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REVIEW

Precise nanoscale fabrication technologies, the "last mile" of medicinal development

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Abstract Nanotechnologies seek to overcome inherent deficiencies of conventional diagnosis and treatment, which attracted sustained attention and a limited number of nanomedicines approved by the FDA. However, the critical gaps in clinical translation remain, and nanomedicines that were initially heralded as magic bullets have yet to reach their realistic potential. The major obstacles of fabrication technologies may be overlooked in the nanoparticles' journey. Suboptimal manufacturing strategies partly hampered the inefficient transformation. In this review, we discuss the nanoparticle manufacturing strategies of "Top-Down" and "Bottom-Up" on precise nanoscale fabrication, including artificial intelligence introduced to guided nanomedicine fabrication for accelerating the transformation. Re-engineering existing nanomedicine fabrication, individual manufacturing, and modular technology might highlight the dilemmas of nanomedicines to meet their initial expectations.

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1. Introduction

"It was the best of times; it was the worst of times …", the APS Meeting in 1959 opened a new chapter in nanotechnology. Over the past two decades, the field of nano therapy has witnessed remarkable progress in addressing the inherent limitations of conventional medicine (Fig. 1). Therapeutic nanomedicine platforms, such as nanocrystals, liposomes, albumin nanoparticles, and polymeric nanoparticles, present a promising avenue for delivering active pharmaceutical ingredients (APIs) to target cells. This targeted delivery approach addresses limitations associated with conventional APIs, such as low solubility, narrow therapeutic indices, and suboptimal pharmacokinetic and pharmacodynamic profiles. The establishment of the U.S. National Nanotechnology Initiative in 2000 marked a significant milestone in the development of nanomedicines. Subsequent public and private investments, driven by a few fundamental mechanistic principles, have fueled the field's growth. These concerted efforts by academia and industry have led to the successful translation of over 60 nanomedicines from bench research to commercially available formulations, with notable examples including Doxil®, Abraxane®, Onpattro®, and Vyxeos®. Furthermore, numerous nanomedicines are currently undergoing clinical trials (Table 1). Modern nanomedicines can be categorized into three categories: nanodrug, nanocarrier, and engineering cell-derived nanoplatforms. The ability to precisely manipulate and tailor nanomaterials at the biological environment is crucial for various therapeutic modalities, particularly in overcoming physiological barriers for applications like genome editing and RNA therapy[1]. Nanoscale fabrication technologies offer this exquisite control over chemical composition, structure, and biological ligand-functionalization, enabling selective targeting and controlled APIs release. Despite the plethora of nanomedicine fabrication technologies developed within academia, translational success rates remain modest. This discrepancy stems from immature manufacturing processes that lack adequate control of quality attributes.

Nanomedicine manufacturing technologies typically employ "Top-Down," "Bottom-Up," or combinative strategies, often involving high-energy and/or precipitation processes. In industrial settings, nanodrugs are commonly manufactured using "Top-Down" approaches. For instance, particle replication in non-wetting template (PRINT) technology enables the precise fabrication of nanoparticles. In contrast, the fabrication of more complex nanocarriers often utilizes "Bottom-Up" approaches, such as solvent diffusion or evaporation. This approach laid the foundation for nanomedicine development, marked by the first published liposome structure in 1964 and the subsequent U.S. Food and Drug Administration (FDA) approval of Doxil®, a liposomal formulation, in 1995. Nanomedicine manufacturing processes can be categorized as either batch-to-batch or continuous. Continuous manufacturing offers advantages for scale-up, enabling precise control over production scale without altering formulation parameters. Consequently, scale-independent technologies like microfluidics have emerged as promising tools for transforming complex nanoformulations. Microfluidics offers several advantages, including a smaller factory footprint, reduced capital expenditure, and easier compliance with Good Manufacturing Practice (GMP) requirements. These benefits create significant opportunities for modular production setup. Furthermore, the expanding understanding of nano-bio interactions has led to a rapid diversification of nanomedicine platforms. Notably, cell-derived nanomedicines emerged in 2011 as a strategy to circumvent immune responses[2,3].

In the future, individualized medicine demands increasingly precise nanomedicine structures, posing significant challenges for nanofabrication techniques. Nanomedicine delivery targets can be categorized into three levels-primary, secondary, and tertiary-based on release dynamics and target specificity. Primary targeting, largely determined by size, aims to reach specific organs or penetrate the blood−brain barrier (BBB). However, size discrimination limitations can be overcome by meticulously engineering physicochemical properties such as molecular composition, surface charge, and mechanical characteristics. Achieving efficient secondary and tertiary targeting, which aims to localize to specific cells or subcellular structures, necessitates sophisticated nanomedicine manufacturing processes. The first targeted and

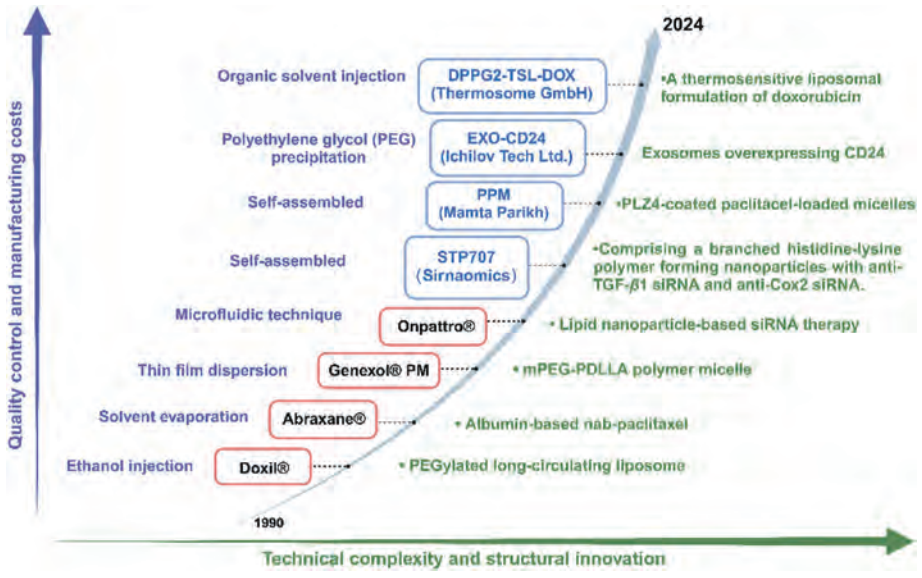

Figure 1 Life science and nanotechnology offer continuous opportunities for nanomedicine development. Refined and functionalized nanomedicine is proportional to process complexity, and manufacturing costs will increase accordingly. A mature marketed nanomedicine requires numerous process manufacturing optimizations and multiple clinical trials.

Table 1 The representative nanomedicines under clinical evaluation approved by clinicaltrials.gov.

Formulation	Example	Active ingredient	Fabrication technology	Applicant	Indication	Size	Clinical phase	NCT	Quote
Targeting nanoparticles	STP707	mRNA and siRNA	Self-assembled	Sirnaomics, Inc.	Solid tumor	100–200 nm	I	05037149	Patent: NO. 20210324384 and 9642873
Stimuli-responsive liposomes	DPPG2-TSL-DOX	Doxorubicin	Organic solvent injection	Thermosome GmbH	Sarcoma	100–150 nm	I	05858710	Patent: NO. 20240173257
Micelles	PPM	Paclitaxel	Conjugate self-assembled	Mamta Parikh	Recurrent non-muscle invasive bladder carcinoma	10–100 nm	I	06173349	Patent: NO. 11219692
Exosomes	EXO-CD24	CD24	Polyethylene glycol precipitation	Nano24med	ARDS	80–220 nm	II	05947747	Patent: NO. 20240002810

programmed polymeric nanoparticles entered clinical trials in 2011 (BIND-014). Subsequently, in 2014, ThermoDox®, a stimuli-responsive nanomedicine based on lysolipid thermally sensitive liposome technology, entered clinical trials for treating primary hepatic carcinoma. The manufacture of nanomedicines should be discussed in three interdependent dimensions: functional realization *in vivo*, quality control (QC), and manufacturing complexity. These dimensions should be viewed as components of an interactive network rather than isolated elements. As nanosystem complexity and biological performance increase in quality-oriented manufacturing, the uncertainty surrounding product attributes also tends to rise. This uncertainty has sparked debate regarding safety, efficacy, biological barrier permeability, and cellular uptake.

Despite the considerable promise of nanomedicines for cancer therapy highlighted in numerous studies, only a limited number have received regulatory approval. This discrepancy between theoretical potential and clinical reality raises a crucial question: what factors contribute to this translational gap? The COVID-19 pandemic underscored the value of sustained investment in nanotechnology. Although the concept of using mRNA as a protein therapy was proven in 1990, it wasn't until 2020, with the approval of the first mRNA COVID-19 vaccines, that nanomedicines experienced a resurgence. Despite its potential, nanomedicine is often characterized by a low rate of clinical translation. Transforming a novel concept into a commercially viable pharmaceutical product necessitates sophisticated design and manufacturing techniques. Moreover, the development process is often hindered by high costs, complex regulatory landscapes, and lengthy timelines, collectively contributing to a challenging translational landscape often referred to as "Death Valley". This translational gap underscores the need for patience when developing novel technologies, particularly complex ones, as the journey from bench to bedside is often protracted and resource-intensive. However, the emergence of advanced manufacturing technologies, including microfluidic platforms, three-dimensional printing, DNA origami cages, and engineered cell-derived nanoplatforms, offers renewed optimism for the future of nanomedicines.

This review discusses the scale-up fabrication technologies for precise nanomedicines from an industrial perspective. The analysis encompasses both established nanoscale fabrication methods and emerging technologies, considering technical aspects and scientific advancements in parallel.

2. Requirements of next-generation nanomedicine manufacturing

A promising strategy for overcoming nano-engineering challenges involves developing advanced platforms capable of precisely manipulating both the chemical and biological components of nanomedicines using scalable, high-throughput methods (Fig. 2).

2.1. Acceptable quality limits: controlled shape and size, colloid stability

While the functional realization of commercial nanomedicines is inherently linked to their composition and design, manufacturing technologies play a crucial role in achieving desired functionalities. From a therapeutic perspective, the size, shape, charge, composition, structure, targeting ligand, and surface features of

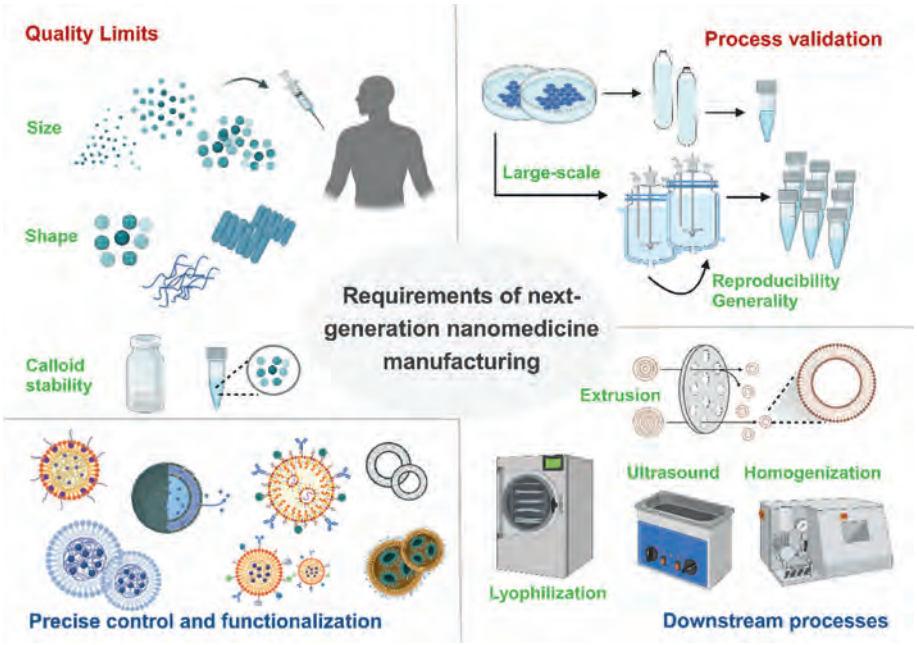

Figure 2 Requirements of next-generation nanomedicine manufacturing.

nanomedicines are critical quality attributes that can influence nanoparticle interactions with proteins and cell membranes *in vivo*.

The particle size and polydispersity index (PDI) of nanomedicines influence not only the drug loading and release behavior of APIs but also the pharmacokinetics, biodistribution, and clearance pathways. This underscores the importance of precise size control during standardized nanomedicine fabrication. According to the National Institutes of Health, the nanoscale ranges from 1 to 100 nm in at least one dimension. However, for intravenous administration, the maximum acceptable diameter of nanomedicines is distinctly below 5 μm to avoid embolism, as the minimum diameter of a blood capillary is approximately 5−6 μm[4]. The development of effective nanomedicines necessitates addressing various biological barriers inherent to applications such as molecular imaging, cell tracking, diagnostics, and drug/gene delivery. Overcoming these barriers necessitates engineering nanomedicines with specific capabilities, including prolonged circulation time, efficient tumor accumulation and penetration, enhanced cellular uptake, and successful endosomal escape. Nanoparticle size represents a critical design parameter, significantly influencing nanomedicine biodistribution, with renal filtration playing a crucial role. Renal filtration establishes a minimum effective cutoff size of approximately 10 nm for soluble nanomedicines. Leveraging the enhanced permeability and retention (EPR) effect is a rational approach for nanomedicine design. Particles in the 100−200 nm range are optimal for enhanced permeation. Conversely, nanomedicines exceeding 200 nm in diameter are susceptible to rapid clearance by the reticuloendothelial system (RES), resulting in nonspecific accumulation in the liver and/or spleen[5]. The current consensus suggests that nanoparticles within the 50−200 nm diameter range exhibit prolonged circulation times and enhanced passive accumulation in tumor tissues. Dense stroma within the tumor microenvironment presents a significant obstacle to targeted APIs delivery, particularly in poorly permeable tumors. Smaller nanoparticles exhibit longer diffusion distances within tumors. Cabral et al.[6] found that only nanomedicines smaller than 50 nm could effectively penetrate hypo-vascular, poorly permeable pancreatic tumors, thereby enhancing the anti-tumor effect.

Beyond size-dependent biodistribution, the efficacy of nanomedicines is further influenced by the subprocesses of cellular uptake. Numerous studies employing various complex models have been conducted to elucidate the relationship between nanoparticle uptake pathways and particle size[7]. Nanoparticles with diameters between 30 nm and 50 nm consistently demonstrate the most efficient cellular internalization across multiple studies[8]. This optimal size range likely arises from the interplay between various endocytic pathways and their inherent size constraints. Cellular internalization of nanoparticles occurs *via* endocytosis, a complex process driven by dynamic physicochemical interactions and a series of kinetic events regulated by clathrin-dependent, caveolin-dependent, and clathrin/caveolin-independent mechanisms, such as receptor-mediated endocytosis. Despite extensive research, a comprehensive understanding of the specific uptake mechanisms governing engineered nanoparticles remains elusive. Larger particles, ranging from 500 nm to 3 μm, are internalized *via* phagocytosis, a process that does not rely on intermediate pathways. The interaction between nanoparticles and cell membranes critically influences nanoparticle internalization. Upon attaching to cell membranes, nanoparticles create a heterogeneous interface that alters the energy landscapes governing interaction forces[7]. These interactions, encompassing both specific (*e.g.*, ligand−receptor binding) and nonspecific (*e.g.*, van der Waals forces, electrostatic interactions, and hydrophobic effects) forces, contribute to the overall adhesion forces. Nanoparticle endocytosis induces membrane bending, leading to membrane tension at the wrapping site. This membrane bending arises from the inherent asymmetry of the lipid bilayer and the presence of transmembrane proteins, particularly in clathrin/caveolin-mediated endocytosis. In contrast to nonspecific interactions, receptor-mediated endocytosis introduces a time delay, as receptor diffusion to the binding site precedes membrane wrapping, ultimately influencing the kinetics of endocytosis. The internalization of lipid nanovesicles, which

dock onto cell membranes *via* ligand−receptor interactions, is influenced by several factors, including interfacial hydrodynamics, the diffusivity of membrane-bound tethers, and nanoparticle size. Larger nanoparticles, however, exhibit reduced lateral diffusion distances[9]. A characteristic length scale (λ), defined as $\lambda = (2B/\sigma)^{1/2}$, where B represents bending energy and σ represents stretching energy, characterizes the relative contributions of bending and stretching energies during membrane wrapping. For nanoparticles with radii smaller than a typical λ value of 50 nm, bending energy dominates the wrapping process. Conversely, as nanoparticle radius surpasses λ, membrane tension plays an increasingly significant role[7]. Clathrin/caveolin-mediated endocytosis and phagocytosis typically occur over a timescale of 30 s to several minutes[10]. Interestingly, a significant portion of nanoparticles desorb from the membrane and are internalized rapidly within seconds. This rapid internalization may occur through pre-existing endocytic events rather than triggering *de novo* internalization, and it appears to be independent of individual nanoparticle size. Nanoparticle agglomeration, both *in vitro* and *in vivo*, presents a significant challenge in nanomedicine design, as it can alter initial particle size and consequently affect internalization pathways[11]. Precise control over nanoparticle size during manufacturing is therefore crucial, particularly for complex nanomedicines. Nanoparticle shape, size, and interface curvature with the cell membrane are critical determinants of cellular internalization efficiency. Therefore, precise nanofabrication techniques can be employed to optimize these morphological parameters, ultimately enhancing cellular loading *via* phagocytosis.

In industrial-scale manufacturing, nanomedicines with a PDI value below 0.1 are considered monodisperse, while a PDI of 0.05 represents a high monodispersity criteria. In practice, a PDI of 0.2 and 0.3 is generally acceptable for polymeric micelles and liposomes, respectively[12,13].

The geometrical characteristics of nanomedicines significantly influence the desired outcome of various *in vivo* processes, including blood circulation, biodistribution, extravasation, target specificity, and cellular uptake, ultimately affecting overall therapeutic efficacy. In recent years, non-spherical nanomedicines, with their unique properties, have emerged as a novel strategy for targeted drug delivery through rational design. Common shapes of nanomaterials include spheroid, polyhedron, rod-like, and fibrous. Due to their altered hydrodynamic behavior in the bloodstream and differential phagocytosis rates, non-spherical nanomedicines often exhibit distinct circulation half-lives compared to their conventional spherical counterparts[14]. Lee et al.[15] presented a general mathematical model to predict the transport behavior of nanoparticles with varying material properties, sizes, and shapes within a linear laminar flow. This model demonstrated that nanoparticles could experience lateral drift, known as hydrodynamic margination, due to the combined effects of inertial and hydrodynamic forces. Within the tortuous geometry of blood vessels, nanoscale medicines tend to follow streamlined flow patterns, as volume forces are negligible compared to hydrodynamic forces[16].

The lateral drift velocity of anisotropic nanoparticles, determined by the particle Stokes number (S_{ta}), correlates positively with the density, size, and rotational inertia of the nanoparticles. Discoidal particles with a low aspect ratio exhibit the greatest margination propensity and preferential targeting to the diseased microvasculature. The shear rate represents another crucial parameter influencing the lateral migration of nanoparticles. Non-spherical particles typically oscillate around their trajectory at shear rates below 10^2/s, except in the presence of external force fields. In contrast, at shear rates between 10^2/s and 10^4/s, they attain a drift velocity of approximately 1−10 μm/s[15]. Shah et al.[17] further employed computational modeling to investigate the influence of ligand density, shape, and shear rate on the adhesion kinetics of non-spherical nanoparticles to vessel walls. Under a shear rate of 8/s, nanorods exhibit a three-fold higher binding probability compared to nanospheres of the same volume. This enhanced binding likely arises from the tumbling motion of nanorods, which facilitates increased surface contact with vessel walls compared to their spherical counterparts. While tissues appear to exhibit distinct preferences for different nanoparticle shapes, a definitive understanding and consensus regarding this phenomenon remain elusive. Nanoparticle shape significantly influences cellular uptake and intracellular transport. Nanoparticles with sharp morphologies generally exhibit enhanced internalization, with local curvature acting as a key determinant of the initial membrane-wrapping rate. Studies have demonstrated an uptake efficiency order of rod > disc > sphere[18]. Consequently, optimizing nanoparticle uptake efficiency necessitates careful consideration of size, shape, targeting ligand, and uptake pathway. Furthermore, the shape of nanomedicines exerts a complex influence on *in vivo* toxicity, impacting both biodistribution and cellular uptake. For instance, the mononuclear phagocyte system (MPS) can reduce or delay the phagocytosis of anisotropic nanoparticles, thereby altering their biodistribution. Moreover, anisotropic immobilized pendant polymers on the surface of nanoparticles have been shown to inhibit macrophage adhesion[19]. Compared to spherical nanoparticles, disk- or rod-shaped polystyrene nanoparticles induced significantly fewer changes in nanocarrier-mediated cardiopulmonary responses, including systemic and pulmonary arterial pressure. This difference is attributed to the delayed uptake of these anisotropic shapes by pulmonary intravascular macrophages.

Colloidal stability is a critical quality attribute of nanomedicines, influencing the choice of formulation (*e.g.*, suspension or lyophilized powder) for FDA-approved products. The limited availability of polymeric micelle formulations for intravenous administration, particularly in certain geographical regions, can be partly attributed to concerns regarding their *in vivo* colloidal stability.

The mechanical properties of nanomaterials, particularly their elasticity, influence their *in vivo* fate by modulating interactions with cells, which are sensitive to mechanical cues. Numerous studies have demonstrated that, compared to stiffer counterparts, soft nanoparticles exhibit prolonged blood circulation, enhanced BBB penetration, and superior tumor cell uptake[20,21]. Soft nanoparticles prolong their circulation time by evading macrophage recognition, thereby reducing splenic clearance. Soft nanoparticles exhibit enhanced adhesion to cells, promoting uptake under flow conditions. Moreover, evidence suggests that soft nanoparticles facilitate rapid lysosomal transport[21,22]. The shift in nanoparticles' elasticity from stiff to soft appears to alter the mechanism of cellular internalization, transitioning from energy-intensive endocytosis to a less energy-dependent membrane fusion process[23]. However, the literature reports inconsistent findings regarding the impact of nanoparticles' elasticity on cellular uptake. This inconsistency may stem from the complex interplay between nanoparticles' elasticity and other factors such as material properties, size, ligand−receptor interactions, and tumor heterogeneity. Additionally, current studies often employ nanoparticles with a limited range of elasticity, making it challenging to comprehensively assess the impact of elasticity on

tumor uptake[24,25]. The inherent low modulus of extracellular vesicles (EVs) may contribute to their efficient cellular uptake and the protection of their contents from endosome/lysosome degradation.

Of course, the observed biodistribution and cellular uptake, which ultimately dictate biological activity, are influenced by a complex interplay of nanoparticle properties, including size, morphology, surface chemistry, and internal nanostructure[26−28]. Given the dependence of nanomedicine performance on factors such as APIs properties, delivery routes, and clinical demands, establishing universally applicable control standards and drawing definitive conclusions remain challenging. The advanced understanding of how morphology influences nanoparticle delivery presents both challenges and opportunities for precise nanomedicine manufacturing[29].

2.2. Process validation of large-scale, reproducibility and generality

Strategic aspects underpinning the commercialization of nanomedicines necessitate a synergistic interplay between market demand ("pull") and technological advancements ("push"). The manufacture of nanomedicines for commercialization presents numerous challenges, encompassing technology transfer, adherence to Chemistry, Manufacturing, and Control regulations and GMP, as well as the development of controllable, reproducible, and scalable synthesis methods. The inherent complexity of certain nanomedicines, particularly those integrating features such as multi-drug encapsulation, biological targeting ligands, multiple functional units, and stimulus-response elements, often necessitates intricate multi-step fabrication processes, significantly escalating the challenges associated with clinical translation. Furthermore, limitations inherent in current preparative methods and their scalability pose a significant hurdle to clinical translation, as a stable and reliable process is paramount even for pilot-scale batches[2]. Limited methodologies, including nanoprecipitation and emulsion-based techniques, demonstrate suitability for industrial-scale nanomedicine production due to their inherent scalability. However, the principles of industrial factorial design, crucial for optimizing nanofabrication processes, are often overlooked during the launch of new nanomedicine products in the laboratory. Therefore, to effectively address the challenges inherent in scaling up nanomedicine production, the adoption of robust and practical experimental designs is essential.

2.3. Precise control and functionalization of complex systems

The increasing demand for sophisticated targeting mechanisms and enhanced biological performance necessitates increasingly complex nanomedicine designs. This complexity, in turn, requires greater precision in manufacturing within acceptable quality limits, posing significant challenges for production units and manufacturing processes. Nanomedicines involving multi-step or complex fabrication processes present significant challenges for achieving large-scale, reproducible manufacturing. Precisely controlling the structure and surface functionalization of complex nanomedicines is crucial for achieving desired therapeutic effects *in vivo*. The transition from laboratory to clinical practice often necessitates optimization of formulation parameters and even process modifications. Therefore, incorporating considerations for scalable manufacturing into the early design stages of nanomedicines is paramount.

2.4. Simple downstream processes

Downstream processing of nanomedicines, encompassing solvent removal, purification, particle size control, concentration, and sterilization, is equally critical. These downstream operations need to be amenable to industrial scale-up. Downstream processing techniques, such as ultrasound, extrusion, freeze−thaw cycles, homogenization, or a combination thereof, can be employed to standardize nanomedicine size or reduce liposome lamellarity. Extrusion and high-pressure homogenization (HPH) represent commonly employed techniques for controlling nanomedicine size, although they are not suitable for rigid structures. Manufacturing sterile nanomedicines is an absolute prerequisite for human and animal applications. Sterile manufacturing, typically requiring high-grade cleanroom environments or subsequent sterilization, presents challenges. These include potential stability issues for APIs and/or lipids, as well as significant losses of active ingredients during filtration[30]. The agglomeration state of nanomedicines significantly impacts therapeutic efficacy, necessitating strict control over uniformity and reproducibility during manufacturing to minimize within-batch and batch-to-batch variability. Controlled sonication within a multi-transducer vessel generates stable, reproducible cavitation fields that facilitate controlled deagglomeration of plasmid delivery systems, either before or after plasmid loading, while also safeguarding DNA plasmids from degradation[31]. Ideally, nanomedicine manufacturing technologies should strive to simplify downstream processing, thereby minimizing labor and cost. This can be achieved through approaches such as organic solvent-free synthesis technology or precise particle size control during manufacturing.

3. Nanoscale fabrication technologies

In this review, we focus on nanomedicines administered *via* intravenous injection. The major classical approaches to manufacturing nanoscale colloidal particles can be broadly categorized into three types: "Bottom-Up" and "Top-Down," as well as combinative techniques.

"Top-Down" manufacturing techniques leverage external mechanical forces to break down macroscopic structures or deform drug compounds into nanomaterials suitable for various drug delivery methods. This approach encompasses three primary categories: (1) milling and homogenization, yielding nanocrystals with narrow size distributions and adaptable batch processing; (2) mechanical stretching, transforming uniformly shaped nanoparticles into those with diverse morphologies and elasticities; and (3) lithography and other shaping techniques, enabling large-scale particle replication with surface modifications to enhance drug delivery efficiency and bioactivity. "Top-Down" strategies offer several advantages, including batch uniformity, scalability, straightforward drug design, and process controllability. However, these methods often present limitations, including incompatibility with thermosensitive drugs, potential for drug degradation, and significant production losses. Consequently, their applicability remains limited to a subset of drugs, and they offer restricted design flexibility.

Conversely, "Bottom-Up" fabrication techniques assemble nanomedicine carriers from the molecular level, rendering them suitable for diverse drug delivery applications. These techniques often involve designing drug carriers that mimic cellular structures or inclusions, thereby prolonging drug release and enhancing

retention within the body. Early "Bottom-Up" methods encountered limitations, including high purification costs, non-uniform nanoparticle sizes, poor stability, and scalability challenges, as industrialization needs robust, reproducible processes supported by comprehensive data[32]. Recent advancements, however, have mitigated some of these limitations. Microfluidic platforms, for example, offer the potential for high-throughput production and optimized large-scale processes. Furthermore, stimulating cells to secrete nanoscale vesicles represents another "Bottom-Up" approach, yielding biopharmaceutical carriers with enhanced biocompatibility. Achieving the ideal fabrication of EVs—characterized by uniformity, high drug load capacity, high encapsulation efficiency, scalability, and suitability for high-throughput downstream processing—will remain a major milestone toward realizing precise nanoscale manufacturing techniques.

For the successful commercialization of nanomedicines, a hybrid approach that leverages the strengths of both "Top-Down" and "Bottom-Up" techniques may be essential. While "Top-Down" methods excel in ensuring uniformity and scalability, "Bottom-Up" approaches offer superior biocompatibility and the potential for developing innovative drug delivery systems. By capitalizing on the complementary advantages of both fabrication paradigms, this integrative strategy holds significant promise for driving the successful commercialization of nanomedicine.

3.1. "Top-Down" techniques

The "Top-Down" strategy starts from macroscopic structures and transforms them into nanomaterials through external chemical or mechanical control, inducing severe plastic deformation. Examples of such techniques include high-pressure homogenizers, extrusion, and milling. This "Top-Down" approach is particularly suitable for relatively simple nanomedicine designs, offering advantages such as good scale-up transferability, batch homogeneity, and batch-to-batch consistency, which are crucial for QC.

3.1.1. Classical "Top-Down" techniques

Classical "Top-Down" strategies, primarily media milling and HPH techniques (Table 2), are widely employed to process nanocrystals, offering a narrow particle size distribution and flexibility in handling batch quantities. Despite their less frequent use in intravenous administration, nanocrystals offer a significant advantage in enhancing dissolution rates by reducing particle size. Wet stirred media milling and HPH have established techniques for the commercial production of nanomaterials using "Top-Down" approaches, with the former being the most widely employed method for obtaining nanosuspensions. A significant challenge in the milling process is contamination from residue generated by the abrasion and breakage of the grinding medium. Spherical D-mannitol beads have emerged as a promising contamination-free milling media, considered safe for ingestion[33]. In addition, maintaining a specific milling temperature is crucial, as many pharmaceutical compounds are heat-sensitive. To date, the FDA has only approved two nanocrystals for intravenous administration, both of which were manufactured using "Top-Down" strategies. Meloxicam (ANJESO® injection) was milled using a NanoMill.RTM. Milling system with polymeric media composed of polystyrene or cross-linked polystyrene to produce 150 nm nanocrystals, stabilized with 5% polyvinyl pyrrolidone (U.S. Pat. No. 6431478 and 10709713). HPH is another general technique for fabricating nanodrugs. Its working principle is primarily categorized into piston-gap and jet-stream homogenization (U.S. Pat. No. 20130065888). The choice between piston-gap homogenization (French Press) and jet-stream homogenization (microfluidizers) depends on the desired dispersion degree, concentration, and aggregate state of the materials. Microfluidization is particularly favored for nanoscale drug manufacturing. Ryanodex® was prepared using a Model 110 L Microfluidizer at a pump pressure of 15,000 psi for four cycles of 1.5 min each, resulting in a particle size of approximately 400 nm (U S. Pat. No. 8685460). Compared to media milling, microfluidization offers advantages in terms of processing time and drug loading while achieving similar physical properties such as amorphization or polymorphic conversion[34].

3.1.2. Anisotropic nanomedicine manufacture

Nanoparticles, whether possessing rigid or flexible structures, are often depicted as spherical to ensure minimizing interfacial tension. While the anisotropic shape of rigid nanoparticles is permanent, flexible lipid-based nanoparticles, due to the dynamic nature of their lipid membranes, can undergo anisotropic shape transformations within very short timescales[35]. The unique margination dynamics exhibited by anisotropic nanoparticles, characterized by distinct rolling and tumbling motions, influence tissue penetration and cellular uptake, leading to increased interest in biomedical applications. Manufacturing techniques for non-spherical nanoparticles with defined geometries encompass both standard preparation methods, such as self-assembly, nanoprecipitation, solvent evaporation, and mechanical stretching, as well as advanced techniques like PRINT and film lithography. Self-assembly can lead to the formation of non-spherical particles with various morphologies, including rods, worms, fibers, and other filamentous structures of micelles, as well as even more complex shapes and surface textures. Conventional "Bottom-Up" strategies for manufacturing anisotropic nanomedicines involved self-assembly, nanoprecipitation, phase separation, or solvent evaporation. These methods rely on driving forces, such as van der Waals forces, electrostatic interactions, hydrogen bonding, or hydrophobic interactions governed by thermodynamic changes in entropy and enthalpy, to guide the assembly of building blocks. Mechanical stretching, a method applying weak stress at the nanoscale, has been explored as a means to drive mechanical

Table 2 Classical "Top-Down" techniques.

Technique	Advantage	Limitation
Milling	Simple craft, narrow size distribution, be suit for heat unstable and insoluble APIs	Residual of solvent and medium
High-pressure homogenization	Less pollution, small average particle size, narrow particle size distribution, and good reproducibility	High energy consumption
Laser ablation and crushing	Organic solvent free	Causing oxidative degradation and crystal change of some sensitive APIs by shock wave or heat

deformation, enabling the fabrication of non-spherical nanoparticles ranging from the nanoscale to the micro-scale. Spherical particles are embedded into polymeric films of predetermined thickness and flexibility. Subsequently, the films are subjected to stretching to induce particle deformation. Fabrication methods for non-spherical particles often involve a sequential combination of liquefaction and stretching steps. Nanoparticles are first liquefied, either by the addition of a solvent or by heating above the glass transition temperature. Subsequently, the films are stretched unidirectionally (1D) or bidirectionally (2D), resulting in distinct final particle shapes (Fig. 3, Scheme A)[36]. This process is followed by the re-solidification of the liquefied particles, rendering them plastic[37]. In Scheme B, PVA films are initially stretched to create voids around the particles. Subsequently, these voids are filled by liquefying the particles. For Scheme C, prior to nanoparticle liquefaction, the polymer film is stretched and removed from the stretcher, this process generates wrinkles at the film−void interface to form an additional texture feature on the particles' surface in subsequent procedure. The surface roughness can minimize repulsive electrostatic and hydrophilic interactions, thereby influencing nanoparticles uptake[38]. Following stretching, the embedded non-spherical nanoparticles are extracted by dissolving the polymer membrane. This recovery process requires ensuring that particles remain insoluble in the solvent used for membrane dissolution.

Nanoparticles embedded in poly(vinyl alcohol) film were subjected to multiaxial stretching below the glass transition temperature to induce deformation into non-spherical micro-/nanoparticles[14]. The resulting stretched anisotropic particles within a single film exhibit heterogeneity in geometry and dimension, likely due to variations in strain applied across different regions of the film. Moreover, the stretching procedure necessitates extended processing times. In addition, the multi-step nature and stringent preparation conditions associated with mechanical stretching limit its applicability for loading sensitive biomolecules. Despite these limitations, mechanical deformation or stretching technology presents a potential advantage for large-scale production due to its amenability to automation, provided that challenges related to the thermal stability of biopharmaceuticals during processing can be addressed.

3.1.3. Lithography and other molding techniques

Lithography techniques offer a promising alternative to traditional fabrication methods, enabling the creation of biomedical devices with homogeneous sizes and intricate designs. The presence of wrinkles on non-spherical particles has been shown to significantly enhance cell attachment, offering potential benefits for various biomedical applications without requiring surface modification. Li et al.[39] reported a method for the rapid fabrication of wrinkled non-spherical particles using a combination to mimic natural textures on the surface. In their method, non-spherical particles are first fabricated using flow lithography and coated with a partially cured polymer layer within a microfluidic channel. Subsequently, the coated particles are subjected to plasma treatment, which induces rapid buckling of the polymer layer, resulting in a wrinkled surface. Furthermore, the surface morphology of the particles can be tailored by adjusting the ultraviolet exposure time and the washing process. This technology appears to be amenable to application in nanofabrication.

PRINT is a continuous, high-resolution, plug-and-play molding technology for the design and synthesis of precisely defined nanoparticles. This "Top-Down" GMP-compliant platform utilizes a fluorocarbon mold and is amenable to large-scale particle fabrication. It allows for unprecedented tailoring of physicochemical parameters, including particle chemical composition, cargo, size, shape, and surface functionalization. Standard photolithographic techniques are employed to fabricate master templates on silicon wafers featuring uniform particle sizes down to 50 nm. These templates enable the creation of multiphasic and region-specifically functionalized particles, including biphasic Janus particles, end-labeled particles, and multi-phasic shape-specific particles. Subsequently, a photocurable, non-wetting perfluoropolyether polymer, characterized by extremely low surface energy, low modulus, and high gas permeability, is deposited onto the master templates to form the mold[40]. The non-wetting nature of the mold facilitates the creation of isolated micro/

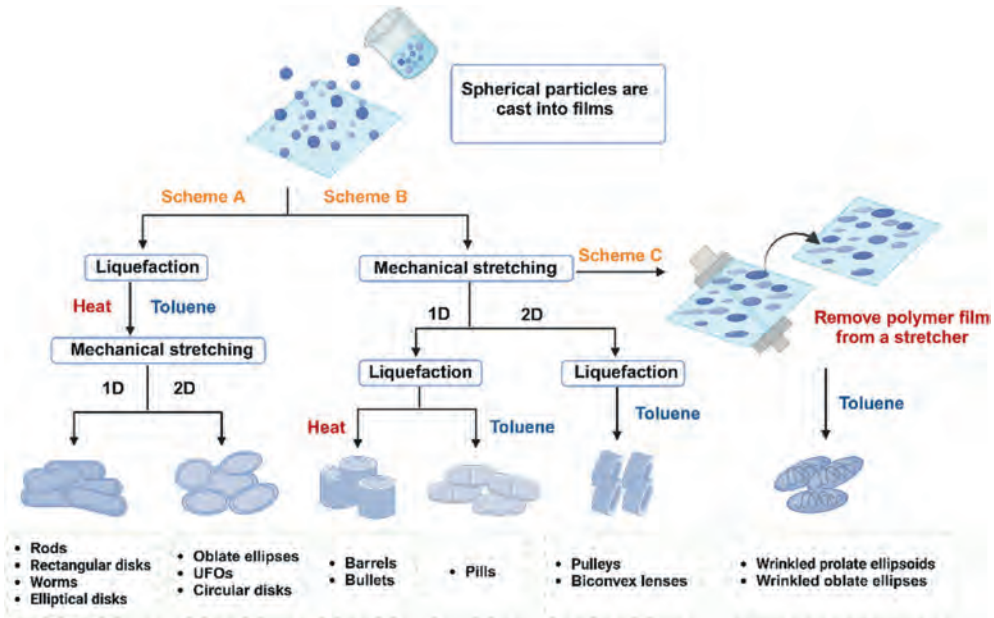

Figure 3 A schematic of non-spherical particles manufacturing by mechanical stretching.

nano-sized particles capable of high APIs loadings (20%)[41]. Ferreira et al.[42] investigated the drug loading capacity of PRINT-based nanoparticles using various loading strategies. A template-based fabrication approach was employed to generate 1000 × 400 nm discoidal nanoparticles composed of poly(lactic-co-glycolic acid) (PLGA) and poly (ethylene glycol)-diacrylate, crosslinked *via* ultraviolet-light polymerization. Two distinct loading strategies, "direct loading" and "absorption loading" were employed to encapsulate APIs within the nanoparticles. In the "direct loading" approach, polymeric materials and APIs were directly mixed within the template to form nanoparticles. Conversely, the "absorption loading" method involved rehydrating lyophilized nanoparticles in an APIs solution. The encapsulation efficiency of the "absorption loading" method was markedly higher than that of the "direct loading" strategy. This suggests that "direct loading" may not be suitable for encapsulating moderately hydrophobic compounds with low molecular weights within PRINT-based nanoparticles, as evidenced by the low encapsulation efficiency (lower than 1%).

While surface chemistry is a critical factor influencing the physiological behavior of nanoparticles, many existing nanomedicine platform technologies exhibit limitations in their drug loading and surface modification capabilities. Morton et al.[43] proposed a high-throughput, rapid spray-combined PRINT platform for analyzing diverse surface functionalities and APIs combinations. This platform utilizes spray-assisted layer-by-layer deposition, complementing PRINT-based roll-to-roll technology, to generate customizable functional nanoparticles (Fig. 4)[44]. Layer-by-layer polyelectrolyte deposition is a promising approach for enhancing the serum stability of biologics, enabling synergistic co-delivery, and achieving sustained release of therapeutics within a single nanoscopic platform. This technique facilitates surface coating, allowing for the programmable loading of multiple drugs within PRINT-based nanoparticles. While PRINT technology is currently considered experimental, the potential cost barrier is significantly mitigated by employing continuous roll-to-roll molding in scalable particle manufacturing processes[44]. Understanding the baseline immune responses of nanoparticles is crucial for clinical application design. In this regard, the innate immune responses of hydrogel nanoparticles fabricated using PRINT technology were evaluated in a humanized mouse model with cross-validation[45]. Notably, PRINT-based non-spherical nanoparticles demonstrate an ideal profile for APIs delivery, as they elicit minimal inflammatory or cytokine responses and do not activate complement upon uptake by human $CD14^+$ monocytes.

A scalable and rapid emulsification approach employing shear mixing or sonication of the continuous phase, followed by solidification of the dispersed phase, has been widely used for nanoparticle fabrication. However, this method often results in the formation of irregular clumps due to droplet coalescence prior to solidification. To address this challenge, Kim et al.[46] developed a universal static-state particle fabrication method based on the rapid vitrification of a thixotropic medium. This technique effectively eliminates droplet deformation and coalescence. In this approach, a thixotropic continuous phase, such as gelatinized corn starch in water, is employed. Thixotropic fluids exhibit shear-thinning behavior, meaning their viscosity decreases under increasing shear stress. Upon cessation of shearing, the thixotropic

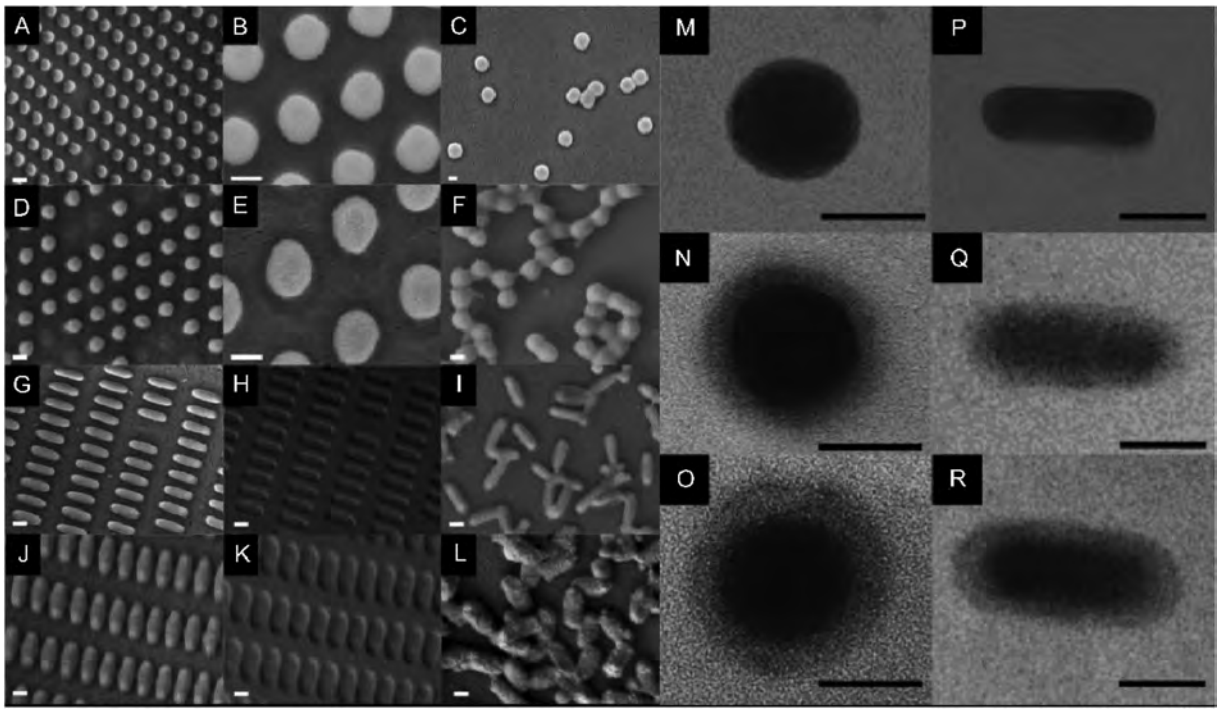

Figure 4 SEM, TEM characterization of particles pre/post−spray layer-by-layer functionalization. SEM of (A, B, G, H) two sizes of uncoated, crosslinked PLGA nanoparticles array; (C, I) two sizes of purified uncoated, crosslinked PLGA nanoparticles; (D, E, J, K) two sizes of coated PLGA nanoparticles array $[(PLL/HA)_3]$; (F, L) two sizes of purified coated PLGA nanoparticles $[(PLL/HA)_3]$. White scale bar = 200 nm. TEM of (M, P) two sizes of uncoated, crosslinked PLGA nanoparticles; (N, Q) two sizes of purified coated PLGA nanoparticles $[(PLL/HA)_3]$; (O, R) two sizes of purified coated PLGA nanoparticles $[(Chit/HA)_5]$. Black scale bar = 100 nm. Reprinted with the permission from Ref. 44. Copyright © 2013 Advanced Materials (Deerfield Beach, Fla).

medium undergoes rapid solidification through vitrification. Notably, the solidified matrix remains permeable to energetic fields, enabling further manipulation of the embedded nanoparticles. This allows for the fabrication of anisotropic nanoparticles with programmed functionalities, including self-propelling Janus nanoparticles, metallic nanoparticles with low melting points, and unidirectionally-magnetized robotic nanoparticles.

3.2. *"Bottom-Up" techniques*

Classical "Bottom-Up" technologies have been intensively applied in industrial-scale fabrication, achieving significant success in delivering APIs within acceptable quality limits. "Bottom-Up" techniques involve the assembly of complex nanocarriers from simpler chemical constituents in a molecule-by-molecule fashion. In contrast to "Top-Down" methods, which are inherently limited by the molecular properties of APIs, "Bottom-Up" approaches offer significant design flexibility, enabling the creation of complex nanomedicines with greater versatility. Several methods fall under the umbrella of "Bottom-Up" approaches, including solvent evaporation, precipitation, salting-out, emulsification-diffusion, solvent displacement, supercritical precipitation, electro-spraying, spray-drying, chemical conjugation, crosslinking, and others, as shown in Table 3[47–54]. Despite the versatility of "Bottom-Up" approaches, achieving successful industrial-scale translation necessitates additional processing steps, such as homogenization and extrusion, to refine particle size. For instance, ethanol injection following extrusion is a common method for commercially producing liposomes with desirable particle size and PDI. This technique highlights the preference for solvent diffusion over solvent evaporation in certain applications. However, the "Bottom-Up" synthesis of nanomedicines often results in impurities such as residual solvents, unreacted monomers, excess surfactants, and initiators. Purification from these impurities can be laborious and time-consuming. Furthermore, random self-assembly between monomeric nanomaterials and APIs often leads to a wide particle size distribution. Achieving a desired PDI necessitates additional processing steps such as extrusion, high shear homogenization, or HPH.

Self-assembly, a ubiquitous method for "Bottom-Up" nanoparticle fabrication in industrial settings, requires a comprehensive understanding and precise control across multiple length scales. A wide array of amphipathic building blocks, varying in molecular weight, charge, hydrogen bonding capacity, and hydrophobic group ratio, are now available for the colloidal self-assembly process. In a significant development, Hua et al.[55] described a general mechanism for the fabrication of highly anisotropic nanoparticles with controlled dimensions. This method, based on supramolecular bond formation-driven morphological transformation, holds promise for the large-scale production of polymer/nanoparticle systems. The introduction of a high concentration of polymer to isotropic nanoparticle seeds induces a rapid transformation to anisotropic structures driven by supramolecular bond formation. The extent of anisotropic growth is directly proportional to the concentration of polymer added.

Despite their prominence in commercial nanomedicines, liposomes present a significant challenge for the encapsulation of hydrophilic APIs. HPH offers a potential solution by enabling the use of higher lipid concentrations to enhance APIs encapsulation efficiency. This method exploits the order of magnitude difference between the external and internal aqueous phases of the liposomes to effectively entrap hydrophilic APIs (U.S. Pat. No. 10662060). However, liposome preparation necessitates temperatures that reach the phase transition temperature of the lipid membrane, potentially compromising the stability of certain APIs. Conventional fabrication methods, such as heating, stirring, and sonication under a nitrogen atmosphere, offer a potentially viable alternative by mitigating APIs degradation[56]. To circumvent the issue of organic solvent residues, supercritical or near-critical fluids present a viable alternative for producing organic solvent-free multilamellar vesicles (MLVs) encapsulating hydrophobic drugs. This process leverages pressure reduction to facilitate drug encapsulation. Notably, MLVs can serve as precursors for the production of single-compartment liposomes, including small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) (U.S. Pat. No. 5776486).

Table 3 Classical "Bottom-Up" techniques.

Technique	Advantage	Limitation	Ref.
High-pressure homogenization	Hot or cold HPH, coupled with other techniques, large-scale, organic solvent-free	High temperature and pressure might be a challenge, incomplete homogenization for expensive and sensitive substances, especially difficult to produce small sample amounts required, high operating costs and time	47,48
Solvent injection method	A simple, fast manufacturing technique	Residual organic solvent and low APIs concentration	49
Phase inversion	Low energy input and avoidance of organic solvent	Poor stabilities	50
Emulsification-solvent evaporation	A simple, fast manufacturing technique	Residual organic solvent and poor PDI	50
Nanoprecipitation	Low energy consumption and easy operation	Broad particle size distribution, low physical stability, and batch-to-batch variability	51
Spray drying	Continuous and quick process	Size control, applying stress to lipid membranes and the encapsulated active APIs, low-yield	52
Supercritical carbon dioxide technology	A mild process to retain the bioactivity of sensitive biomolecules	High production costs, low dissolving capacity of CO_2	53
Co-axial electrospray	Address the components incompatibilities	Sub-micrometers to micrometers particle	54

Once formed, liposome particle size can be further tailored through downstream processing. Bath or probe tip sonication effectively produces SUVs, while conventional extrusion typically involves passing the liposome suspension through a series of polycarbonate filters with decreasing pore sizes (*e.g.*, 0.8 to 0.1 μm) under high pressure (up to 500 psi). Modified extrusion methods have been developed to simplify production and enhance yield. These methods utilize a single-stage process with a single-sized filter (*e.g.*, a 0.14 μm ceramic membrane) and involve multiple passes (12−18) under lower pressure (approximately 90 psi) and controlled temperature (68 °C). This streamlined approach is particularly amenable to large-scale production (U.S. Pat. No. 10265269). Furthermore, dual asymmetric centrifugation has emerged as an alternative method for the fabrication of lipid-based nanoparticles. In this technique, vesicle size is modulated by adjusting the centrifugation speed, with higher G-force and/or lower counter-rotation ratios resulting in smaller particle sizes. This method eliminates the need for extrusion and enables the production of liposomes with minimal amounts of particles larger than one μm, making it particularly well-suited for "bedside preparation" immediately prior to patient administration (U.S. Pat. No. 10662060).

Janus nanoparticles, characterized by their distinctive two-faced structure, exhibit unique anisotropic properties. This asymmetry allows for sophisticated drug delivery strategies by leveraging the distinct properties of each compartment, such as selective degradation. Janus nanoparticles can be composed of polymeric, inorganic, or hybrid polymeric-inorganic materials, and their fabrication can be achieved through various methods, including surface nucleation and seeded growth, phase separation, microfluidic synthesis, covalent conjugation, and Pickering emulsion interfacial synthesis[54]. A fluidic nanoprecipitation system was successfully employed for the one-step fabrication of biocompatible Janus PLGA nanoparticles. These nanoparticles were designed to encapsulate both hydrophilic doxorubicin hydrochloride (DOX) and hydrophobic paclitaxel (PTX) within their distinct compartments[57]. The distinct physicochemical properties of the two compartments within Janus nanoparticles enable decoupled release kinetics of encapsulated agents governed by factors such as hydrophobic or electrostatic interactions. Hwang et al.[58] developed a multi-compartmentalized anisotropic nanostructure for the encapsulation of two oppositely charged biomacromolecules using electrohydrodynamic jetting (Fig. 5)[59]. Enrichment of hydrophilic biomacromolecules within the nanoparticles was achieved through a hydrophobic ion-pairing method, enhancing their solubility in polar organic solvents by complexation with oppositely charged polymers.

The jetting device, equipped with dual inlets for each particle half and a dispersing stream, facilitates the controlled formation of Janus nanoparticles. This advanced spray drying technique, utilizing double solvent channels, effectively addresses component incompatibility issues and shows promise for scalable production[59].

3.2.1. *Microfluidic platform*

Microfluidics, a technology enabling precise manipulation of fluids at the microscale, exploits the dominance of inertial and capillary forces within microchannels[60]. Within these miniaturized fluidic systems, flow resistance is significantly reduced, resulting in a constant flow rate that is directly proportional to the applied pressure[28] Unlike macroscale systems characterized by turbulent flow, microscale fluidic networks predominantly exhibit laminar flow. Microfluidic technology offers several advantages, including precise control over trace multiphase fluids, reduced diffusion distances within microchannels, precise control over reaction times, continuous flow capabilities, and excellent temperature controllability[61]. The precise control afforded by microfluidics extends to the fabrication of nanomedicines, enabling fine-tuning of nanoparticle properties and facilitating both sequential and parallel processing steps.

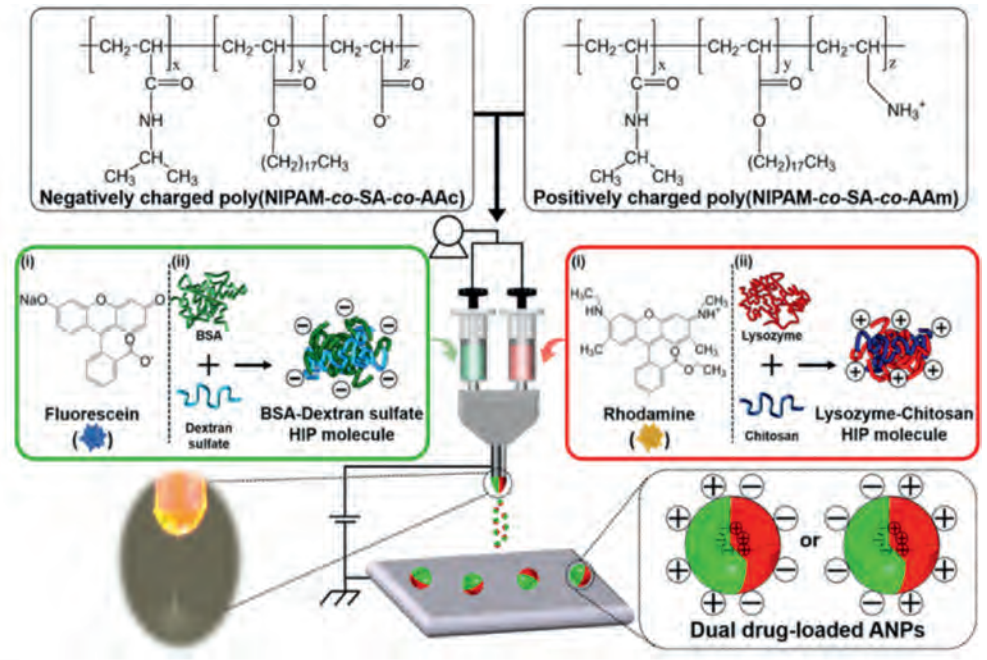

Figure 5 Schematic diagram of oppositely charged Janus nanoparticles preparation by electrohydrodynamic co-jetting consisted of a side-by-side needle geometry, the dual-drug co-delivered d anisotropic nanoparticles with decoupled drug release behavior. Reprinted with the permission from Ref. 58. Copyright © ACS applied materials & interfaces.

In contrast to conventional bulk extrusion mixing methods for nanoparticle synthesis, microfluidics offers a significantly more controlled and consistent reaction environment. This advantage stems from the rapid and controllable mixing of reagents, enabling precise manipulation of mixing sequences and reaction pathways. Consequently, microfluidics facilitates the reproducible and scalable production of nanoparticles with exceptional uniformity in size and physical properties. Table 4 summarizes the advantages and limitations associated with nanomedicine manufacturing using microfluidic devices. The widespread adoption of microfluidics in pharmaceutical nanotechnology is evident in various commercially available products. Moreover, microfluidics has played a crucial role in scaling up the production of vaccine adjuvant nanoemulsions. Pfizer's successful production of mRNA-loaded lipid nanoparticles for their COVID-19 vaccine exemplifies the scalability of microfluidic technology. Their approach utilized an impingement jet mixer, which combines lipid solvent and mRNA solution from opposing inlets under high pressure. The parallelization of these mixers enabled a remarkable production capacity of 30 million vaccine doses per day. Given the significant achievements of microfluidics in the biomedical field, this review will further delve into its feasibility and versatility, summarizing key applications in nanoparticle synthesis, drug encapsulation, and targeted delivery. Microfluidic devices for nanofabrication can be engineered using various strategies, as outlined in Table 5, which summarizes the evolution of microfluidic systems. The design of microfluidic devices is often tailored to specific nanoparticle synthesis methods. For example, nanoprecipitation, a widely used technique for producing organic nanoparticles, relies on the controlled mixing of a solvent containing the organic precursor (*e.g.*, lipids, polymers) with an antisolvent, leading to nanoparticle self-assembly through a liquid-liquid-solid process. This method encompasses variations such as rapid mixing-induced nanoprecipitation and sequential nanoprecipitation. A key challenge in nanoprecipitation is channel blockage due to premature nanoparticle precipitation. Consequently, microfluidic device design must prioritize efficient mixing to mitigate this issue.

Several microfluidic designs are commonly employed for nanoparticle synthesis, each offering unique advantages. These include hydrodynamic flow focusing (HFF), staggered herringbone micromixers (SHM), simple geometric configurations like T-shaped junctions, microfluidic platforms integrating external fields (*e.g.*, Acoustofluidics, magnetic fields), confined impinging jet mixers, and multi-inlet vortex mixers (MIVM). Achieving rapid fluid mixing necessitates incorporating specific design elements that promote turbulence or enhance diffusion. From a fluid dynamics perspective, the ideal design maintains optimal mixing efficiency even at high flow rates. Precision *NanoSystems* (Vancouver, BC, Canada) addressed this challenge by incorporating Dean vortices into their NanoAssemblr Ignite system. This innovative design leverages Dean vortices to enhance mixing efficiency, expanding the applicability of microfluidic platforms to high-throughput nanoparticle production. Since 2004, HFF has gained significant traction in producing liposomes, lipid nanoparticles, and polymeric nanoparticles with superior size uniformity and control over particle size. HFF operates under laminar flow conditions, exploiting the controlled diffusion between a stream of nanoparticle precursors in solvent and a perpendicularly introduced antisolvent stream to initiate nanoparticle formation. HFF offers remarkable tunability in nanoparticle size distribution, which can be precisely controlled by adjusting channel dimensions and flow rates. However, achieving smaller nanoparticle sizes using HFF often necessitates narrower channels and higher flow rates, potentially leading to longer processing times and diluted nanoparticle concentrations at the outlet. To address these limitations, researchers have explored alternative strategies, such as SHM or the integration of external forces (*e.g.*, acoustic or magnetic fields), to induce turbulence and achieve significantly faster mixing.

Table 4 Advantages and limitations of nanomedicine manufacture by microfluidic devices.

Advantage	Limitation
Controllable quality, more excellent uniformity	The yield of a single microfluidic reactor is low, and a large-scale parallelization design is required
Elimination of batch effect	Finite lifespan
Fabricate nanomedicines with complex structures	Limited scalability
Integration miniaturization and automation, high throughput	Require meticulous optimization of various parameters

High-throughput production *via* microfluidics: This subsection delves into the design principles of diverse microfluidic systems engineered for the scalable, high-throughput production of nanoparticles, with a focus on critical performance parameters, including throughput, QC, nanoparticle stability, encapsulation efficiency, and drug delivery efficacy (Fig. 6).

The low throughput of HFF microfluidics, a consequence of small channel dimensions, presents a significant obstacle to large-scale manufacturing. Numerous methods have been proposed to enhance throughput. For instance, optimizing the design parameters of microfluidic channels, such as channel structure, aspect ratios, and depth, has demonstrated potential for throughput enhancement. One study reported a hundredfold increase in throughput (up to 288 mg/h) for a 2D HFF device with optimized channel dimensions compared to traditional HFF devices[62]. Hood et al.[63] demonstrated a similar throughput enhancement by increasing the aspect ratio of a vertical HFF device, achieving a production rate of 96 mg/h for monodisperse liposomes. The incorporation of multiple inlet channels has also proven effective in augmenting throughput for the fabrication of polymer-lipid hybrid nanoparticles. For example, Kim et al.[64] developed a three-inlet microfluidic chip that utilized a controllable microvortex, achieving a throughput of up to 0.3 g/h. Microvortex characteristics can be precisely controlled by adjusting parameters such as the Reynolds number, flow rate, and flow rate ratios between the outer and inner solutions. This control enables tailored mixing dynamics for nanoparticle synthesis. Yang et al.[65] demonstrated this principle by fabricating D-α-tocopheryl polyethylene glycol succinate (TPGS)-PLGA hybrid nanoparticles with a high production rate using a three-inlet microfluidic chip. The chip design facilitated rapid mixing between the PLGA solvent in the inner channel and the polymer TPGS in the outer channel. Furthermore, incorporating serpentine or herringbone geometries within the microfluidic channel can substantially enhance mixing efficiency, thereby enabling high production rates. For instance, the commercially available NanoAssemblr platform utilizes a staggered herringbone micromixer for the large-scale manufacturing of nanoparticles[66]. HFF devices are commonly fabricated using polydimethylsiloxane (PDMS). However, PDMS

Table 5 The engineering of microfluidic devices.

Mode	Microfluidic reactor	Annotation
Passive mode: simply using the geometry or fluid properties to produce the mixing effect, the mixing does not rely on any external forces other than the force driving the fluid flow	T- and Y-shaped micromixers	In the simplest and most basic micromixer design, two fluids are mixed, and the diffusion of the interface in the flow channel depends on them. The limitations of T- and Y-shaped micromixers' slow mixing rate may be addressed by reducing the channel diameter, adding obstacles or roughening the channel walls, and increasing the high flow rate
	Parallel lamination micromixers	The parallel lamination micromixer subdivides the two fluids to be mixed into multiple laminations by the physical structure of the micromixer, makes them interact with each other, and finally converges into one lamination. Increasing the contact surface area between the two fluids and decreasing the diffusion length
	Sequential lamination micromixers	The series laminated micromixer means that the inlet two fluids first form a fluid flow in the horizontal state and then are vertically divided, then horizontally merged, vertically divided, and so on
	Focusing enhanced mixers	Hydrodynamic focusing is another pathway to reducing the mixing path. The central sample solution flows within the sheath of the outer fluid, which laterally constrains the internal sample flow to achieve a smaller stream and a thinner laminate width
	Chaotic advection micromixers	The chaotic convection micromixer modified the internal or external structure of the mixing microchannel in a certain form so that the fluid layer was divided, elongated, and folded. They can generate transverse flow components, which cause the interface area to increase exponentially and strengthen the mixing process
	Droplet micromixers	The droplet micromixer flows the two liquids to be mixed, and the inert liquid is sandwiched between them to prevent them from contacting in advance into an immiscible oily liquid to form a droplet, which is mixed inside the droplet. The two mixed liquids react quickly in the droplet by turbulent flow, and there is no dispersion during the flow
Active mode: with the help of magnetic force, electric force, sound field, and other external forces to achieve mixing	Electrokinetic disturbance	The fluctuating electric field is employed to induce fluidic mixing in microfluidic channels or chambers, leading to rapid stretching of fluid interfaces and agitation of the fluid stream within a highly laminar flow regime
	Magneto-hydrodynamic disturbance	The Lorentz force, resulting from coupling the electric and magnetic fields, stretches the fluidic interface to enhance the mixing rate
	Ultrasound disturbance	Another active mixing method using the sound field as an external force is ultrasonic vibration. Ultrasonic vibration mixing has high mixing efficiency, but ultrasonic can cause local temperature increase, so it is not suitable for biological samples

exhibits limited solvent compatibility and is susceptible to deformation under high-pressure conditions. In contrast, borosilicate glass offers superior solvent compatibility and demonstrates greater resilience to deformation during high-pressure operation. The feasibility of constructing intricate 3D flow geometries in borosilicate glass further enhances its suitability for HFF applications. These 3D geometries promote increased interaction between reagents and mitigate channel fouling. Lorenz et al.[67] demonstrated the superior stability and reproducibility of glass-based HFF devices compared to their polymer-based counterparts. Beyond HFF devices, glass capillaries have been integrated into other turbulent and vortex-based mixing platforms to enhance throughput and reliability[68,69]. For example, gold nanoparticles and liposomes with controlled sizes have been synthesized using co-flow glass capillary microfluidic devices[70]. The transformation of 2D HFF devices into 3D configurations has also been explored as a strategy to enhance production rates. This approach leverages the inherent advantages of 3D structures, which minimize deposition and channel clogging[71]. In one such study, an eight-channel 3D HFF device was developed for the production of PLGA-polyethylene glycol (PEG) nanoparticles[72]. The parallel production capability of the eight-channel device facilitated a high production rate of 84 mg/h, while the 3D architecture ensured high reproducibility and controllability. Similarly, Chen et al.[73] reported a liposome production rate of up to 240 mg/h using a single 3D HFF device. Hood et al.[74] achieved significant throughput enhancement by developing a 3D glass multicapillary HFF array, which comprised seven small capillaries embedded within a larger capillary. This modified 3D HFF device yielded a production rate of 97 mg/h, demonstrating a substantial improvement over the control 2D device, which produced only 0.04 mg/h. Beyond the HFF device, 3D flow geometry has proven effective in other platforms, such as polyimide film microreactors. These devices, composed of seven layers of hydrophobic and non-stick fluoropolymer-coated polyimide film, have demonstrated the ability to generate PLGA-PEG nanoparticles at 10 g/h while mitigating particle aggregation and adsorption on the channel surface[75]. The efficacy of 3D structures in mitigating clogging, enhancing throughput, and improving solvent compatibility has been demonstrated in other tubing microfluidic devices[76,77]. Beyond structural, material, and geometric considerations in microfluidics, the integration of acoustic flow presents a compelling avenue for

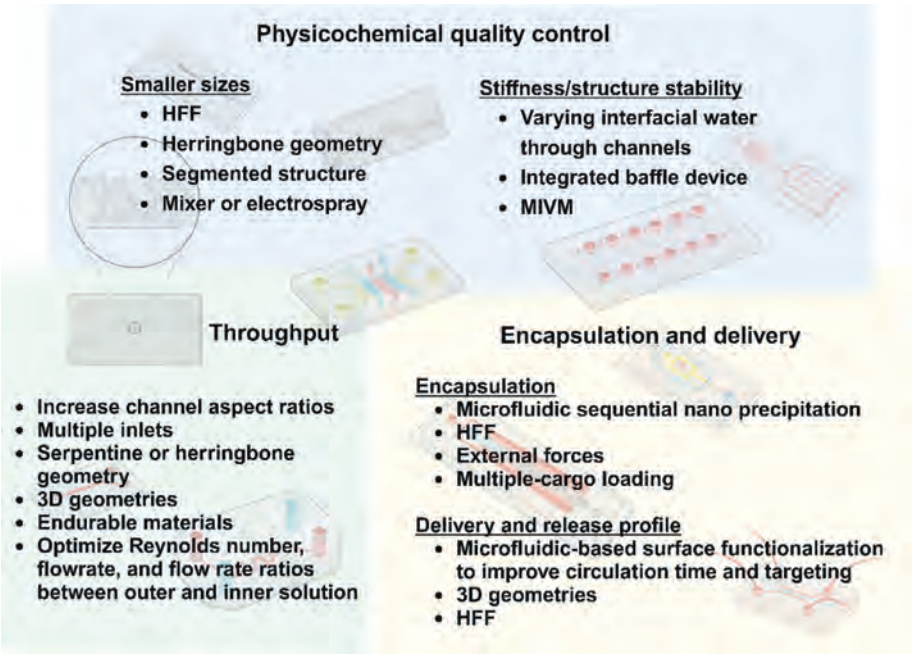

Figure 6 Overview of the strategies of high-throughput microfluidic nanoparticle manufacturing. Three subsections were discussed: throughput, physiochemical quality control, and encapsulation and delivery.

enhancing production rates. Huang et al.[78] demonstrated this principle by employing a piezoelectric actuator to induce an actuating frequency that effectively disrupted the laminar flow regime, thereby achieving rapid mixing of two solutions. The high driving voltage (55 Vpp) enabled the maintenance of uniform, small particle sizes even at high flow rates, thereby facilitating a significantly higher production throughput. Furthermore, the microfluidic device is amenable to parallel operation with other modules, enabling high-throughput nanoparticle production. Previous work by our group employed a microfluidic electroporation approach, utilizing a combination of nano- and millisecond pulses to achieve large-scale EVs generation. This method yielded a substantial increase in EVs production of 45-fold in mouse embryonic fibroblasts and 32-fold in human embryonic kidney 293T cell lines (relative to controls)[79].

Physicochemical property control of nanoparticles production: HFF has been shown to produce nanoparticles with significantly greater size uniformity and smaller average diameters compared to conventional bulk synthesis methods[80,81]. Othman et al.[82] demonstrated that HFF devices yield nanoparticles with smaller average sizes compared to co-flow devices utilizing nanoprecipitation. The integration of electrospray techniques with HFF devices has been shown to further minimize nanoparticle size distribution[83]. Furthermore, studies employing SHM have reported the production of lipid nanoparticles with diameters around 100 nm and a narrow size distribution[84,85]. Segmented flow microfluidic reactors offer superior size control compared to laminar or continuous flow microreactors. This advantage stems from their ability to eliminate axial dispersion, ensuring uniform residence times for all segmented batches[86,87]. Adding a mixer is another method to reduce particle size. For instance, Hibino et al.[88] incorporated a baffled mixer into microfluidics to produce homogenous small-sized liposomes. Beyond size control, microfluidic platforms offer precise control over a range of nanoparticle properties, including compactness, rigidity, structure, and uniformity. Chitosan nanoparticles synthesized using HFF devices exhibited greater uniformity and a more spherical morphology compared to those synthesized *via* conventional bulk methods, which often exhibit significant heterogeneity[81]. Furthermore, Martins et al.[89] further reported that microfluidic synthesis yielded nanoparticles exhibiting a less negative zeta potential while maintaining comparable polydispersity to those produced *via* bulk methods. Stability represents a critical consideration in drug delivery systems. An integrated baffle device has been developed to facilitate the rapid dilution of the post-formulation solvent, enhancing stability by mitigating Ostwald ripening (a process leading to lipid fusion and aggregation). This rapid dilution minimizes aggregation, thereby promoting stability[90,91]. Studies employing microfluidic systems with independently controlled inlets (*e.g.*, MIVMs) have demonstrated enhanced lipid stability. These systems facilitate flash nanoprecipitation and enable precise control over saturation levels and product formulation by independently adjusting flow rates[92,93].

Drug encapsulation: Microfluidic platforms have emerged as promising tools for the production of nanoparticles with enhanced drug-loading capabilities. Employing the HFF device, Hasani-Sadrabadi et al.[94] successfully fabricated PTX-loaded nanoparticles *via* self-assembly. This approach yielded nanoparticles exhibiting a 9.9% loading efficiency, uniform sizes below 200 nm, and a sustained release profile. Furthermore, the integration of external forces with microfluidic systems has demonstrated potential for enhancing drug loading efficiency[95]. Zeng et al.[83] reported the successful fabrication of PTX-loaded polymer nanoparticles exhibiting an encapsulation efficiency exceeding 90% and a drug loading of 7.5% through the application of an electric field within a microfluidic chip. Moreover, microfluidic platforms offer the capability for multiple-cargo loading, a feature that holds significant promise for improving therapeutic efficacy. As a notable example, HFF devices have been employed to encapsulate PTX, along with PLGA chains conjugated with

radioactive bisphosphonate tracer molecules, into superparamagnetic iron oxide nanoparticles. This multifaceted system enables targeted cancer drug delivery, MRI-based diagnosis, and hyperthermia treatment[96]. Valencia et al.[97] demonstrated further advancements in this area by conjugating cisplatin to the backbone of a polymer and subsequently loading irinotecan into the resulting polymer nanoparticles using a microfluidic device. This method yielded nanoparticles with a remarkably low PDI, attributed to the rapid mixing capabilities of microfluidic systems.

Microfluidic platforms have further exhibited significant advantages in the precise manipulation of nanoparticle surface chemistry. This level of control has profound implications for optimizing nanoparticle pharmacokinetics, biodistribution, and drug release profiles[91,92]. Numerous studies have explored the use of microfluidics for nanoparticle surface modification to enhance therapeutic efficacy. For instance, PEGylation, achieved through microfluidic methods, has been shown to effectively prolong nanoparticle circulation time. In a notable study by Han et al.[98], nanoparticles were coated with natural cellular membranes using a novel approach that combined microfluidic sonication with cholesterol-modified aptamer functionalization. This innovative technique resulted in a significant extension of nanoparticle circulation time. Moreover, HFF devices have proven particularly effective in the fabrication of siRNA-loaded nanoparticles. These nanoparticles exhibit prolonged blood circulation time, enhanced bloodstream stability, and superior anti-tumor efficacy compared to those prepared using conventional bulk mixing methods[99,100]. The targeted delivery of nanoparticles can be further enhanced through the microfluidic-assisted decoration of their surfaces with ligands or other targeting moieties. For example, an HFF device was employed to achieve the dual-coating of polymer nanoparticles with hyaluronic acid and folic acid, resulting in enhanced targeting specificity[101]. In a separate study, HFF devices facilitated the fabrication of liposomes decorated with either single or dual ligands. Subsequent screening of various ligand combinations revealed that the combination of folic acid and the cell-penetrating peptide, transactivating transcriptional activator (TAT), yielded the highest targeting efficiency[102].

The ability to sustain a controlled drug release profile represents a critical aspect of effective drug delivery systems. In an innovative approach, Baby et al.[103] employed a 3D tubing microfluidic device to synthesize curcumin-loaded shellac nanoparticles. The shellac matrix exhibits pH−responsive properties, effectively retaining curcumin for a minimum of 10 days under acidic conditions (pH 4.5) while facilitating its release at a neutral pH. Previous research has demonstrated that specific nanoparticle formulations, particularly chitosan-based nanoparticles synthesized using HFF devices, exhibit superior sustained release profiles compared to those produced *via* conventional bulk methods[104]. Furthermore, the ability of microfluidic synthesis to precisely control nanoparticle compactness presents a significant advantage, as compact nanocarriers have been shown to exhibit enhanced long-term circulation stability[105]. In one study, researchers employed an HFF device to encapsulate PTX within nanoparticles, achieving a low−release profile at a relatively neutral pH[106]. The development of pH stimuli-responsive drug delivery systems is of paramount importance in various therapeutic applications. In this context, researchers have successfully utilized a Tesla micromixer to coat nanoparticles produced by an HFF device with a pH-sensitive layer. This innovative approach resulted in enhanced cellular uptake in response to pH changes compared to nanoparticles prepared using traditional bulk methods[107].

Commercialization and future perspectives of microfluidic devices: commercially available microfluidic platforms for nanoparticle production typically consist of pumps, syringes, disposable chips, and interface controls, often designed for modularity and ease of use through plug-and-play chip replacement. A notable example is the NanoAssemblr platform, which facilitates the scalable manufacturing of liposomes and polymeric nanoparticles through the utilization of parallelized microfluidic units. Pfizer has demonstrated the scalability of microfluidic technology by replicating the impingement jet mixer design, incorporating up to 100 static mixers for the parallelized and high-throughput production of mRNA vaccines. Despite these advancements, the widespread adoption of microfluidic systems for commercial nanoparticle manufacturing is hindered by the relatively high cost of these platforms. While numerous proof-of-concept microfluidic chips have been developed in academic settings, their translation to commercial manufacturing faces significant hurdles, including stringent regulatory requirements for scaling up from laboratory to industrial production. These requirements encompass the establishment of pilot manufacturing facilities, adherence to specialized GMP guidelines, comprehensive equipment characterization and integration, and rigorous quality assessment and control protocols for therapeutic nanomedicine products. The inherent characteristics of microfluidic systems, particularly their small length scales and rapid reaction times, pose significant challenges for implementing effective QC measures. Consequently, the development of novel automated systems specifically designed for QC in microfluidic manufacturing is crucial. Furthermore, advancements in chip manufacturing processes are essential to meet the growing demand for microfluidic chips. The development of more affordable and standardized chips is paramount, and 3D printing has emerged as a promising technology for achieving this goal. 3D printing offers the capability for integrated chip fabrication, enabling the incorporation of diverse materials and complex structures. Integrating 3D printing with robust quality assessment systems has the potential to significantly improve chip reproducibility, minimize batch-to-batch variations, and facilitate the development of personalized nanomedicine formulations. In conclusion, while microfluidic platforms offer compelling advantages for nanoparticle synthesis in drug delivery applications compared to conventional bulk mixing methods, their widespread commercialization necessitates further investment in areas such as cost reduction, standardization, QC, and scalable manufacturing processes.

3.2.2. DNA origami

DNA origami has emerged as a powerful strategy for the "Bottom-Up" fabrication of nanostructures, offering precise control over size within a range spanning tens of nanometers to submicrometers[108]. Beyond its role as genetic material, DNA serves as a versatile building block capable of self-assembly through the predictable and specific base pairing dictated by the Watson-Crick principle[109]. This inherent property enables the efficient and precise construction of DNA nanostructures through the controlled hybridization of complementary sequences[110]. Various methods exist for creating DNA nanostructures of diverse complexities. One approach involves the self-assembly of DNA or RNA tiles or bricks from single strands, potentially leading to the formation of hierarchical structures[111]. Alternatively, DNA origami nanostructures can be created by folding a long, single-stranded DNA scaffold strand into a desired shape using complementary synthetic "staple" strands, often derived from the

M13 bacteriophage genome[112]. Compared to tile-based assembly, DNA origami synthesis offers greater robustness, higher yields, and the ability to generate complex, non-periodic shapes. This advantage stems in part from the intricate interplay between multiple scaffolds and staple strands during the origami folding process. DNA origami has advanced to enable the synthesis of virtually any arbitrary 1D to 3D shape, including those with user-defined asymmetry, cavities, and curvatures[113]. Recent advancements in DNA origami have led to the realization of dynamic structures, single-stranded origami, and the hierarchical assembly of supramolecular architectures. Sophisticated software tools such as SARSE-DNA[114,115], caDNAno[116], and CanDo[117] have been developed to facilitate the design of increasingly complex 2D and 3D DNA nanostructures. The actual construction of DNA nanostructures is typically straightforward. It often involves simply mixing the designed DNA strands and subjecting them to annealing.

In recent years, growing interest has been in exploring DNA origami structures as promising carriers for drug delivery applications. DNA's inherent biodegradability and minimal cytotoxicity in low doses make it a highly attractive material for drug delivery vehicles. Furthermore, the versatility of DNA in interacting with therapeutic molecules through various mechanisms, such as intercalation, base pairing, and covalent binding, has enabled the successful loading of these molecules into DNA origami carriers[118]. A key advantage of DNA origami structures lies in their ability to provide designated cavities or internal regions that act as secure docking sites for therapeutic payloads, effectively shielding them from premature degradation or interactions with the surrounding biological environment[119]. The exceptional flexibility and uniformity of DNA origami structures contribute to their ability to selectively penetrate specific biological barriers based on their *in vivo* distribution patterns[120]. This targeted localization capability further enhances their potential for controlled drug delivery, enabling researchers to precisely direct therapeutic agents to specific tissues or cells[121]. The versatility of DNA origami as a drug delivery platform is evident in its successful application in delivering a wide range of therapeutic payloads, including inorganic nanoparticles, small-molecule chemotherapeutics, proteins, gene molecules, and their combinations. These DNA origami-based systems have shown promise in various therapeutic areas such as photoacoustic imaging, immunology, gene therapy, chemotherapy, and photothermal therapy[122].

Despite the remarkable progress in developing multifunctional DNA origami nanoplatforms for drug delivery, several challenges remain before their widespread *in vivo* application can be realized. A deeper understanding of the systemic pharmacokinetics of DNA origami nanocarriers is crucial. This includes elucidating the influence of size and shape on their internal circulation, biodistribution, metabolism, and overall fate within the body[123]. Equally important is a thorough investigation of the endocytosis mechanisms by which different DNA origami nanocarriers enter cells and their subsequent intracellular trafficking and fate. Furthermore, careful consideration must be given to potential immune responses elicited by these exogenous DNA-based materials[124]. Despite these challenges, the inherent programmability and versatility of DNA origami, particularly its potential for personalized medicine, position it as a powerful contender in the field of drug delivery. With continued advancements in DNA origami nanotechnology, we envision the development of increasingly sophisticated and intelligent nanoplatforms capable of revolutionizing disease therapy and diagnosis.

3.2.3. 3D printing

3D printing technology has witnessed remarkable advancements, enabling the fabrication of objects with intricate geometries from a wide range of materials, including polymers, metals, and ceramics, through a layer-by-layer deposition process[125]. While seven distinct 3D printing technologies exist, each with its own advantages and limitations, not all are equally suitable for pharmaceutical applications, particularly in the context of drug delivery and personalized medicine[126]. Among these, the convergence of nanotechnology and 3D printing has emerged as a particularly promising avenue for revolutionizing drug delivery[127]. Two primary approaches have been explored: (1) directly printing nanostructures embedded with therapeutic agents, leveraging the unique properties of nanomaterials to enhance drug efficacy, and (2) incorporating drug-loaded nanocarriers onto the 3D-printed scaffold either during or after the printing process[128]. This synergistic combination empowers the creation of innovative drug delivery systems, including personalized medicines tailored to individual patient needs, novel dosage forms, and drug formulations with enhanced therapeutic profiles.

Doxorubicin, a widely used chemotherapy drug, has shown particular promise in conjunction with 3D printing and nanotechnology for targeted cancer therapy[127]. Zhang et al.[129] demonstrated this potential by fabricating 3D-printed scaffolds composed of mesoporous bioactive glass and polycaprolactone (PCL) embedded with magnetic nanoparticles and doxorubicin. These scaffolds, with a porosity of 60%, not only provided structural support for bone tissue regeneration (as evidenced by their ability to stimulate hBMSC differentiation and proliferation *in vitro*) but also enabled localized hyperthermia therapy through the magnetic nanoparticles. Similarly, another study explored titanium alloy implants created using selective laser melting, incorporating titanium nanotubes on their surface for drug delivery. These nanotubes were loaded with both doxorubicin and an apoptosis-inducing ligand, aiming to enhance the cytotoxic effect on cancer cells. *In vitro* assessments confirmed the implant's biocompatibility, as evidenced by its ability to support cell adhesion and proliferation, suggesting good osseointegration potential. Furthermore, the implant demonstrated significant anticancer activity, reducing tumor cell viability by 16.4%, highlighting its capacity for effective drug release and localized cancer treatment[130]. Ahangar et al.[131] presented another innovative approach using nanoporous polymer disks loaded with doxorubicin for treating bone metastasis. This strategy aimed to provide both localized chemotherapy and structural support for bone replacement. *In vitro* studies demonstrated sustained drug release over seven days and significant inhibition of metastasis cell migration, metabolism, proliferation, and growth, underscoring the therapeutic potential of this approach.

Beyond doxorubicin, the synergy of 3D printing and nanostructured materials has paved the way for novel drug delivery systems incorporating various other antitumor agents. For instance, inkjet printing was employed to fabricate bioadhesive films composed of hydroxypropyl cellulose, incorporating both PTX complexed with cyclodextrins for sustained release and cidofovir-encapsulated PEG-PCL nanoparticles for targeted delivery. These films exhibited a biphasic drug release profile, with the majority of PTX released within 8 h and cidofovir released over a more extended period of 16 h. This controlled release system effectively reduced HeLa cell viability by 35% while demonstrating good biocompatibility with fibroblast cell lines, highlighting its therapeutic potential[132]. In another study,

researchers developed 3D-printed scaffolds composed of hydrophobically modified silica nanoparticles and PCL for osteosarcoma treatment, integrating ruthenium-loaded PEGylated liposomes as a novel drug delivery mechanism. Liposome integration was achieved by soaking the scaffolds for 3 h, allowing for efficient loading within the scaffold structure. *In vitro* studies demonstrated the scaffold's ability to induce apoptosis in osteosarcoma cells, likely through the induction of mitochondrial dysfunction. This apoptotic effect was attributed to a biphasic drug release profile, with an initial burst release of liposomes from the scaffold surface followed by a sustained release of liposomes embedded deeper within the scaffold over 48 h[133].

3.2.4. Engineering cell-derived nanoplatforms

EVs are a heterogeneous population of naturally occurring, membrane-bound vesicles secreted by cells and tissues across a wide range of organisms[134]. These vesicles play a crucial role in intercellular communication, acting as messengers by transporting bioactive molecules, such as proteins, lipids, and nucleic acids, between cells. Their involvement in diverse physiological and pathological processes has sparked significant interest in their potential for therapeutic, diagnostic, and prognostic applications[135]. Among the different subtypes of EVs, exosomes, ranging in size from 40 to 200 nm, are of particular interest. These nanosized vesicles originate from endosomes and are released into the extracellular space through the fusion of multivesicular bodies with the plasma membrane.

EVs have emerged as promising therapeutic delivery vehicles, offering several key advantages over conventional synthetic nanocarriers. Their inherent biocompatibility, stemming from their natural origin, minimizes toxicity and immune responses, as evidenced by clinical studies, making them inherently safer than their synthetic counterparts[136]. Furthermore, EVs can overcome multiple drug resistance mechanisms often encountered by other nanocarriers. Their ability to efficiently transfer functional proteins and RNAs to recipient cells allows them to modulate drug resistance phenotypes, enhancing therapeutic efficacy[137]. EVs also exhibit inherent targeting capabilities attributed to their unique membrane protein and lipid composition. These surface molecules can interact with specific receptors on target cells, facilitating precise delivery and enhancing therapeutic efficacy[138]. Their ability to cross the BBB, a significant obstacle for many drugs and drug delivery systems, positions them as particularly promising vehicles for treating brain disorders[139]. Moreover, EVs demonstrate remarkable stability in physiological fluids, even under pathological conditions, ensuring the protection and integrity of encapsulated therapeutic agents during circulation and release[140].

The numerous advantages of EVs have propelled them to the forefront of drug delivery research, leading to a surge in clinical trials and significant interest from the pharmaceutical industry. Clinical trials have demonstrated the therapeutic potential of EVs across various cancer types. For instance, dendritic cell (DC)-derived EVs have shown promise in treating colorectal cancer, demonstrating a T-cell-mediated antitumor effect and successfully completing phase II clinical trials[141]. Similarly, DC-derived exosomal vaccines delivering melanoma antigens are currently being evaluated in Phase I clinical trials for patients with non-small cell lung cancer and metastatic melanoma[142]. Beyond cancer, MSC-derived EVs have also shown potential in treating viral infections, with a phase I clinical trial for COVID-19 already completed and further trials actively recruiting[143]. The pharmaceutical industry has taken note of the therapeutic potential of EVs, with several companies actively pursuing EVs-based therapies. Codiak Biosciences, for example, is currently conducting phase I and II clinical trials for ExoIL-12 and ExoSTING, respectively. These EVs-based therapies target early-stage cutaneous T-cell lymphoma and advanced solid tumors by leveraging the immune-modulating properties of IL-12 and STING agonists[144,145]. Similarly, MD Anderson Cancer Center is exploring engineered EVs loaded with KRAS-G12D siRNA to target cancer cells harboring KRAS mutations[146]. Beyond these examples, numerous other companies, including Anjarium Biosciences and STRM.BIO is developing innovative EVs-based therapies, highlighting the growing investment and rapid pace of development in this field[147,148]. Notably, Eli Lilly's recent investment further underscores the increasing recognition of EVs' therapeutic potential[148].

Despite the remarkable progress of EVs in pre-clinical and clinical settings, several challenges hinder their widespread clinical translation. These challenges primarily revolve around scalable and reproducible manufacturing, encompassing aspects such as large-scale production, efficient drug loading, streamlined downstream processing, precise dose control, and rigorous quality assurance (QA) and QC. To address these manufacturing hurdles, researchers are actively exploring innovative strategies, broadly categorized as "Top-Down" and "Bottom-Up" approaches. The "Top-Down" strategy centers on manipulating endogenous cellular processes through genetic engineering to enhance EVs production or modify their inherent properties. In contrast, the "Bottom-Up" strategy entails exogenous physicochemical interventions, utilizing techniques like microfluidics or nanofabrication to assemble EVs-like nanoparticles with tailored functionalities.

Given the limited availability of natural EVs, there is increasing research interest in developing artificial EVs. This research primarily focuses on "Top-Down", "Bottom-Up" and combinative techniques. "Top-Down" strategies involve the generation of artificial EVs through the manipulation of cell membranes[3], for example, *via* extrusion, nitrogen cavitation, sulfhydryl-blocking, and exposure to alkaline solutions. Conversely, "Bottom-Up" approaches center on assembling fully synthetic EVs from their constituent biomolecules for various biomedical applications[149]. Biohybrid strategies offer a third approach, enabling the fabrication of hybrid EVs by fusing natural EVs with synthetic nanoparticles while preserving the intrinsic characteristics of both components.

Large-scale production of EVs: while EVs are produced by nearly all cell types, they exhibit significant biological heterogeneity and vary in quantity. Therefore, adopting a "Top-Down" strategy is crucial for screening donor cells capable of robust EVs production, as the genomic differences among donor cells significantly influence both the secretion efficiency and biological function of the resulting EVs.

Tumor cells are known to release EVs in greater abundance compared to other cell types, and certain tumor antigens carried by these tumor-derived EVs can elicit immune responses. However, their clinical application remains controversial due to the paradoxical effects of tumor-derived EVs, which have been shown to promote angiogenesis and contribute to tumor metastasis[150]. Promising candidates for EVs-based therapies include DCs, natural killer (NK) cells, B/T lymphoma cells, neutrophils, chimeric antigen receptor (CAR)-T cells, macrophages, and mast cells, owing to their inherent tumor-suppressing properties[151]. For example, EVs derived from DCs are enriched with major histocompatibility

complex (MHC) molecules, which are subsequently internalized by cytotoxic T lymphocytes, leading to the activation of anti-tumor immunity[152]. Moreover, DC-derived EVs inherit communication functions from their parent cells, enhancing antigen presentation and promoting a more robust cytotoxic response for tumor elimination[153]. Similarly, EVs derived from NK cells exhibit anti-tumor activity by delivering perforin, a protein known to induce cell death[154]. However, the limited accessibility of these cell types poses a significant challenge for their large-scale production. Mesenchymal stem cells (MSCs) represent another potential EVs source, particularly for applications in tissue repair, bone regeneration, and inflammation modulation[155]. Research efforts have focused on overcoming the limited expansion capacity of MSCs, potentially paving the way for their scalable production. Adherent cells, such as those previously mentioned, generally exhibit lower scalability compared to suspension cells due to their growth space dependency[156]. HEK293, an immortalized cell line characterized by high growth capacity and suspension culture adaptability, has garnered considerable interest for therapeutic applications. This cell line has been extensively utilized in biopharmaceutical production for over three decades and has been successfully adapted to serum-free media, facilitating large-scale production and downstream processing[157]. Furthermore, the FDA approval of several biopharmaceuticals produced using HEK293 cells underscores their biosafety for clinical-grade biological production. However, concerns remain regarding the potential of HEK293-derived EVs to promote tumor metastasis due to the immortalized nature of the HEK293 cell line[158]. Further research is warranted to mitigate these potential risks.

Beyond leveraging the inherent production capacity of cells, "Bottom-Up" approaches employing exogenous physicochemical stimulation offer an alternative means to enhance EVs production. For instance, modifying the cell culture medium or incorporating specific stimulators represents a viable strategy for enhancing EVs production independent of the cell source[159]. Studies have demonstrated that serum-free media, characterized by reduced nutrient availability, can stimulate EVs secretion[160]. Similarly, the addition of non-toxic sodium iodoacetate (IAA, a glycolysis inhibitor) and 2,4-dinitrophenol (DNP, an oxidative phosphorylation inhibitor) has been shown to enhance EVs secretion by 3- to 16-fold[161]. Furthermore, treating MSCs with a combination of *N*-methyldopamine and norepinephrine resulted in a threefold increase in EVs production without affecting the inherent anti-inflammatory, pro-angiogenic, or collagen-suppressing properties of the MSCs[162]. Additionally, 45S5 Bioglass® (BG) has been shown to enhance EVs production in MSCs by upregulating the expression of nSMase2 and Rab27a, proteins implicated in the EVs biogenesis pathway[163].

Furthermore, the choice of culture platform significantly influences EVs production efficiency. For example, 3D spheroid cultures have demonstrated superior EVs production efficiency compared to traditional monolayer cultures, particularly in the context of MSCs[164]. Moreover, bioreactor systems, such as hollow fiber and stirred tank bioreactors, which impose controlled shear stress, have been reported to enhance EVs secretion compared to static or low-shear culture systems like shake flasks, spinner flasks, and roller bottles[165]. The utilization of large-scale bioreactors, rather than shake flasks, aligns well with GMP regulations. In summary, large-scale EVs production is achievable using cell sources such as HEK293 cells, MSCs, and other cell types with established biopharmaceutical applications. However, optimizing various factors, including cell culture media composition, bioreactor design, and operation parameters, remains crucial for maximizing both EVs yield and drug loading capacity.

Loading efficiency of EVs: Drug loading into EVs is primarily achieved through two main strategies: exogenous loading ("Bottom-Up") and endogenous loading ("Top-Down")[166]. Exogenous loading, also referred to as direct loading, entails the incorporation of therapeutic agents into pre-formed EVs. Commonly employed techniques for exogenous loading include incubation, freeze−thaw cycles, sonication, extrusion, membrane permeabilization, dialysis, and electroporation[167]. For instance, co-incubation has been successfully employed to load the chemotherapeutic drug PTX into EVs, resulting in enhanced cytotoxic effects against autologous prostate cancer cells[168]. Similarly, freeze−thaw cycles have proven effective in loading doxorubicin into EVs[169]. However, these methods often suffer from limitations such as inefficient loading capacity and restricted cargo selectivity, primarily due to reliance on passive diffusion and potential cargo aggregation issues[170]. To address these limitations and enhance loading capacity, physical methods like sonication, extrusion, membrane permeabilization, and electroporation have been explored[171]. For example, sonication has been utilized to successfully load oncogenic siRNAs into EVs without inducing aggregation, and these siRNA-loaded EVs demonstrated potent tumor growth inhibition by targeting HER2 knockdown[172]. For instance, saponin-assisted surface permeabilization has been successfully employed to load hydrophilic porphyrins into EVs, achieving an 11-fold increase in drug loading capacity and a 60% enhancement in cellular uptake. Electroporation represents another widely used method for loading therapeutic agents into EVs by transiently permeabilizing their membranes through the application of an electric field[167]. However, conventional bulk electroporation methods often suffer from low encapsulation efficiency due to potential damage to the EVs membrane structure[173]. Consequently, optimized electroporation protocols involving adjustments to voltage, pulse duration, pulse interval, and buffer composition have been developed to enhance EVs engineering efficiency[174]. For example, studies optimizing electroporation parameters identified that settings of 750 V and ten pulses resulted in substantial miRNA (miR-31 and miR-451a) loading into EVs, leading to enhanced apoptosis in hepatocellular carcinoma (HepG2) cell lines[175]. In another study, KRAS-G12D siRNA was successfully loaded into isolated EVs using the Gene Pulser Xcell Electroporation System, and these KRAS-G12D siRNA-loaded EVs, in conjunction with CD47 peptide modification, demonstrated significant tumor growth inhibition in pancreatic cancer mouse models[176]. The incorporation of protective buffers during electroporation can further enhance EVs stability and loading efficiency[177]. Zhang et al.[178] reported a modified calcium chloride-mediated transfection method for loading miRNAs into isolated EVs, demonstrating enhanced miRNA uptake by recipient cells. While physical methods have significantly improved the encapsulation efficiency of direct loading compared to passive methods like incubation and freeze−thaw cycles, they can potentially compromise EVs membrane integrity. Although some membrane recovery is possible, this can result in reduced EVs recovery rates and suboptimal cargo loading efficiencies[179]. Furthermore, the choice of loading method can influence the bioactivity of the encapsulated therapeutic cargo. Additionally, direct loading methods often exhibit limitations in encapsulating larger molecules, such as mRNAs[180].

Endogenous loading, also referred to as cell-based drug loading, involves the incorporation of therapeutic agents into EVs

during their biogenesis within donor cells[181]. A common approach for endogenous loading involves incubating donor cells with the desired therapeutic agents[182]. For instance, one study demonstrated the successful loading of PTX into microvesicles secreted by bone marrow stromal cells following co-incubation with PTX[183]. A chemotherapeutic agent may induce EVs carrying damage-associated molecular patterns (such as Hsp70) secretion to active innate immune responses. Spatial localization of biomolecules in EVs also affects the biological efficacy, myeloma-derived exosomes engineered with membrane-bound Hsp70 efficiently stimulated DCs maturation, $CD8^+$ CTL- and NK-mediated antitumor immunity than cytoplasmic Hsp70 loaded exosomes[184]. Hsp70/Bag-4 membrane expression pattern was correlated with varying capacities to export these molecules onto the surface of exosomes[185]. However, this co-incubation approach can lead to variable and often suboptimal loading efficiencies, as the incorporation of substances into EVs is influenced by their inherent properties, including zeta potential, charge, and hydrophobicity/hydrophilicity[186]. Physical methods such as inducing cellular stress or transfection have been explored to enhance intracellular loading efficiency[171]. Various cellular stresses, including hypoxia, heat shock, and nutrient deprivation, have been shown to stimulate EVs secretion[187]. For example, EVs derived from cells cultured under hypoxic conditions are enriched in functional miRNAs that target angiogenesis[188]. However, inducing cellular stress has demonstrated limited efficacy in loading exogenous substances into EVs. Transfection, on the other hand, has emerged as a more effective method for loading exogenous cargo into donor cells for subsequent EVs encapsulation[189]. For example, Ohno et al.[190] demonstrated that transfecting donor cells with oligonucleotides encoding Let-7a miRNA resulted in the production of EVs enriched in Let-7a. These EVs effectively targeted epidermal growth factor receptors by engineering fused GE11 peptide, and suppress tumor growth. In addition to vector-based or lipofectamine-mediated transfection, electroporation has emerged as a promising strategy for loading cargo, particularly large molecules like mRNAs and transmembrane proteins, into EVs during their biogenesis[191]. Electroporation also offers the advantage of stimulating substantial EVs production during transfection, facilitating the generation of large quantities of EVs loaded with therapeutic cargo[167]. Despite these advantages, challenges remain in optimizing endogenous loading strategies. Precisely quantifying encapsulation efficiency and loading capacity remains difficult, posing challenges for dose control. Co-incubation and chemical transfection methods can result in low purity of therapeutic EVs and potential cytotoxicity. Electroporation for EVs, while effective, can compromise EVs membrane stability and integrity, potentially reducing EVs yield[192]. Therefore, the development of versatile EVs engineering platforms, coupled with efficient downstream purification methods and robust analytical tools, is crucial for advancing the large-scale manufacturing of therapeutic EVs.

QA and QC of EVs: The development of scalable EVs production for therapeutic applications necessitates isolation methods that are reproducible, efficient, scalable, and cost-effective (Fig. 7)[193]. Crucially, the isolated EVs should exhibit high purity and yield while retaining their biological integrity. Two commonly employed downstream processing methods in the pharmaceutical industry are continuous ultracentrifugation and tangential flow filtration (TFF)[194]. Continuous ultracentrifugation systems can process relatively large sample volumes, up to 8 L per rotor. However, effectively operating these systems requires technical expertise. Moreover, the high centrifugal forces (*e.g.*, $100{,}000\times g$) employed can compromise EVs membrane integrity and result in lower recovery rates, limiting the widespread adoption of this method[195]. TFF, a crossflow filtration method that avoids the formation of a filter cake, is particularly well-suited for large-scale EVs production. TFF encompasses two primary filtration modes: ultrafiltration and diafiltration. Ultrafiltration concentrates the sample into a smaller volume, while diafiltration further purifies the sample through buffer exchange. During TFF, EVs are retained within the system based on their size, typically using a membrane with a molecular weight cutoff of 300 kDa,

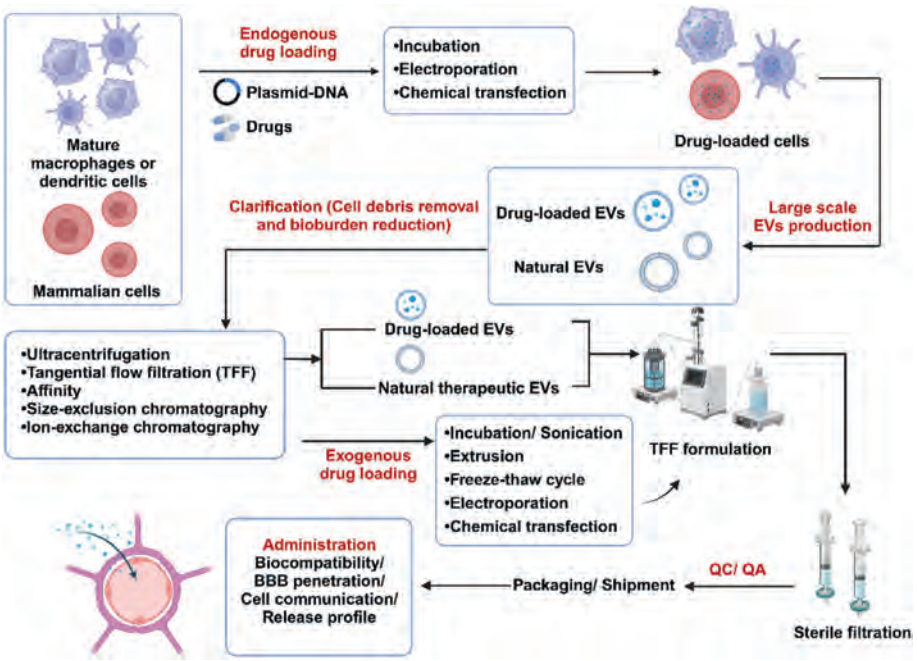

Figure 7 Manufacturing approaches to produce and deliver exosomal drug formulations and their advantages.

while smaller contaminants pass through into the waste stream. However, TFF presents a trade-off between flow rate and EVs recovery. High flow rates can generate shear stress that may damage the fragile lipid bilayer membranes of EVs, while low flow rates, though gentler, result in impractical processing times that can lead to degradation of encapsulated cargo, particularly sensitive molecules like mRNA[196,197].

Downstream processing of EVs faces additional challenges, including differentiating between EVs containing cargo and those that are empty, as well as separating EVs from similarly sized contaminants like lipoproteins. Distinguishing between cargo-laden and empty EVs poses a significant obstacle, as they often exhibit minimal differences in size, density, and isoelectric point. Effectively removing lipoproteins and other similarly sized contaminants is crucial for minimizing potential side effects. Membrane affinity and immunoaffinity techniques, leveraging antigen−antibody interactions, offer promising avenues for addressing these challenges[180]. However, elution following affinity-based purification often requires harsh conditions, such as extremely low pH, which can compromise EVs structural integrity. Furthermore, residual antibodies bound to EVs can interfere with EVs-target interactions, leading to steric hindrance[198]. Current EVs isolation and purification techniques often fall short of the efficiency required for large-scale therapeutic applications. Therefore, exploring alternative methods capable of high-throughput processing, effective cargo preservation, high yield, and accurate differentiation between cargo-laden and empty EVs is paramount for advancing EVs-based therapeutics.

EVs, unlike commercially available nanoparticles, recombinant Adeno-associated viruses (AAV), and other drug delivery systems, exhibit complexities in size, composition, loading capacity, encapsulation efficiency, and drug release kinetics. These complexities stem from their intricate intercellular biogenesis[199]. In particular, the amount of exosomal mRNA encapsulated at different times after transfection varies widely, making precise control of mRNA encapsulation unattainable[200]. This variability in mRNA encapsulation poses a significant challenge for achieving precise control over drug loading. Furthermore, the lack of distinct demarcation between EVs containing cargo and empty EVs hinders efficient downstream separation, potentially leading to the administration of high doses and subsequent side effects.

While the use of EVs as drug carriers holds significant promise, substantial challenges must be addressed before large-scale manufacturing can be realized. The development of systematic QA/QC standards is crucial. These standards must address large-scale production, high encapsulation efficiency, high-throughput downstream processing, empty-full differentiation, dose control, and other relevant pharmaceutical criteria to facilitate the clinical translation of EVs-based therapies.

3.3. Combinative techniques

"Top-Down" technologies excel in establishing a progressive interface, effectively bridging the gap between macroscopic scales (millimeters, microns) and the controlled nanoscale. While the "Bottom-Up" strategy offers precise control over the structural architecture of nanomedicines, the accurate assembly of individual nanoparticles remains a significant challenge due to the time and cost involved. The convergence of "Top-Down" and "Bottom-Up" strategies represent a promising avenue for developing practical nanotechnology products. This integrated approach facilitates both the precise engineering of nanoscale devices and their seamless integration with macroscopic systems while addressing challenges related to complex formation and batch-to-batch reproducibility.

Prolonging the *in vivo* circulation of rapidly metabolized drugs with high plasma protein binding can enhance their therapeutic efficacy. Cheng et al.[201] developed docetaxel-loaded nanocrystals encapsulated within mPEG-PLA micelles (DOC(Nc)@mPEG-PLA) to improve drug loading and pharmacokinetic properties. Docetaxel amorphous nanocrystals (DOC(Nc)) were prepared using HPH and subsequently loaded into mPEG-PLA micelles *via* a thin-film hydration method. The "Bottom-Up" assembly of micelles resulted in a reduction of DOC(Nc) particle size from 168.4 to 72.5 nm. Compared to conventional docetaxel injection, DOC(Nc) and DOC(Nc)@mPEG-PLA demonstrated a 1.94-fold and 2.69-fold increase in bioavailability, respectively. DOC(Nc)@mPEG-PLA exhibited a 2.39-fold increase in elimination half-life ($t_{1/2}$) compared to docetaxel injection, while DOC(Nc) alone did not demonstrate this enhancement. Liang et al.[202] leveraged the combined advantages of nanocrystals and liposomes, including high drug loading, controlled release, prolonged circulation, and high stability, for the delivery of hydrophobic agents. Nanocrystal-loaded liposomes (NC@PEG-Lipo) were composed of a drug nanocrystal core and a liposome shell. The nanocrystal agent was fabricated using wet ball milling followed by probe sonication and subsequently loaded into PEGylated liposomes *via* a film dispersion method. NC@PEG-Lipo exhibited a slight increase in size and demonstrated an 11.6-fold improvement in $AUC_{0\text{-}t}$ compared to nanocrystals alone.

4. Nanofabrication technologies of approved products for commercialization

The development of Doxil®, the first FDA-approved nanodrug (1995), exemplifies how addressing these fabrication challenges can lead to successful clinical translation. Doxil® utilizes a liposomal formulation, where the bilayer, composed of high-transition temperature (53 °C) phosphatidylcholine and cholesterol, adopts a "liquid ordered" phase for enhanced stability. The incorporation of PEG to create PEGylated nano-liposomes further prolongs circulation time and facilitates evasion of RES. Notably, doxorubicin is loaded into these nano-scale stealth liposomes *via* a remote loading method driven by a transmembrane ammonium sulfate gradient. This method ensures high drug encapsulation efficiency and minimizes leakage, contributing to the drug's efficacy and safety profile[203]. Since the FDA approval of Doxil® for tumor therapy, the nanomedicines' market has experienced unprecedented growth, characterized by both high risk and high reward. This growth is projected to reach USD 350.8 billion by 2025 despite ongoing challenges related to safety, cost, regulatory hurdles, manufacturing technology, and scale-up. Commercially available nanomedicines encompass a diverse range of platforms, including liposomes, polymeric micelles, crystalline and polymeric nanoparticles, metal nanoparticles, carbon-based nanoparticles, and dendrimers. These platforms utilize various methods for loading APIs, such as physical embedding, chemical bonding, and electrostatic adsorption. Table 6[203−211] provides a comprehensive list of nanomedicine platform technologies currently under commercial development. Market insights indicate that injectable nanomedicines are predominantly formulated as sterile suspensions or lyophilized powders. While most approved nanomedicines are lipid-based nanoparticles, exceptions include

Table 6 The major commercial injectable nanomedicines approved by the FDA and EMA.

Platform	Proprietary name	Active ingredient	Fabrication technology	Dosage form	Formulation	Average particle size	Indication	Approved time	Patent
Alkermes Nacrimic	Anjeso®	Meloxicam	Milled in a NanoMill. RTM. milling system	Injection	Nanocrystalline	<150 nm	Postoperative analgesia	2020	Patent NO. 10709713 and 6431478
–	Ryanodex®	Dantrolene sodium	HPH by microfluidizer and lyophilization	Injectable suspension	Nanocrystalline	400 nm	Malignant hyperthermia	2014	Patent NO. 9884044and 8685460
Stealth technology	Doxil®	Doxorubicin	Ethanol injection combined with remote drug loading (ammonium sulfate)	Injection	Liposome SUV	About 90 nm[203]	Advanced-stage ovarian cancer	1995	Patent NO. 5192549 and 5013556
Myocet™	Myocet®	Doxorubicin	Freeze-thaw cycle combined remote drug loading (citrate)	Lyophilized powder	Liposome MLV	150 nm[204]	Breast neoplasms	2001	ES2198429T3
–	Onivyde®	Irinotecan	The thin film method combined remote drug loading (Triethylammonium sucrose octasulfate)	Injection	Liposome SUV	110 nm[205]	Metastatic pancreatic cancer	2015	Patent NO. 9782349, 10722508 and 8329213
–	ONPATTRO®	siRNA	Microfluidic technique	Injection	Lipid complex	<100 nm [206]	Transthyretin-mediated amyloidosis	2018	Patent NO. 8058069, 8158601 and 11141378
Nab™ technology	Abraxane®	Paclitaxel	Solvent evaporation technique from an oil-in-water emulsion prepared under conditions of high shear force	Injectable suspension	Albumin based nanoparticles	130 nm[207]	Breast cancer	2005	Patent NO. 8853260, 5916596 and 5439686
CombiPlex	Vyxeos™	Daunorubicin and cytarabine	Thin film method and active drug loading technology	Liposome for injection	Liposome	<250 nm	Secondary acute myeloid leukemia	2017	Patent NO. 8022279
–	AmBisome®	Amphotericin B	Solvent evaporation combined with spray drying	Spray dried powder	Liposome	45–80 nm	Fungal infection	1997	Patent NO. 5965156
–	Genexol® PM	Paclitaxel	Thin film dispersion	Lyophilized powder	mPEG-PDLLA polymer micelle	24 nm[208]	Metastatic breast cancer, non-small cell lung cancer, Ovarian cancer	2012 (Korea)	Patent NO. 11179466
Presome^{R}	Visudyne®	Verteporfin	Thin film dispersion	Lyophilized powder	Unilamellar phospholipid vesicle	150 –300 nm[209]	Choroidal neovascularization	2000	Patent NO. 5707608
XR-17™	Apealea®	Paclitaxel	–	Lyophilized powder	Micelles contained a vitamin A-based substance	20–60 nm[210]	Ovarian cancer, Peritoneal cancer, Fallopian tube cancer	2018 (EMA)	–
OPTISOME technology	Marqibo®	Vincristine sulfate	The thin film method combined remote drug loading (Citric acid)	Injection	Liposome SUV	100 nm[211]	Acute lymphoblastic leukemia	2012	Patent NO. 5543152, 20150290184 and 20120003297

–Not applicable.

polymeric micelles and albumin nanoparticles. Notably, Genexol® PM, developed by the South Korean biopharmaceutical company Samyang Biopharm, represents the first approved polymeric micelle nanomedicine fabricated using a film dispersion method (U.S. Pat. NO. 11179466). Micellar stability poses a significant challenge *in vitro* due to the rapid influx of aqueous environments. Similarly, the complex physiological environment can lead to the disintegration of unstable micelles. Conventional nanomedicine manufacturing methods, such as solvent evaporation, solvent diffusion, and nanoprecipitation, often present challenges in achieving precise control over nanomedicine properties and scalability for large-scale production. Liposomes can passively encapsulate APIs during formation or be actively loaded post-formation. Additionally, several freeze-thawing cycles can enhance the encapsulation efficiency of hydrophilic APIs within MLVs. Subsequent size reduction processes can then transform MLVs into SUVs. Regardless of the production method, achieving a homogeneous mixture of phospholipid and cholesterol is crucial for producing uniform liposomes. However, challenges such as residual organic solvents, cholesterol precipitation, and incomplete hydration can arise, negatively impacting the PDI and batch-to-batch consistency. Developed by Nippon Fine Chemical in the 1990s (U.S. Pat. No. 5096629), "PresomeR" technology addresses these challenges by employing a specialized tube furnace and vacuum chamber. This system facilitates the rapid separation of organic solvents from lipids, resulting in an ordered arrangement of phospholipid and cholesterol molecules. The resulting mixed lipid powder exhibits a high degree of homogeneity, good repeatability, and minimal residual organic solvents. Subsequent hydration of this powder readily yields liposomes. "PresomeR" technology significantly simplifies the production process of Visudyne®, leading to improved product stability and enhanced encapsulation rates. FDA-approved liposomal formulations, including Doxil® and Onivyde®, predominantly utilize the solvent injection method. In this method, lipid materials and lipophilic substances are first dissolved in the water-soluble organic solvent. The organic phase is then injected into an aqueous buffer, leading to the spontaneous formation of SUVs through interfacial turbulence. Notably, this method does not necessitate additional energy input (*e.g.*, ultrasonication or extrusion) for particle size control. However, parameters such as flow rate, solution temperature, lipid concentration, and stirring rate can influence the properties of the resulting liposomes. Modified solvent injection methods utilize devices such as Y-joints, membrane contactors, or tangential flow devices to improve the microscopic mixing of the organic and aqueous phases. A novel non-injectable liposome formulation (U.S. Pat. NO. 7718189) achieves a high drug-to-lipid ratio (greater than 1) for water-soluble drugs. This formulation employs an in-line mixing process, where a lipid solution stream and an amikacin solution stream are combined within a mixing tube connected to a Y-connector. This infusion method has demonstrated scalability for industrial production and yields liposomes with reduced lipid-to-drug ratios and increased encapsulation efficiency. In addition, the obtained sizes of the liposomes increase with the lipid concentration in the ethanol injection process, which is an approach to efficiently control the liposomal size. (U.S. Pat. NO. 11395799). Despite these advantages, solvent injection methods face challenges related to the complete removal of organic solvents. Achieving high lipid concentrations can also be technically demanding. Moreover, the excess aqueous media used in these methods can lead to low encapsulation efficiencies for hydrophilic APIs. This limitation is inherent to liposomal systems, as they tend to encapsulate a smaller proportion of hydrophilic substances within the inner aqueous core compared to the external water phase.

In recent years, the introduction of microfluidic technology has accelerated the clinical transformation of nanomedicines, especially for gene agents' delivery, such as Abraxane® and lipid nanoparticles of Covid-19 vaccines. Microfluidic devices enable the efficient encapsulation of diverse nucleic acid agents—including DNA plasmids, oligonucleotides, siRNA, mRNA, and ribonucleoproteins—into lipid nanoparticles, yielding products with high uniformity and repeatability. Microfluidic-based nanomedicine manufacturing is often applied in laboratory settings due to its advantages in rapid prototyping and cost-effectiveness. Significantly, the optimal preparation conditions identified in the laboratory can be directly translated to large-scale production without significant adjustments. However, scaling up lipid nanoparticle production using microfluidic devices necessitates devices with robust pressure resistance and mechanical strength. Consequently, further development of glass or metal components for these devices is crucial.

The safety, efficacy, and commercial viability of a nanomedicine candidate are inherently linked to its fabrication technologies. Therefore, selecting an appropriate production process that aligns the desired formulation with the chosen fabrication technology and practical considerations is crucial.

5. Computationally guided nanomedicine fabrication

The FDA Modernization Act 2.0, signed into law by President Joe Biden in late December 2022, removes the requirement for new drugs to be evaluated on animals prior to FDA approval. This legislation represents a significant departure from over 80 years of drug safety regulations regarding animal testing. The FDA recommends increased reliance on non-animal methods developed within the last 10−15 years, including computer modeling, "organ chips", and other innovative approaches to drug development. *In silico* medicine, artificial intelligence was utilized to screen potential new drug candidates for a specific target in only 21 days in 2019. Furthermore, the integration of data curation and machine learning has demonstrated considerable promise in advancing nanomedicines development (Fig. 8).

5.1. Predict the self-assembly of nanocomposites

Self-assembled nano-aggregates, formed through drug−drug or drug−carrier interactions, offer a promising strategy for modulating pharmacokinetic behaviors. These nano-aggregates have been investigated for their potential to enhance drug delivery by reducing toxicity and improving bioavailability. However, this approach is not without its challenges. For instance, the non-specific aggregation of small molecules with proteins in solution can lead to false-positive readings in high-throughput screening assays and off-target effects. Additionally, non-specific binding of aggregates to intracellular proteins can induce protein unfolding and disrupt cellular functions[212]. Investigating nano-aggregates with unknown self-assembly mechanisms involves navigating complex, high-dimensional data spaces. Machine learning offers a powerful approach to address this challenge. By leveraging existing datasets, machine learning algorithms can predict the function, structure, and properties of self-assembled nanoparticles without relying on prior knowledge of the underlying

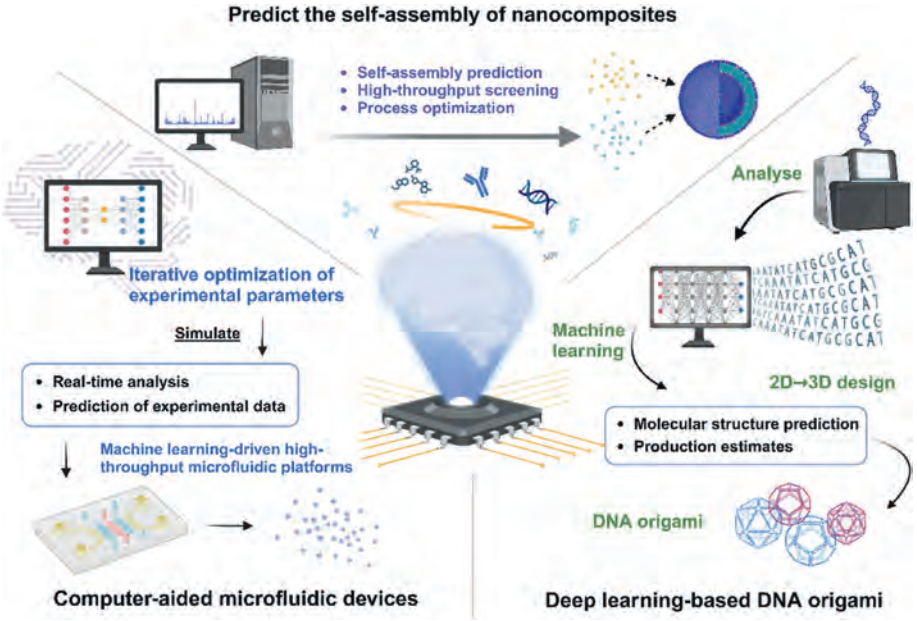

Figure 8 An integrated pipeline for AI-assisted design of nanomedicine iterative synthesis to improve the effective translation.

relationships. This characteristic is particularly valuable when studying complex systems that lack a solid theoretical foundation[213]. The development of targeted drug carriers *via* supramolecular self-assembly and chemical modification often involves complex synthesis schemes that are challenging to predict, execute, and control. In a notable study, Shamay et al.[214] reported a targeted drug delivery system that leverages the predictive power of molecular descriptors to guide self-assembly. Their system accurately and quantitatively predicts nanoparticle formation based on the molecular structure of the precursor molecule. Specifically, they achieved ultra-high drug loading capacities (up to 90%) by assembling a drug with sulfated indocyanine. The researchers employed electron topological molecular descriptors, particularly SpMAX4_Bh(s), to capture information about the molecules' geometrical complexity, bond order, and heteroatoms. This approach allowed them to assess the predictive utility of their model for self-assembly.

Furthermore, machine learning techniques have demonstrated success in predicting the properties of nanocrystals produced by "Top-Down" methods. These methods offer significant advantages in enhancing the dissolution rate of water-insoluble drugs by reducing particle size to the nanometer scale. Researchers have developed predictive models to analyze the factors influencing nanocrystal properties using data from the three primary preparation methods: ball milling, HPH, and antisolvent precipitation. Moreover, machine learning techniques, specifically those employing algorithms like gradient boosting machines, can effectively predict the particle size and PDI of nanocrystals. For instance, a recent study revealed that milling time is a critical factor in ball milling, while the cycle index is crucial for HPH[215]. Machine learning techniques have also demonstrated potential in guiding the "Bottom-Up" assembly of nanomedicines, particularly when strategies are employed to mitigate the impact of low-quality data. Reker et al.[216] addressed this challenge by developing a high-throughput platform for identifying effective co-assembly pairings. Their approach focused on nanoprecipitation to produce nanoparticles with high drug-loading capacities (over 95%) using APIs and small-molecule dyes as excipients. The key challenge lies in efficiently selecting suitable excipient-aided co-assembly combinations from a vast pool of millions of small-molecule candidates. To address this, they integrated machine learning with high-throughput experimentation to screen a vast library of 2.1 million potential self-assembling pairings. This approach successfully identified 100 self-assembling nanomedicines, using particle size as the primary indicator. Their high-throughput platform utilized a 4515-dimensional descriptor for each APIs-excipient pair to simulate nanoparticle structure using a random forest model. A training set of 1440 data points generated from the high-throughput co-aggregation experiments was used to develop the model.

Targeted nanomedicines often necessitate intricate synthetic schemes, encompassing both nanomaterial self-assembly and surface modification. These complex processes pose significant challenges in terms of predictability, simulation, control, and execution. Two promising avenues for addressing these challenges are rational design guided by "evolutionary theory" and unbiased high-throughput screening approaches. Both strategies offer valuable tools for optimizing targeted nanomedicines. Looking forward, we envision that computational design, coupled with these experimental approaches, will play an increasingly pivotal role in accelerating the clinical translation of targeted nanomedicines.

5.2. *Computer-aided microfluidic devices*

The inherent complexity of designing and precisely controlling custom microfluidic devices presents a significant obstacle to their widespread practical application. While microfluidic devices can be designed based on researchers' expertise, this approach is often limited by its inherent subjectivity and lack of consistent reliability[217]. Computer-based numerical modeling offers an alternative approach; However, accurately simulating complex phenomena such as multiphase flows, inertial processes, or biological and chemical performance indicators (*e.g.*, synthesis yields, gene expression, morphology) remains challenging[218,219].

Recent advancements in artificial intelligence (AI) have prompted researchers to explore its potential in facilitating the efficient and rational design of microfluidic devices for nanomaterial synthesis. AI, particularly when coupled with machine learning, leverages trainable statistical models to discern patterns within complex datasets and predict future behavior. This capability has the potential to bridge the knowledge gap between domain experts and end-users, effectively integrating AI's data analysis and predictive power with the precise control and high-throughput nature of microfluidics to enable the intelligent synthesis of nanomaterials[220,221]. Therefore, integrating a rational data collection strategy with machine learning algorithms presents a promising avenue for addressing the challenges inherent in designing and optimizing microfluidic devices for nanoparticle synthesis[222].

Synthesizing nanoparticles with tailored properties often necessitate iterative optimization of experimental parameters, given the multifaceted factors influencing their characteristics. Traditional experimental approaches, however, are inherently time-intensive and present challenges in data processing[223]. Machine learning, conversely, excels in rapidly establishing intricate mapping relationships within datasets. This capacity to accurately predict nanoparticle properties positions machine learning as a potential catalyst for breakthroughs within this intricate research domain. For instance, researchers at the National University of Singapore have proposed a two-step theoretical framework integrating Gaussian process Bayesian optimization algorithms and deep neural networks to realize a machine learning-driven, high-throughput microfluidic platform[224]. In a notable study, Tao et al.[225] demonstrated the intelligent synthesis of nanoparticles by leveraging microfluidic technology for precise reaction control and integrating machine learning algorithms for real-time analysis and prediction of experimental data.

Machine learning offers a transformative approach to enhancing the quality of nanomedicines. Rebollo et al.[226] exemplify the power of machine learning, specifically artificial neural networks (ANNs), in predicting and optimizing the size and PDI of liposomes fabricated *via* a microfluidic system. These findings highlight two key advantages of integrating machine learning into microfluidic liposome production. First, the trained artificial neural network model accurately predicted the size and PDI of liposomes produced under previously untested conditions. This predictive capability can significantly reduce time-consuming and resource-intensive experimental iterations during optimization. Second, by analyzing the relationship between input parameters and output properties, artificial neural network models can guide the selection of optimal process parameters for achieving desired liposomal properties, such as a specific size range, ultimately enhancing drug delivery to tumors. As these technologies continue to mature and research efforts delve deeper into this promising intersection, we anticipate a paradigm shift in the landscape of nanomaterial design and application.

5.3. Deep learning-based DNA origami

The practical efficacy of DNA origami is significantly affected by numerous factors, including DNA sequence, strand length and concentration, and environmental conditions. Machine learning algorithms show considerable promise for DNA origami nanostructure characterization and optimization, enabling the transformation of high-dimensional input descriptors into low-dimensional representations and the extraction of hidden insights from complex scientific datasets. AI models, exemplified by AlphaFold, have revolutionized molecular structure prediction, significantly accelerating protein design[227]. Could AI and machine learning similarly assist in the design of DNA origami nanostructures? However, in contrast to proteins, experimentally or computationally validated structural datasets for DNA origami remain limited. Kim et al.[228] propose a versatile graph neural network to accurately and rapidly predict the 3D conformation of DNA origami. A hybrid data-driven and physics-informed approach is employed to minimize both physics-informed and data-driven loss, thereby overcoming limitations in model training. Furthermore, this model demonstrates the capability to analyze the supramolecular assembly of hundreds of DNA blocks. The utilization of this strategy facilitates real-time virtual prototyping of DNA origami, including applications such as automated inverse design. Mechanical characterization of dynamic DNA origami is essential to biological applications, as deep neural networks accelerate the characterization of dynamic DNA origami and undergo large conformational fluctuations[229]. The integration of deep learning technology enables the detection of multiple DNA origami nanostructures and the estimation of their yield in complex environments, achieving a detection speed within the millisecond range[230].

Moreover, machine learning can monitor and analyze the behavior of DNA origami within biological systems, providing real-time feedback for drug delivery applications. This feedback encompasses predicting drug distribution, metabolism, and toxicity in *vivo* and evaluating the long-term stability and biocompatibility of origami structures within these complex environments[227]. AI can rapidly identify promising origami structures with high translational potential. This capability, in turn, reduces experimental costs and time, broadens the design space of DNA origami, and accelerates the commercialization of research findings.

6. Summaries and perspectives

Nanomedicines hold immense potential for transforming the therapeutic landscape across a wide range of diseases. Over the past few decades, there has been a surge in research and development efforts focused on revolutionary nanomedicines. However, this progress has been predominantly confined to the realm of nanoscience rather than translating into practical nanotechnological applications. Consequently, the translation of precision nanomedicines from the bench to the clinic faces significant hurdles, hindering their ability to fully realize their transformative potential in disease treatment.

Tackling the challenges of nanomedicines: are we ready to ultimately improve clinical outcomes for patients? The nanomedicine industry requires robust, reproducible, and scalable manufacturing processes supported by comprehensive research data. Translating nanomedicines into 'real-world' applications hinges on numerous factors, including a critical assessment of whether enhanced properties justify the associated production cost. Despite the challenges associated with industrial scale-up, precision nanotechnologies remain essential for developing next-generation nanomedicines. A primary focus for advancing nanomedicines translation should be the development of cost-effective, reproducible synthetic routes. Recognizing that nanomedicine encompasses a diverse toolkit of approaches rather than a single industry, future commercialization efforts should prioritize the development of modular, 'plug-and-play' synthetic systems.

In addition, AI, as an 'enabling' tool, offers the unprecedented capability to systematically analyze structure−function

relationships in nanomedicines, providing theoretical and empirical rules for the rational design of advanced materials and the precise manufacturing of nanostructures. Moreover, AI-assisted nanomedicine design holds the potential to uncover novel and unexpected nanostructures with previously unreported properties. However, it is crucial to emphasize that AI should serve as a complement to, rather than a substitute for, the pursuit of fundamental understanding regarding the interplay between the reaction mechanisms, structure, and properties in nanomedicines.

The vast potential of nanomedicine fabrication, aptly described by the phrase "plenty of room at the bottom", is being unlocked by revolutionary technologies that provide unprecedented insights into precision nanostructures. Rationally designed, digitally enabled nanomedicines hold the key to overcoming current limitations, such as poor efficacy, and bridging the translational gap between benchtop discoveries and bedside applications.

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Conflicts of interest

The authors declare no conflict of interest.

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