occurs actually *in vivo* is usually different with that observed *in vitro* [255, 256]. For example, most *in vitro* studies were performed in 2D monoculture of cells, which is a static model with limited level of complexity compared to 3D dynamic *in vivo* environments. Organs consist of multiple cell types organized in a 3D architecture with their specific functions, whereas monoculture might result in the loss of intercellular communication between different cell types and could not recapitulate the native 3D tissue architecture and cellular polarization [257]. Another critical difference between *in vitro* and *in vivo* applications is the complex mixture of extracellular proteins. Extracellular serum proteins in blood will adsorb onto the surface of NPs to form a "protein corona", thereby influence biodistribution and function of NPs [258]. In addition, NPs have to face more challenges in *in vivo* applications. For instance, in order to achieve an efficient *in vivo* gene delivery to target cells via systemic administration, a series of extracellular barriers must be conquered including DNA degradation in blood plasma, DNA clearance by mononuclear phagocyte system, crossing microvessel wall and transport in extracellular matrix, which are not present for *in vitro* gene delivery[154, 259]. Therefore, there is a necessity to investigate the NPs and their interaction with stem cells both *in vitro* and *in vivo*, to make the comparison, to find out the reason and to address the issue.

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