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Advancing intestinal organoid technology to decipher nano–intestine interactions and treat intestinal disease

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ABSTRACT

With research burgeoning in nanoscience and nanotechnology, there is an urgent need to develop new biological models that can simulate native structure, function, and genetic properties of tissues to evaluate the adverse or beneficial effects of nanomaterials on a host. Among the current biological models, three-dimensional (3D) organoids have developed as powerful tools in the study of nanomaterial–biology (nano–bio) interactions, since these models can overcome many of the limitations of cell and animal models. A deep understanding of organoid techniques will facilitate the development of more efficient nanomedicines and further the fields of tissue engineering and personalized medicine. Herein, we summarize the recent progress in intestinal organoids culture systems with a focus on our understanding of the nature and influencing factors of intestinal organoid growth. We also discuss biomimetic extracellular matrices (ECMs) coupled with nanotechnology. In particular, we analyze the application prospects for intestinal organoids in investigating nano–intestine interactions. By integrating nanotechnology and organoid technology, this recently developed model will fill the gaps left due to the deficiencies of traditional cell and animal models, thus accelerating both our understanding of intestine-related nanotoxicity and the development of nanomedicines.

KEYWORDS

intestinal organoid, biomimetic extracellular matrices (ECMs), intestinal tissue engineering, nano–intestine interaction

1 Introduction

Nanomaterials are widely present in common consumer products, including food additives, antibacterial agents, medicines, and cosmetics [1, 2]. With the ever-growing use and disposal of such products, significant amounts of nanomaterials inevitably enter the human body over the course of a lifetime [3, 4]. The entry of nanomaterials through the intestine via an oral route is a particularly important pathway, which includes acting on the intestine itself and inducing adverse or beneficial effects [5]. To date, several lines of evidence have confirmed that the safety risks of nanomaterials to the intestine are not insubstantial [6, 7]. Apart from nano safety concerns, therapeutic systems based on nanomaterials have shown great potential for applications in intestine-related diseases, since such formulations can easily enter the target organ [8, 9]. From the perspective of toxicology and nanomedicine, there is an urgent need to understand nano–intestine interactions and their underlying mechanisms, to both avoid the risks and broaden the application of nanomaterials. Thus far, studies on the nano–intestine interaction have been dominated by traditional two-dimensional (2D) cell models and animal models [10]. However, 2D cell models cannot reproduce the three-dimensional (3D) structure of the native intestine, while animal models are limited due to the complexity of the "3R" rules (reduction, replacement, and refinement) [10–12]. Based on these drawbacks, there is significant room for novel biological models to investigate nano–intestine interactions.

In recent years, with the improvement of culture systems, intestinal organoid models have emerged as a novel tool in the field of intestinal biology [13], especially in investigations of nano–intestine interactions [14]. Of note, with the help of nanotechnology, several new generations of biomimetic extracellular matrices (ECMs) have been developed for the culture of intestinal organoids, effectively solving many problems of animal-derived traditional ECMs (such as poor reproducibility, complex components, and immunogenicity), thereby promoting the translation of intestinal organoid research to clinical applications [15]. This review summarizes the recent advances in the development and construction of intestinal organoid culture systems, introduces novel biomimetic ECMs based on nanotechnology for the culture of intestinal organoids, and presents an outlook into the future applications of intestinal organoids in nano–intestine interactions.

2 The construction of 3D intestinal organoids

Since Hans Clevers' lab successfully established a thorough culture system of murine intestinal organoids in 2009, organoid models have been a major focus of biological research [16]. Intestinal

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organoids, also known as "mini-intestines", are *in vitro* 3D tissue structures cultured from intestinal stem cells (ISCs) and intestinal epithelial cells (IECs) derived from the former [11]. In contrast to traditional 2D cell models, intestinal organoids can more reliably represent the native structures of intestinal tissue, their genetic characteristics, and their microenvironment, thus making up for the drawbacks of 2D models (Fig. 1) [17, 18].

At this point, various types of intestinal organoids from humans and other animals have been constructed [19]. One such model is based on the leucine-rich repeat-containing, G protein-coupled receptor 5 positive (Lgr5$^+$) ISCs in the intestinal crypt (Fig. 2(a)), which can be divided into enteroids and colonoids (i.e., derived from the small intestine and colon, respectively), according to the intestinal region utilized. Another type of organoid is cultured from induced pluripotent stem cells or embryonic stem cells (iPSCs/ESCs; Fig. 2(b)) [11, 20–22]. Both systems exploit the proliferation and differentiation potential of stem cells to trigger the formation of intestinal organoids. In this section, we will introduce the process of generating intestinal organoids.

2.1 The categories of intestinal organoids based on Lgr5$^+$ ISCs

Normally, the fast regeneration rate (5–7 days) of the intestine mainly relies on Lgr5$^+$ ISCs [23]. Lgr5$^+$ ISCs are located in the basement of intestinal crypt, and their proliferation and differentiation are dynamic processes along the crypt–villus axis. Functionally, Lgr5$^+$ ISCs in the crypt niche proliferate and divide into transit-amplifying cells (TACs), which then differentiate into Paneth cells into the basement of the crypt. Meanwhile, TACs differentiate into absorptive IECs and various secretory IECs (goblet cells, enteroendocrine cells, and cluster cells) along the villus. Eventually, senescent/apoptotic IECs shed from the villus tip, and new IECs are constantly renewed from Lgr5$^+$ ISCs [19, 24].

The proliferation and differentiation of Lgr5$^+$ ISCs are strictly regulated by the following four classical signaling pathways: wingless/integrated (Wnt), Notch, epidermal growth factor (EGF), and bone morphogenetic protein (BMP) [25]. As a starting point, the Wnt signal provides the "fuel" for the proliferation of Lgr5$^+$ ISCs, while the Notch signal maintains Lgr5$^+$ ISCs in the undifferentiated state (if the Notch signal is blocked, Lgr5$^+$ ISCs will differentiate into secretory IECs). The EGF signal activates tyrosine kinase receptors and promotes mitosis of ISCs and TACs. Notably, the BMP signal plays an important role in maintaining the stemness of ISCs [26–28]. These four signaling pathways exert different functions in the formation of mature intestine along the crypt–villus axis. Interestingly, the activity of three of these pathways (Wnt, Notch, and EGF) remains at a high level in the crypt but decreases along the crypt–villus axis. In contrast, the BMP signaling pathway shows the opposite pattern along this axis. It is this difference in the spatial expression of different signaling pathways that results in the crypt–villus structure of the intestinal lumen.

Intestinal organoids are constructed from Lgr5$^+$ ISCs or intestinal crypts, obtained surgically or endoscopically, and encapsulated in ECM [29]. Two factors are essential for the construction of 3D intestinal organoids. One is medium that contains the nutrients and cytokines necessary for the development of the organoids. Based on the different organoid functions, this can be divided into amplification medium and differentiation medium (Fig. 2). The amplification medium contains Wnt3a, R-Spondin-1 (ligand of Lgr5; associated with Wnt signaling pathway), Noggin (inhibitor of BMP), and EGF, which is mainly used for the amplification and long-term culture of intestinal organoids. Based on the amplification medium, the differentiation medium, which is mainly used for the differentiation of the intestinal organoid into a crypt–villus structure, lacks Wnt3a. The other is an ECM that provides a 3D scaffold for the development of the intestinal organoid [20, 29].

As per the source tissue, intestinal organoids based on Lgr5$^+$ ISCs can be divided into enteroids and colonoids, which not only reproduce the structures, genetic characteristics, and specific functions of the native intestine *in vitro*, but also possess the property of "infinite amplification". Even after being passaged weekly for several years, their morphological structures and genetic characteristics remain stable (Fig. 2(c)) [16, 20]. In addition, these 3D structures also exhibit the specificity of the donor sources. The gene expression profiles of intestinal organoids based on Lgr5$^+$ ISCs derived from different intestinal regions, intestinal diseases, or individuals or species retain their unique characteristics [30, 31]. These properties make them extremely versatile tools in numerous research fields, such as personalized medicine.

2.2 The categories of intestinal organoids based on iPSCs/ESCs

The method for generating induced intestinal organoids, specifically cultured from iPSCs/ESCs, was first reported by Spence et al. in 2011. This process can be briefly divided into the following three stages (Fig. 2(b)) [21]. (1) iPSCs/ESCs are induced to form a definitive endoderm by activin A stimulation. (2) The definitive endoderm is then induced to differentiate into midgut/hindgut tissue, which requires stimulation with fibroblast growth factor (FGF) and Wnt signaling. (3) The sphere grown from midgut/hindgut is finally encapsulated in ECM for 3D culture, which is similar to the differentiation culture stage of enteroids and colonoids. Compared with enteroids and colonoids, the induced intestinal organoids take several weeks to form mature crypt–villus structure, whereas enteroids and colonoids take only one week. Moreover, the composition of induced intestinal organoids is also quite different. They contain not only IECs but also intestinal mesenchyme, such as fibroblasts and smooth muscle cells (SMCs), which develop from mesoderm

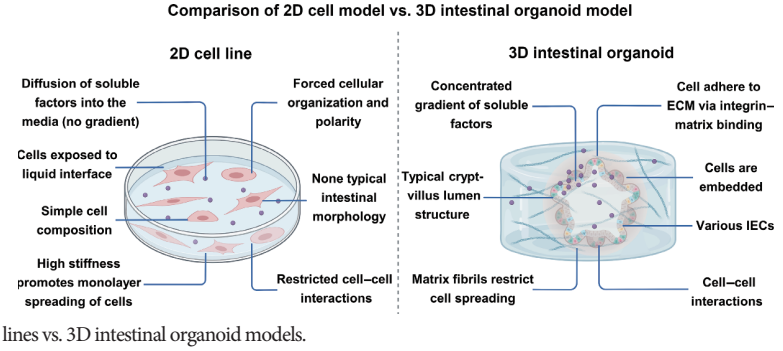

Figure 1 Comparison of 2D cell lines vs. 3D intestinal organoid models.

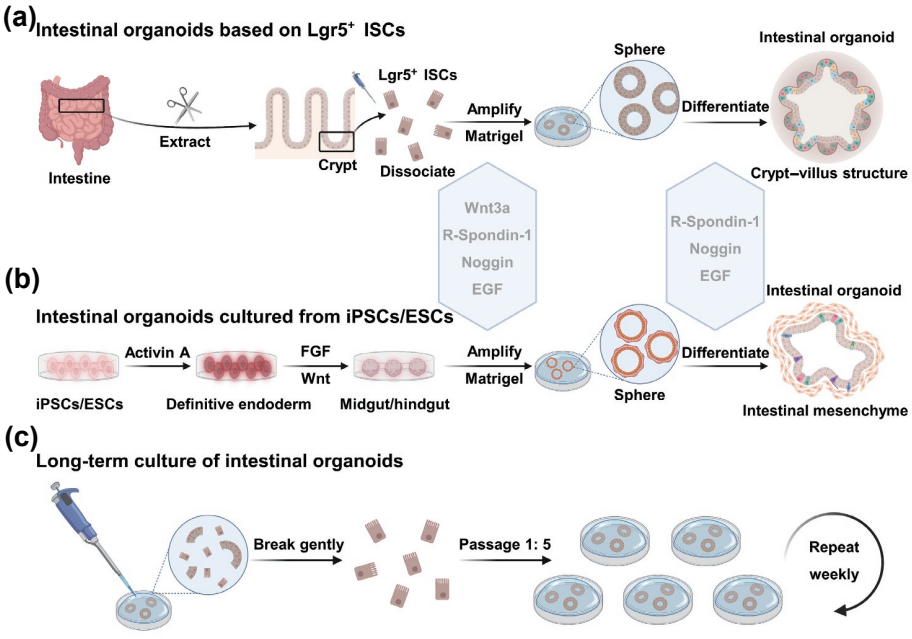

Figure 2 Culture system of 3D intestinal organoids. (a) Intestinal organoids based on Lgr5⁺ ISCs. (b) Intestinal organoids cultured from iPSCs/ESCs. (c) The passage and long-term culture of intestinal organoids.

remnants [32]. Although induced intestinal organoids show the same advantages as enteroids and colonoids in organizational structures and functional characteristics, they do not have the potential for infinite amplification and can only be passaged a limited number of times. The differences between these two intestinal organoid culture systems are listed in Table 1.

Table 1 Differences between the two intestinal organoid culture systems

Characteristics	Enteroids/colonoids	Induced intestinal organoids
Source	Lgr5⁺ ISCs	iPSCs/ESCs
Cell composition	IECs	IECs and intestinal mesenchymal cells
Crypt–villus structure	Yes	Yes
Specificity of donor source	Yes	No
Infinite amplification	Yes	No
Culture cycle	Short	Long

3 Extracellular matrices for the culture of intestinal organoids

ECMs, used to form the three-dimensional scaffold for the culture of intestinal cells, are indispensable factors in the construction of intestinal organoids. At this time, two classical ECMs, animal-derived native ECMs (e.g., Matrigel) and biomimetic ECMs based on nanotechnology (e.g., poly(ethylene glycol) (PEG) hydrogels), have been used in the construction of 3D intestinal organoids [33, 34]. In this section, we will discuss the two types of ECMs in detail.

3.1 Animal-derived native extracellular matrices

In the early stages of organoid technology, animal-derived native ECMs usually served as the 3D scaffold for the development of 3D intestinal organoids. Among these ECMs, Matrigel has proven to be one of the most effective ECMs for the culture of intestinal organoids. Normally, Matrigel is isolated from Engelbreth–Holm–Swarm (EHS) murine sarcoma and comprises collagen IV, laminin-111, nestin, heparin sulfate glycoproteins, growth factors, and matrix metalloproteinases (MMPs) [35]. At room temperature, Matrigel can aggregate to form a 3D scaffold for the growth of intestinal organoids and reproduce the tissue structures and physicochemical properties of native ECMs, thus realistically simulating the developmental process of the intestine. However, in recent years, Matrigel has presented some shortcomings, such as complex composition, large differences between preparations, immunogenicity, and sensitivity to temperature and/or proteases. These issues limit the application of Matrigel in organoid technology, especially when it comes to clinical translation. In order to overcome these obstacles, Clevers et al. described a biomimetic ECM in which ISCs can gradually develop in a collagen I gel [36]. Furthermore, Broguiere et al. exploited fibrin-based ECMs (including laminin-111) for the culture of intestinal organoids [37]. It is worth noting that, although these biomimetic ECMs, synthesized only from the main components of native ECMs, are more explicitly defined and controllable, their performance in culturing intestinal organoids is incomparable to Matrigel.

3.2 Biomimetic extracellular matrices based on nanotechnology

In order to overcome the limitations of animal-derived ECMs and further the development of intestinal organoids, additional biomimetic ECMs based on nanotechnology have emerged [38, 39]. There are three key conditions for the function of native ECMs (Fig. 3(a)). First, ECMs must provide anchor sites for intestinal organoids, among which the Arg-Gly-Asp (RGD) sequence is the most common. Second, laminin-111, as one of the important components of Matrigel, is needed to generate biological signals to promote the development of intestinal organoids. Third, the ECMs used for 3D intestinal organoid culture must have a certain mechanical strength. For instance, in the early stage of intestinal organoid development (days 1–3), the stiff ECM is more conducive to the sphere formation. But in the later stage (day 5), the soft matrix is more beneficial to the formation of mature crypt–villus intestinal lumen structure for intestinal organoids. The hardness change of ECM reflects the different mechanical strength demand during intestinal organoid development [37, 40]. Biomimetic ECMs based on nanotechnology utilizes the above mechanisms to simulate the biological actions of native ECMs *in vitro* [41]. Compared with

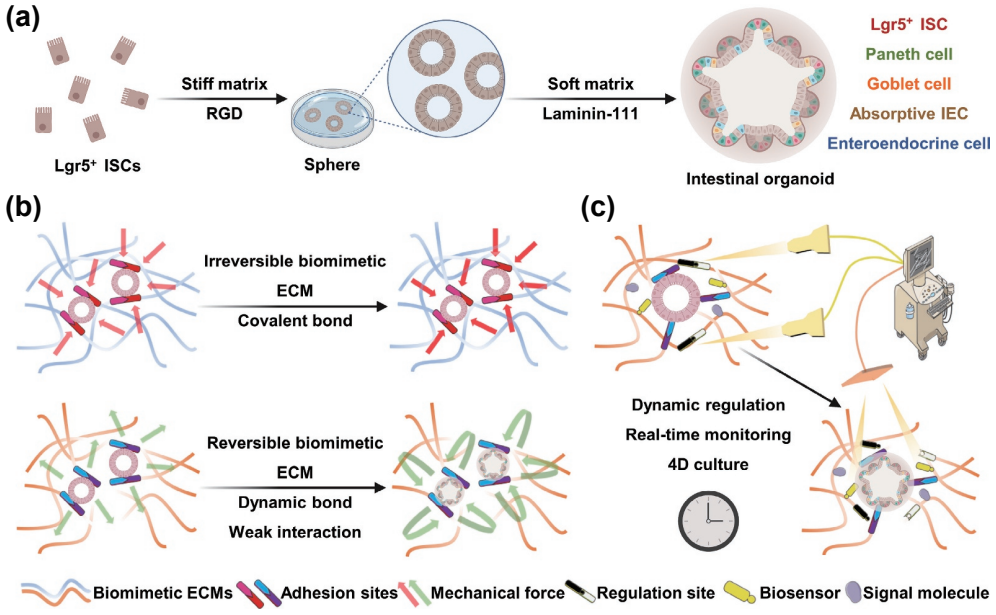

Figure 3 Biomimetic ECMs based on nanotechnology for the culture of intestinal organoids. (a) Design principles of biomimetic ECMs: anchor sites (e.g., RGD), biological signals (e.g., laminin-111), and mechanical strength. (b) Mechanisms of irreversible and reversible biomimetic ECMs. (c) Mechanisms of dynamic and adjustable second-generation biomimetic ECMs.

Matrigel, the composition of synthetic biomimetic ECMs is more defined and controllable.

In recent decade, with the booming nanotechnology, nano-biomimetic materials have made great progresses, which further promotes the development of biomimetic ECMs for intestinal organoid related fields. For example, Gjorevski et al. synthesized the first well-defined biomimetic hydrogel for intestinal organoid culture using PEG [40]. PEG containing eight vinyl sulfone (VS; eight-arm PEG-VS) was modified with Gln and Lys transglutaminase factor XIII (FXIIIa) to cross-link the PEG, and the RGD sequence was coupled to the PEG gel skeleton using the same principle. The report's experimental results indicate that the PEG-RGD matrix could successfully support the amplification of ISCs and intestinal crypts under an elastic modulus of 1 kPa, but could not effectively support the differentiation of intestinal organoids into mature crypt–villus lumen structures. The authors then modified the PEG-RGD matrix to soften the gel by adding PEG-acrylate (PEG-Acr) groups, whose ester bonds could be hydrolyzed to the gel system, and added laminin-111. In this PEG-RGD-LAM matrix, intestinal organoids could form mature crypt–villus lumen structures. Therefore, although laminin is not a necessary condition for the amplification of ISCs, it is essential for the formation of mature intestinal organoids. Moreover, the ECM composition used for intestinal organoid culture is not static but changes dynamically during the development of intestinal organoids. In particular, the pressure release of ECMs is critical for the sustained activation of the transcriptional regulator yes-associated protein 1 (Yap1) and its mediation of the formation of crypt–villus lumen structure at the later stage of maturity. In another report, Cruz-Acuña et al. used a similar method to synthesize a terminal maleimide modified four-arm PEG hydrogel for intestinal organoid culture. In addition, the group also demonstrated that the hydrogel could be used as a carrier for intestinal organoid transplantation *in vivo*. The intestinal organoids, which were transplanted with the PEG hydrogels, effectively improved colonic wound repair [42]. In addition, Capeling et al. designed a simple hydrogel out of unmodified alginate, which served only as a simple physical scaffold for 3D intestinal organoid culture [15]. The alginate hydrogel exhibited similar behavior as Matrigel, in both *in vitro* culture and *in vivo* transplantation, and was able to generate ideal intestinal organoids with embryonic intestinal phenotypes. However, this non-adhesive alginate hydrogel was not amenable to the culture of intestinal organoids based on Lgr5⁺ ISCs. This distinction was because intestinal organoids based on iPSCs/ESCs contain mesenchymal components, while those based on Lgr5⁺ ISCs only contain IECs, indicating that with intestinal mesenchyme can buttress the simplest 3D physical scaffolds in the culture of intestinal organoids.

Unlike the above methods of promoting the differentiation of ISCs into mature lumen structures by adding biological components (such as laminin-111 and intestinal mesenchyme) to synthetic biomimetic hydrogels, Dosh et al. achieved the same effect by a method known as injury stimulation [43]. The authors first synthesized a non-biodegradable N-isopropylacrylamide (NIPAM) hydrogel, and then encapsulated the intestinal crypts or Lgr5⁺ ISCs into the NIPAM hydrogel. However, only simple spherical intestinal organoids were formed with this technique. The group then dissociated the spherical intestinal organoids into fragments or individual cells and encapsulated them in the NIPAM hydrogel. Mature intestinal lumen structures could then be formed, along with various types of differentiated IECs. Although this simple injury stimulation strategy does not require the assistance of any biological component to obtain mature intestinal organoids, the multi-step culture method and injury intervention inevitably introduce confounding factors into the experimental results. Moreover, the mechanism of intestinal damage and repair in this approach is still not clear. A method to effectively combine the mechanism of intestinal damage and repair with 3D intestinal organoid culture remains unknown and requires additional focus in future work. Overall, although these first-generation biomimetic ECMs with covalent crosslinking have addressed the shortcomings of animal-derived native ECMs (such as their complex composition, poor repeatability, high cost, immunogenicity, and tumorigenic potential), they cannot be dynamically regulated with the development of intestinal organoids and remain inferior to Matrigel in inducing ISCs to differentiate into mature crypt–villus lumen structures.

In order to more faithfully simulate native ECMs, dynamic and adjustable second-generation biomimetic ECMs emerged. In general, the first-generation biomimetic ECMs are covalently crosslinked hydrogels, with irreversible formation and breakage of

chemical bonds. To meet the requirement for dynamic changes in the ECM at different stages of intestinal organoid proliferation and differentiation, second-generation biomimetic ECMs that form reversible hydrogels through weak interactions (such as hydrogen bonding, ionic bonding, and hydrophobic interactions) or dynamic covalent bonding have emerged (Fig. 3(b)) [44]. In the initial stage of intestinal organoid formation, amplification is dominant, and spheroids are formed without obvious anisotropy. At this point, the reversible hydrogel exhibits a high elastic modulus. Subsequently, intestinal organoids begin to differentiate into mature crypt–villus lumen structures, at which time intestinal organoids exhibit apparent anisotropy, and the reversible hydrogel bonds break, due to the elevation of local stress, to meet the mechanical ECM environment requirements of intestinal organoids [45].

Based on the above principles, Chrisnandy et al. recently designed a dynamic hydrogel mediated by reversible hydrogen bonding to promote degradation-independent development of intestinal organoids *in vitro* [46]. The hydrogel used eight-arm PEG (8-PEG) as the backbone, in which 50% of the end groups were cytosine, which formed triple hydrogen bonding interactions with its tautomers, and the other 50% of the end groups were vinyl sulfone, through Michael addition to form covalent bonds. Reversible hydrogen bonds can meet the dynamic mechanical pressure requirements during intestinal organoid development, while interspersed covalent bonds can effectively ensure gel stability to avoid adverse degradation. The dynamic hydrogel Hybrid50 exhibits stress relaxation properties similar to Matrigel in the middle and late stages of intestinal organoid development, thereby effectively activating Yap1, which is more conducive to the symmetry breaking of intestinal organoids. To further expand the application scope and achieve long-term culture of intestinal organoids, Chrisnandy et al. introduced a transpeptidation approach mediated by Sortase-A (SrtA) into Hybrid50 [47]. The SS-Hybrid50 hydrogel, degraded by adding SrtA and soluble triglycine, can achieve at least five stable passages of intestinal organoids.

The feature of dynamic change endows reversible hydrogels with numerous application prospects in the delivery of intestinal organoids *in vivo*. The required intestinal organoids can be encapsulated into a reversible hydrogel, and then the application of shear force can shift the reversible hydrogel into sol behavior. After injection into the target site, the reversible hydrogel can then recuperate its mechanical properties, thereby preventing leakage of the intestinal organoids [48]. In addition, to better achieve dynamic regulation and monitoring, the second-generation biomimetic ECMs can be artificially manipulated through external stimuli (such as light, electricity, magnetism, and temperature) [49, 50], and can perform real-time monitoring of various parameters (such as pH, electrolytes, and functional proteins) in the process of organoid development (Fig. 3(c)) [51]. For example, O'Donnell et al. designed a 3D scaffold for intestinal organoid culture and pH monitoring [52]. They began by designing a biosensor consisting of a cellulose binding domain (CBD) and a pH sensitive-enhanced cyan fluorescent protein (ECFP), which was then attached to a cellulose matrix and imaged by multi-parameter fluorescence intensity and lifetime imaging microscopy (FLIM). The 3D cellulose scaffold not only was able to accurately reflect pH changes during the development of intestinal organoids, but also exhibited the potential to achieve multi-parameter detection.

Although many remarkable achievements have been made in recent years in the development of second-generation biomimetic ECMs, there are still many problems to be addressed. These matrices differ from native ECMs, the latter provide a suitable environment for the growth of intestinal organoids through biological action, while the second-generation biomimetic ECMs achieve the dynamic manipulation of matrix stiffness mainly through chemical and physical mechanisms. Whether there are deviations between the second-generation biomimetic and native ECMs in their effects in culture and time scale still remains to be determined. The four-dimensional (4D) culture model that takes the time dimension into account is an important trend in the development of novel biomimetic ECMs for intestinal organoid culture [53]. There are also two culture systems for intestinal organoids. Enteroids and colonoids with only IECs have stricter and more complex requirements for biomimetic ECMs, while induced intestinal organoids with IECs and intestinal mesenchyme have relatively fewer requirements and simple 3D physical scaffolds can achieve good culture effects, as the intestinal mesenchyme plays a biological role similar to native ECMs during the development of intestinal organoids [15]. Therefore, it is necessary to fully consider the differences in the culture systems of different intestinal organoids when designing biomimetic ECMs. In addition, when using external physical methods to regulate biomimetic ECMs, attention must be paid to the cytotoxicity of the stimulus. For example, although light-controlled biomimetic ECMs are relatively well-characterized, many short-wavelength light signals, especially near-ultraviolet light, will adversely affect the growth of organoids. Thus, establishing quantitative evaluation standards for adequate organoid support will improve the future development of novel ECMs [44]. Moreover, although the combination of biological sensors and biomimetic ECMs can achieve real-time detection of a biochemical index during the development of intestinal organoids, the accuracy of the detection results still requires optimization. Klotz et al. synthesized a mixed hydrogel based on PEG and gelatin for tissue engineering. This mixed hydrogel with medium biological complexity could effectively support the normal development of organoids and showed better results in inducing cell differentiation than Matrigel [54]. However, whether mixed hydrogels with medium biological complexity can be used for intestinal organoid culture with excellent performance and whether their biological safety can meet the requirements of clinical application still require experimental verification.

4 The application prospects for intestinal organoids in nano–intestine interactions

The continuous development of nanotechnology has now ushered in its widespread use in the fields of food and medicine [55]. Correspondingly, the intestinal exposure to nanomaterials has gradually increased [56]. Therefore, it has become increasingly important to scrutinize the biological effects of nanomaterials on the intestine. When nanomaterials enter intestine, they may not only interact with IECs, gut-associated lymphoid tissue (GALT), and intestinal microbiota, but also have distal effects on the brain, liver, and lung through intestine-governed delivery to the broader organism [9, 57, 58]. In addition, various nano-therapeutic systems have achieved remarkable results in the treatment of inflammatory bowel disease (IBD) and colorectal cancer (CRC) [59, 60]; tissue engineering via nanotechnology and stem cell technology has introduced a new dawn for regenerative medicine [61]. However, as nano-intestine research has matured, traditional 2D cell models and animal models have become increasingly limiting to progress. As a 2D *in vitro* model, the cell line cannot veritably reflect the native situation, while animal models are resource intensive, often exhibit large differences between animals, and face bioethical limitations. The emerging 3D *in vitro* models of intestinal organoids effectively solve the problems associated with the above two traditional biological models. *In vitro* 3D

intestinal organoids can not only reproduce the native structure, function, and genetic characteristics of the tissue *in vitro*, but also are amenable to high-throughput screening and long-term amplification. In recent years, intestinal organoids have been a novel tool for nano-intestine research (Fig. 4) [14, 37].

4.1 Intestinal physiology and pathology models

A comprehensive understanding of the effects of nanomaterials on the intestine, as a critical target of ingested pollutants, is urgently needed. This is especially important in regards to the development of ISCs. It is well known that intestinal homeostasis depends heavily on the rapid regeneration rate of the intestinal epithelium via ISCs. A recent study revealed that carbon nanotubes with different modifications promoted the development of intestinal organoids by regulating extracellular matrix viscoelasticity and intracellular energy metabolism [10], indicating that intestinal organoids can be used as a physiological model to study the biological effects of nanomaterials. Unlike cell lines, intestinal organoids contain various IECs and the ability of long-term *in vitro* amplification, making them ideal systems for studying the physiological characteristics of IECs and the mechanisms of cell interaction *in vitro*. For example, the microfold cell (M cell) is a highly specialized IEC covering Peyer's patches (PPs). M cells absorb and deliver antigens and nanoparticles, playing a key role in intestinal mucosal immunity. Although several studies have shown that M cells originate from Lgr5$^+$ ISCs [62, 63], the mechanism of formation of M cells is not fully understood. With an eye towards this gap in our knowledge, Kanaya et al. used murine intestinal organoids (MIOs) to reveal that the NF-κB signaling pathway, mediated by TNF receptor associated factor 6 (TRAF6), is central to the development of M cells [64]. In addition, intestinal organoids can also be used to explore the interactions between different IECs. Paneth cells are secretory IECs located at the basement of crypts. These cells not only secrete antibacterial peptides, but also constitute the niche required for the development of ISCs [65]. Sato et al. demonstrated that Paneth cells can secrete EGF, TGF-α, Wnt3, Notch ligand Dll4, and other niche signals to maintain the normal development of ISCs [66]. In another study, Rodriguez-Colman et al. also used MIOs to demonstrate that Paneth cells can enhance mitochondrial oxidative phosphorylation in Lgr5$^+$ ISCs by providing lactic acid [67]. The reactive oxygen species (ROS) produced by mitochondrial oxidative phosphorylation further activated the p38/MAPK signaling pathway, thereby promoting the proliferation and differentiation of ISCs. The researchers thus used MIOs to reveal the interaction between Paneth cells and Lgr5$^+$ ISCs from a metabolic perspective. Given above studies, intestinal organoids have shown great potential to explore the intestinal biological effects and structure–activity relationships of nanomaterials as a novel physiological model [68, 69].

Recently, an increasing number of reports have shown that nanomaterials may elicit distal effects on the brain through the gut–brain axis [9, 70]. However, the complex gut–brain axis structure and multiple influencing factors in animal models limit the advancement of this field. Fortunately, intestinal organoids and nanotechnology make it possible to establish a representative gut–brain axis model *in vitro*. For example, Kaelberer et al. successfully constructed a gut–brain axis model with nerve conduction function *in vitro* by co-cultivating MIOs or purified enteroendocrine cells with vagus neurons. With the help of optogenetics and whole-cell patch-clamp recording technology, they achieved real-time monitoring of nerve electrical signal speed of transduction [71]. Using this model, they found that intestinal endocrine cells form synapses with vagal nodose neurons through presynaptic adhesion proteins, thus linking the intestine with the brain stem. Intestinal endocrine cells use glutamate as a neurotransmitter to transduce intestinal food stimulus signals to the brain within a few milliseconds, thereby broadcasting dietary

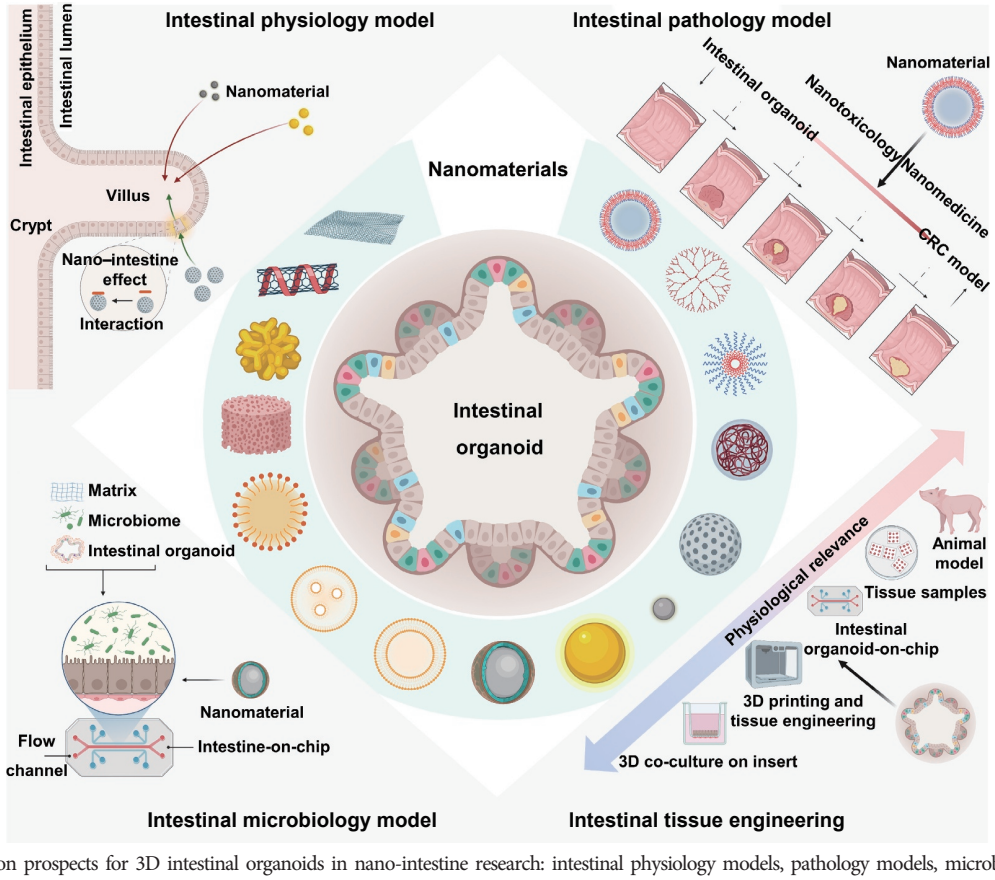

Figure 4 Application prospects for 3D intestinal organoids in nano-intestine research: intestinal physiology models, pathology models, microbiology models, and tissue engineering.

information to the brain. In order to simulate the physiological structure of the native gut–brain axis more realistically, there remains a need for an integration of representative circulatory, immune, and nervous systems to connect intestinal organoids with brain organoids, which is undoubtedly a formidable challenge. It is worth noting that, although some studies have begun to unravel the complex interactions between nanomaterials and typical IECs, research on rare populations of IECs is scarce. Combining the intestinal organoid model with single-cell sequencing technology may be a powerful tool for advancing this area [72, 73].

It is well known that IBD is difficult disease to model. Because patient samples usually cannot be amplified *in vitro* for an extended period of time, while artificial IBD models often fail to reflect the heterogeneity of native IBD. Although several clinical drugs have been developed for the treatment of IBD, patients often experience relapse and exhibit drug resistance after multiple administrations, and some patients even show resistance to traditional treatment methods from the outset [74]. For example, infliximab is only effective in 60%–87% patients, and 23%–46% patients relapse after treatment, exhibiting tolerance [75]. The refractory nature of IBD in only a subset of cases exemplifies the heterogeneity of patients and complicated pathogenic mechanisms, such as intestinal flora imbalance, allergic reactions, pattern recognition receptor-associated gene polymorphisms (such as *NOD2* and *ATG16L1*), cell autophagy, and physical barrier defects [76]. Each pathogenesis has its own characteristics and IBD is often induced by multiple pathogeneses, which is a root cause for the difficulty in both complete recovery and the establishment of representative models [77]. Moreover, some recent reports have demonstrated that nanomaterials can both induce and treat IBD [9, 78, 79]. On the one hand, intestinal exposure to nanomaterials may induce IBD by destroying the intestinal physical and chemical barriers, disrupting the intestinal flora, activating the intestinal mucosal immune response, etc. In consideration of these mechanisms, intestinal organoids can be used as normal physiological models to study the potential nano-toxicity and associated mechanisms of different nanomaterials. On the other hand, a large number of nano-therapeutic systems have achieved remarkable results in the treatment of IBD. To this end, intestinal organoids can be used as pathological models of IBD to study the therapeutic effects of different nano-therapeutic systems. Unlike traditional cell lines and animal models of IBD, intestinal organoid models derived from patients can reproduce the intestinal tissue structure and genetic characteristics of patients *in vitro*, enabling high-throughput screening of treatment options, thus meeting the needs of personalized medicine [80]. However, intestinal organoid models are still inadequate in recapitulating three features of IBD *in vitro*: (1) inducing functional M cells, (2) building a complete immune system, and (3) harboring a representative intestinal microbiome. To address these challenges, Rouch et al. successfully used recombinant RANKL protein, an NF-κB ligand that induces the expression of SpiB transcription factor and drives the differentiation of M cells, to induce a full complement of functional M cells in the human intestinal organoid (HIO) model [81]. However, there still remains the difficulty for constructing an *in vitro* intestinal organoid model with a comprehensive immune system and intestinal microbiome.

Intestinal organoids are extremely suitable for gene editing. With the recent rise of CRISPR/Cas9-related gene editing technology, it has become possible to perform precise gene editing of intestinal organoids [82]. Genetic polymorphisms, such as alleles of *NOD2* and *ATG16L1*, are a major feature of IBD [83]. Gene editing can be used to disrupt or overexpress specific genes to study their mechanisms of action and/or the relationships between different genes involved in IBD [84]. Additionally, intestinal organoids can be used to study the intestinal fibrosis caused by IBD. The damaged tissues in IBD are mainly repaired by mesenchymal stem cells (MSCs) and the ECM. If ECM aggregation is lacking, ulcers or fistulas can readily form. In contrast, if ECM aggregates excessively, hyperplastic connective tissue may develop [85]. Many traditional *in vitro* models cannot simulate this process due to the absence of intestinal mesenchyme. However, iPSCs/ESCs-based induced intestinal organoids are able to model intestinal fibrosis *in vitro*, because they have both IECs and intestinal mesenchymal components [86].

With the extensive exploration conducted over the past two decades, nano-systems with complex structures and physicochemical properties, and unique advantages over traditional methods, have been utilized to realize breakthrough results in the prevention, diagnosis, and treatment of CRC [87–89]. However, numerous nanomaterials have exhibited biosafety problems, especially carcinogenic risks. For example, the international chemical secretariat (ChemSec) has added carbon nanotubes (CNTs) to the substitute it now (SIN) list in 2019 because they are carcinogenic and persistent, and may damage the reproductive system [90]. In fact, CNTs became the first nanomaterial on the SIN list [91].

The construction of a representative CRC model has long been a research hotspot in the study of cancer. Cell lines and patient-derived tumor xenograft (PDTX) models are currently the most common models for CRC research [92]. However, the cell line model comprises only a single component and cannot accurately reproduce the biological and genetic characteristics of the primary tumor. PDTX models are limited by species differences, long modeling periods, and high cost [93]. Again, the intestinal organoid model can address the problems of these models. Intestinal organoid models based on patient-derived ISCs can recapitulate the tumor heterogeneity to the greatest extent, with the advantages of short culture period, low cost, facile construction, and high-throughput screening [94]. In addition, the combination of CRISPR/Cas9-related gene editing technology and intestinal organoid technology enables the investigation of the roles of specific genes in the development and progression of CRC, and the generation CRC models containing specific genetic mutations. For example, Matano et al. used CRISPR/Cas9 technology to introduce common CRC mutations, such as those in *APC*, *SMAD4*, *TP53*, *KRAS*, and *PIK3CA*, into normal HIOs to obtain CRC models with different phenotypes [95]. Furthermore, Fumagalli et al. transplanted intestinal organoids containing different mutant genes *in situ* to simulate the evolution from adenoma to colon cancer, thus revealing the roles of different signaling pathways (Wnt, EGFR, P53, and TGF-β) in tumorigenesis and cancer progression [96]. The above research presents encouraging results that intestinal organoids can provide a promising platform to study CRC, however it remains a challenge for this model to fully reconstruct the tumor microenvironment *in vitro*.

Although intestinal organoids possess great potential to model intestinal physiology and pathology, taking full advantage of their high-throughput screening potential to widen their applications has still not been adequately accomplished. The complexity and diversity of intestinal organoid phenotypes complicate the mapping of clear pharmacodynamic relationships. To address this, Lukonin et al. developed an imaging-based screening platform to characterize the phenotypic landscape of intestinal organoid development and infer functional genetic interactions [97]. The platform was able to screen 301 compounds with strong phenotypes with high reproducibility scores from 2,789 compounds, corresponding to 207 unique target genes. Using this

approach, they discovered a retinoid X receptor inhibitor that improves intestinal regeneration *in vivo*.

Artificial intelligence and machine learning methods based on big data have been widely used in the screening of the biological effects of nanomaterials [98]. Thus, novel nanomaterial-intestinal organoid screening platforms can be established, and the biological effects of nanomaterials in other biological models can be mutually verified, so as to guide the experimental design and the establishment of evaluation criteria. However, several obstacles, such as the lack of high-quality data for training and validate models, and how to encode the complex phenotypes of intestinal organoids and different physicochemical properties of nanomaterials into mathematical descriptors, remain to be overcome. Nonetheless, applying machine learning to the high-throughput screening of nanomaterial-intestinal organoid biological effects are likely to provide valuable new findings in the near future.

4.2 Nano–intestinal microbiota interactions

Under the right circumstances, microbes can readily form regional or global infectious disease. In 2019, a new coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), brought about a global health crisis in only a few weeks. The virus causes coronavirus disease-2019 (COVID-19), which gradually develops from mild flu symptoms to lung damage and multiple organ failure to death [99]. The COVID-19 pandemic has sounded the alarm for the research of high-risk infectious diseases caused by microbes—more than trillions of microbes reside in the intestine [100, 101]. Numerous reports have shown that nanomaterials can affect the composition and function of intestinal microbiota [102]. In addition, many nano-therapeutic systems have shown great potential for wide applicability in intestine-related diseases caused by pathogenic microbes; some nanomaterials (such as Ag) are even inherently antibacterial [9]. Studying intestinal microbiota in animal models is extremely susceptible to interference from external conditions, with significant individual differences. Similarly, cell models do not represent the situation in the body. Thus, there is an urgent need for a representative *in vitro* model of intestinal microbiota. In recent years, the intestinal organoid model has emerged as a prominent strategy in fields focused on intestinal microbiota [103]. On the one hand, these organoids contain a complete intestinal epithelial system and lumen structure, similar to the native intestine. On the other hand, these models reflect the specificity of the donor source, which is extremely important to understanding the intestinal microbiota in patients or susceptible populations. Microbes are typically implanted into the lumen structure of intestinal organoids by microinjection. This modeling method is not only convenient and fast, but also capable of achieving stable repeat amplification [104]. Currently, intestinal organoids used in the study of intestinal microbiota research are mainly used to examine three types of gut inhabitants: bacteria, viruses, and parasites.

Intestinal organoids have been used in the research of common intestinal pathogenic bacteria, such as *Salmonella*, *Escherichia coli*, and *Clostridium difficile*. *Salmonella* is a gram-negative enteric pathogen responsible for greater than one million cases of gastrointestinal diseases worldwide every year [105]. Wilson et al. found that α-defensin secreted into the intestinal lumen can effectively inhibit the growth of *Salmonella* by microinjecting *Salmonella* into the lumen of MIOs [106]. In addition, Forbester et al. established a HIO model of *Salmonella* that exhibited specific transcriptomic changes [107]. Like *Salmonella*, *E. coli*, as a common intestinal inhabitant, can also pose health risks. Tse et al. used the intestinal organoid model to demonstrate that extracellular serine protease P (EspP) can induce enteropathogenic *E. coli* (EHEC) diarrhea through calcium ion mediated induction [108]. In addition, Rajan et al. used the HIO model to demonstrate that enteroaggregative *E. coli* exhibits different adhesion characteristics depending on the intestinal section from which the HIO is derived [109]. Thus, the specific characteristics of the donor source can be used to design intestinal organoid models from different intestinal sites and/or cases to accurately study the pathogenic mechanisms of *E. coli*. Intestinal organoids can also be used to investigate anaerobic bacteria. *C. difficile* is a common anaerobic bacterium that causes antibiotic-associated diarrhea. Engevik et al. used the HIO model to find that *C. difficile* infection reduces Na^+/H^+ exchanger 3 (NHE3) and mucin-MUC2 expression, thereby disrupting the homeostasis of the intestinal microbiota [110].

Intestinal organoids have also been used in the research of enteroviruses, such as human rotavirus (a double-stranded RNA virus) and human norovirus (a single-stranded RNA virus). These two viruses are the most important viruses in cases of acute gastroenteritis. Compared with cell models, the replication rate of human rotavirus in intestinal organoids can be up to tenfold greater [111]. Additionally, human rotavirus has shown to be more infectious in differentiated IECs [112]. The intestinal organoid model has also been used to reveal that interferon can efficiently inhibit the replication of human rotavirus [112–114]. Importantly, human rotavirus exhibited different degrees of replication in intestinal organoid models from different patients, indicating that this model may be well-suited to personalized medicine [115]. Human norovirus exhibits poor replication capacity in cell models, and the lack of an ideal *in vitro* model limits research on this pathogen. However, the virus has shown good replication in intestinal organoid models, predominantly replicating in IECs, and also shows specificity to different intestinal sections. Some human noroviruses require a special intestinal environment (such as bile) to replicate well [116, 117]. Compared with intestinal bacteria and enteroviruses, the health problems caused by intestinal parasites are relatively few, but, once established, intestinal parasites exhibit strong survivability, so this health hazards still warrants research attention. Heo et al. examined *Cryptosporidium* using HIOs, finding that *Cryptosporidium* parasitized mainly absorptive IECs, with the infection activating intestinal innate immune-associated genes [118].

Recently, Monteil et al. used engineered human blood vessel organoids and human kidney organoids to demonstrate that human recombinant soluble angiotensin converting enzyme 2 (hrsACE2) can inhibit early infections of SARS-CoV-2 [99]. Indeed, differentiated IECs also highly express the SARS-CoV-2 receptor, angiotensin converting enzyme 2 (ACE2). Hans Clevers et al. used the HIO model to show that SARS-CoV and SARS-CoV-2 can readily infect IECs and replicate, indicating that the intestine may be another target organ of these viruses [119]. Similarly, Zhou et al. co-incubated SARS-COV-2 isolated from the feces of COVID-19 patients with bat and human intestinal organoids *in vitro* to show that SARS-COV-2 can infect bat and human IECs and replicate robustly, further suggesting that the intestinal tract is a likely transmission route of SARS-COV-2 [120]. Therefore, organoid models clearly possess great prospects for applications in the study of global microbial infectious diseases. However, to make intestinal organoids truly an ideal *in vitro* model for studying the interactions between nanomaterials and intestinal microbiota, several limitations remain to be resolved. First, existing models mainly inoculate the microbes into the lumen of intestinal organoids by microinjection. Although this method can effectively eliminate interference and improve the reliability of results, the composition of intestinal microbiota in a

bona fide intestine is extremely complicated, so the experimental results obtained using the intestinal organoid model may differ significantly from the actual situation, however this has not been adequately tested. Second, intestinal mucosal immunity plays a key role in the process of intestinal microbial infection, but current intestinal organoid models do not include a complete immune system. Were it possible to successfully construct the immune system in an intestinal organoid model? This would bring us one step closer to having an ideal model. Finally, in order to use the intestinal organoid model to research the interactions between nanomaterials and intestinal microbiota, it is also necessary to fully understand ideal introduction methods of nanomaterials. Should they be microinjected with the intestinal microbiota or on their own? Or should the nanomaterials be mixed into the culture medium? How the different exposure methods may affect the experimental results remains to be explored. Only by establishing standardized operating procedures can the experimental results of different laboratories be objectively compared.

4.3 Intestinal tissue engineering

Intestinal organoids can recapitulate the composition and structure of the intestine, thus presenting a therapy strategy for intestinal tissue regeneration. Indeed, two powerful methods have been developed to use intestinal organoids in this way. The first is the direct implantation of intestinal organoids into the damaged intestinal sites to achieve tissue repair. This method is suitable for local intestinal damage caused by IBD (Fig. 5(a)). The second method entails the construction of a biocompatible intestinal 3D scaffold *in vitro*, with the help of nanotechnology, followed by inoculation of the intestinal organoids onto the surface of the scaffold to form a complete intestinal tissue *in vitro* prior to transplantation into the body. This latter approach is suitable in the treatment of the loss of large segments of healthy intestine, such as short bowel syndrome (SBS; Fig. 5(b)). In order to achieve intestinal tissue regeneration, the simple intestinal organoid technology is far from sufficient and needs the support of nanotechnology, such as biomimetic ECMs and 3D scaffolds, for more complete tissue engineering.

In general, IBD can be categorized into two general forms: ulcerative colitis (UC) and Crohn's disease (CD). Intestinal mucosal injury and ulcer formation are common phenomena in IBD patients. In mild cases, current treatments have achieved acceptable therapeutic effects, but for more severe cases, traditional treatments failed to achieve remission, largely because ISCs cannot accomplish self-healing in the damaged areas. Furthermore, cumulative mutation driven by damage carries the risk of inducing IBD-associated cancer [74]. In 2012, Yui et al. attempted to repair IBD injury using intestinal organoids [121]. To accomplish this, the authors delivered GFP^+ colonoids into the damaged intestinal lumen by enema in a dextran sulfate sodium (DSS)-induced acute colitis mouse model (immune-impaired *Rag2*$^{-/-}$). They found that the transplanted colonoids efficiently integrated into the damaged colon to form a monolayer of colonic epithelium which presented the same structure as the surrounding healthy tissue after four weeks. Using a similar transplantation protocol, small intestinal organoids from fetal mice or induced intestinal organoids successfully colonized the colon and exhibited a colonic phenotype [122]. Interestingly, when enteroids (based on $Lgr5^+$ ISCs from adult mice) were transplanted into the murine colon, they grew normally but displayed a mature small intestine phenotype rather than that of the colon [123]. Their findings indicate that the mesenchymal component of the damaged colon can provide a scaffold and the developmental signals that mediate the integration of the transplanted intestinal organoids into the recipient organ. These signals induced immature cells to differentiate into colonic epithelial cells but not to such a degree that they interfere with the adult small intestinal cells. In another study, Sugimoto et al. successfully transplanted human colonoids into the damaged colon of immunodeficient mice, ultimately confirming the feasibility of using intestinal organoids for intestinal tissue regeneration [124]. However, to fully translate intestinal organoid technology into clinical application, the mysteries of Matrigel must be unlocked. In addition, current transplantation methods mainly deliver intestinal organoids into the lumen by enema or endoscope, requiring that the anus be sealed to effectively retain the intestinal organoids in the lumen, which is obviously undesirable. It is expected that nanotechnology can be judiciously employed to solve the above problems. Biomimetic ECMs based on nanotechnology can not only replace Matrigel, but also assist in the colonization of intestinal organoids

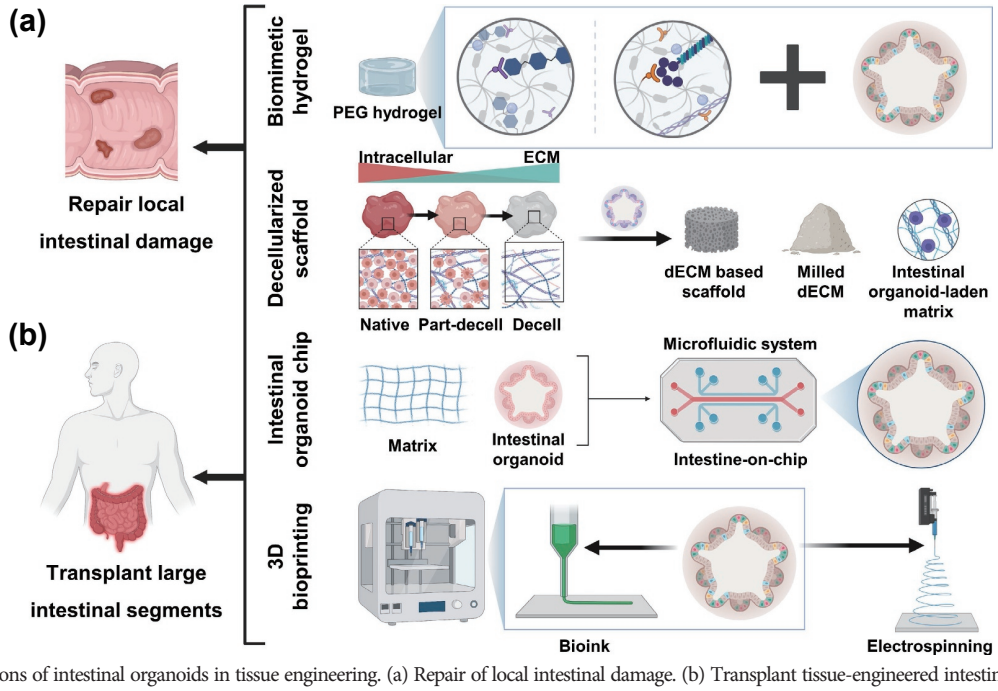

Figure 5 Applications of intestinal organoids in tissue engineering. (a) Repair of local intestinal damage. (b) Transplant tissue-engineered intestine to treat the loss of large segments of healthy intestine.

at the damaged site, and, with the targeting capability of nanotechnology, intestinal organoids can be delivered to specific sites of the intestine, thus improving the efficiency and feasibility of transplantation.

It is clear that transplanting intestinal organoids directly into the intestinal lumen can repair local damage or ulcers, however this strategy is insufficient for patients who have lost a large portion of healthy intestine (such as SBS). In these cases, the survival rate after intestinal organoid transplantation is relatively low and the prognosis is poor. Therefore, it is necessary to develop new therapeutic methods for SBS and other similar disorders [125]. Recently, tissue-engineered intestines based on intestinal organoids and nanotechnology have emerged and shown great therapeutic potential. Among them, 3D printing technology was used to design a biologically inert external mold conforming to the intestinal structure. A synthetic biological scaffold and intestinal organoids can be inoculated onto the surface of the mold, or the intestinal organoids may be directly inoculated onto the synthetic biological scaffold [126, 127]. Finally, the mature and tissue-engineered intestine is transplanted into the body, or the initial tissue-engineered intestine is first transplanted into the abdomen of immunodeficient animals, so that it develops into a complete intestine, and then transplanted to the desired location in the patient. For example, Finkbeiner et al. inoculated HIOs onto synthetic polyglycolic/poly L-lactic acid (PGA/PLLA) scaffolds and found that the constructs were able to develop into complete intestinal tissue, which was almost identical to the adult intestine, whereas a decellularized pig intestinal matrix was inadequate to generate such tissue [128]. Their data reflect the unique advantages of bio-scaffolds synthesized using nanotechnology in the field of intestinal tissue regeneration. However, several improvements are still required to fully realize wide clinical application of tissue-engineered intestine.

First, it is necessary to consider the influence of biomechanics to rationally approach tissue engineering design [129]. Even without biochemical gradients, micro/nanofabrication can drive intestinal organoids to form controllable tissue structures by judiciously altering the geometry of the scaffold. Intestinal stem cells tend to aggregate at the inwardly concave ends of the lumen, which is consistent with the physiology of intestinal crypts, while the protruding ends form the villus structure. The patterned design can direct intestinal organoid budding, preceding the differentiation of Paneth cells and subsequent differentiation. The heterogeneity of Yap1 activity ultimately dictates the "villus" and "crypt" domains by inhibiting stem cell fate and localizing Notch-mediated Paneth cell differentiation, respectively. The topology of the scaffold enables the formation of different ECM mechanical properties in different parts of the intestinal organoids, thus governing the symmetry breaking, thereby facilitating standardization [130]. Nikolaev et al. designed a scaffold capable of long-term *in vitro* culture of intestinal organoids, and thus generated a tissue-engineered intestine [131]. Using a microfluidic system, the mini-intestine was able to self-renew and maintain viability for several weeks, without passaging. In addition, the mini-intestine was able to reproduce some rare and functionally important, specialized cell types that are difficult to obtain in conventional intestinal organoid culture models. This construct was also able to perfectly mimic intestinal injury-repair processes and microbial infection. Second, the construction of blood vessels, immune system, and nervous system on the tissue-engineered intestine requires further advancement. Several studies have shown that the co-culture of endothelial cells, immune cells, and nerve cells with intestinal organoids, or an *in vivo* intestinal structure can be imitated on an *in vitro* mold by etching followed by transplantation of cells into the pores for co-culture, and can build blood vessels, immune system, and nervous system *in vitro* [132–134]. Although these early attempts have achieved initial success, this work is still in its early days and standards for the constructions of tissue-engineered intestines have not yet been realized.

5 Conclusions and prospects

The limitations of traditional cell and animal models make them inadequate for the research of nano–intestine interactions. Cell models cannot reproduce the structure and composition of the intestine *in vitro* and animal models are overly complex in their construction, exhibit large individual differences, and are constrained by ethical considerations. To overcome these problems, the 3D intestinal organoid model, with its unique advantages, has emerged as an ideal tool for the research of nano–intestine interactions [135]. In particular, enteroids/colonoids have the specificity of donor source, and patient-derived HIOs in particular can guide the personalized medicine based on a nano-therapeutic system. It is noteworthy that induced intestinal organoids possess the phenotype of the embryonic intestine, containing intestinal mesenchymal components, providing a unique platform to study the mechanisms of the interactions between nanomaterials and embryonic intestine or intestinal mesenchyme.

Although great progresses have been made towards understanding the construction and further application of intestinal organoids, many challenges remain before clinical adoption of the technology (Fig. 6). For instance, the intestinal organoid model lacks complex blood vessels, immune systems, nervous systems, gut microbiota, and interactions with distant organ. Furthermore, the alternative well-defined biomimetic ECM is also a major hurdle to clinical translation. In general, Matrigel is the most widely used native ECM, with its optimal support in culture, however the large differences between batches, poor stability, immunogenicity, and tumorigenic risks restrict the wide application of this ECM. Alternative biomimetic ECMs that can replace the current culture scaffold are thus required. Through the study of native ECMs, it has been found that anchor points, developmental signals, and mechanical strength are the key considerations in designing ECMs that can play the role of 3D scaffolds. With this goal, biomimetic ECMs based on nanotechnology have been designed. For instance, the first-generation biomimetic ECMs solved many shortcomings of native ECMs, but these materials cannot be dynamically regulated with the development of intestinal organoids. Consequently, "smarter" second-generation biomimetic ECMs have been designed to achieve reversible changes through weak interactions or dynamic covalent bonds. These matrices perfectly meet the dynamic requirements for the mechanical strength of ECMs at different developmental stages of intestinal organoid maturity. In addition, nanotechnology can be used to achieve real-time monitoring of parameters during the development of intestinal organoids. Furthermore, biomimetic ECMs can be artificially adjusted by external stimulation to realize the optimal growth conditions. With the improvements in nanotechnology, greater requirements for biomimetic ECMs have been proposed. The 4D culture model that incorporates the dimension of time is now a developing trend in the design of biomimetic ECMs.

Although intestinal organoids can be used as a physiological or pathological model to study nano–intestine interactions, there are still many considerations that require more thorough understanding. One such example is the method of introducing nanomaterials into organoids. Different methods of exposure to

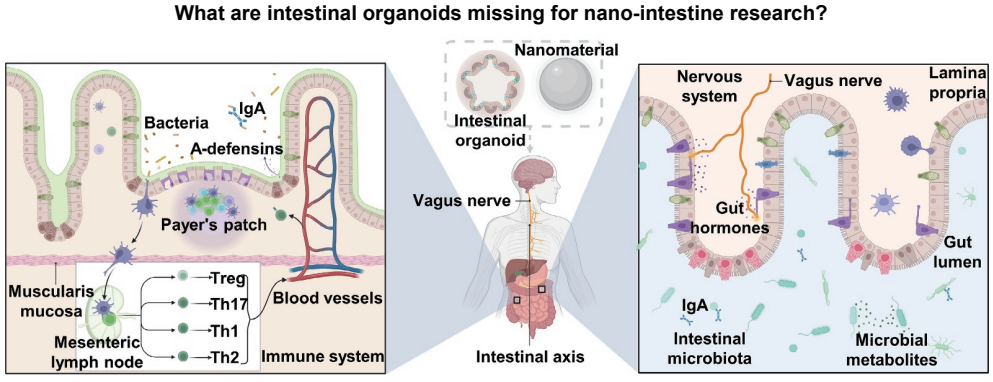

Figure 6 The challenges facing the application of intestinal organoids in the research of nano–intestine interactions. The intestinal organoid model lacks complex blood vessels, an immune system, a nervous system, intestinal microbiota, and interactions with distant organs.

the nanomaterials (e.g., nanomaterials included in the ECM or in the medium) affect their behavior in the intestinal organoids. Additionally, the binding of a gelatinous ECM to nanomaterials affects the scaffold hardness of the ECM and effective exposure dose of nanomaterials. If only by establishing standardized operating procedures and evaluation criteria, can the results of different laboratories be objectively compared, to clarify, for example, the dose-effect relationship between nanomaterials and the intestine? Moreover, the fusion of nanotechnology and intestinal organoids opens up new avenues for intestinal regenerative medicine research and treatment. Further development of tissue-engineered 3D scaffolds generated by micro/nanofabrication technology will soon realize standardized intestinal organoid fabrication, which will close the gap between research and clinical application. Considerable focus is now on building a tissue-engineered intestine that contains blood vessels, an immune system, and a nervous system, however it is exceptionally difficult to artificially construct such a complex biological system *in vitro* using the current technology. Additionally, the interactive network between the various systems of the intestine is extremely complicated; *in vitro* simulations of these interactions should be considered in future work.

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