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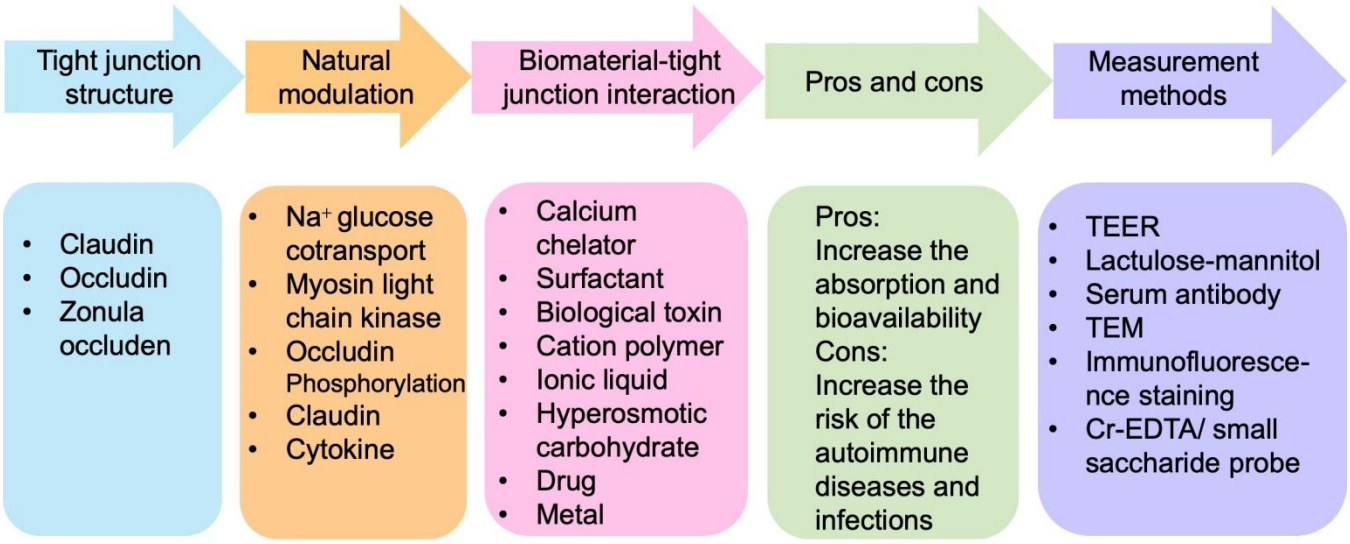

Biomaterial-tight junction (TJ) interactions: Analyses of the TJ structure and natural modulation, interaction mechanism, potential impact and measuring methods.

Biomaterial-tight junction interaction and potential impacts

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Abstract
The active pharmaceutical ingredients (API) have to cross the natural barriers and get into the blood to perform the pharmacological effects. The tight junctions (TJs) between the epithelial cells serve as the major selectively permeable barriers and control the paracellular transport of majority hydrophilic drugs, in particular, peptides and proteins. TJs perfectly balance the targeted transport and the exclusion of other unexpected pathogens under the normal condition. Many biomaterials have shown the capability to open the TJs and improve the oral bioavailability and targeting efficacy of the API. Nevertheless, there is a limited understanding of the biomaterial-TJ interactions. The opening of the TJs further poses the risk of autoimmune diseases and infections. This review article summarizes the most updated literature and pre-sents insights on the TJ structure, the biomaterial-TJ interaction mechanism, the benefits and drawbacks of TJ disruption, and methods for evaluating such interactions.

1. Introduction

Transportation across the biological membrane has been investigated for decades to improve bioavailability and efficacy of the API. There are three major natural barriers in human bodies — intestine, skin and blood-brain barrier, which control the mass exchange between the interior and exterior environment. Oral, transdermal and brain-targeting formulations of API has been developed to address these barriers respectively, enabling APIs to cross these obstacles and arrive at the systemic circulation or get into the acting sites.

In general, there are two pathways to transport or deliver APIs: transcellular and paracellular. For the transcellular way, the API molecules pass the epithelial/endothelial cell membrane either through the endocytosis or transporter mediated pathway, depending on their molecular characteristics.[1] Most hydrophobic APIs cross the membrane through this way, especially for those with small molecular weights. For the hydrophilic APIs such as peptides and proteins that attract increasing attention, the most common route is paracellular, majorly by going through the TJs of the biological barriers.

Many biomaterials have been tested for facilitating the delivery of APIs, in particular, peptides and proteins. These biomaterials played a significant role by interacting with the TJs, transiently opening the biological barriers, and allowing the APIs to reach the systemic circulation or the brain region. For example, GIPET® technology has been used to develop the oral insulin and polypeptides formulation. This platform is based on mixing APIs with the intermediate-chain fatty acids such as caprate sodium to enhance the duodenum absorption. The TJs can be opened transiently, allowing insulin or peptides to cross the intestinal barrier and reach the systemic circulation. The negative effect of opening the TJs, however, has not been fully understood. A successfully developed API formulation is expected to have higher bioavailability, better pharmacological performance and decreased side effects with controlled interaction with the TJs.

This review article focused on biomaterial-TJ interaction and its potential impact on paracellular transportation, and peptide and protein delivery (outline illustrated in Figure 1). Mechanisms for biomaterials to interact with TJs were summarized, and the pros and cons of opening the TJs were discussed. This article further presented insights on relevant reliable measurement methods used in labs and clinics to characterize TJ opening, which is critical for the research in this area, and further development of delivery systems with improved clinical outcomes.

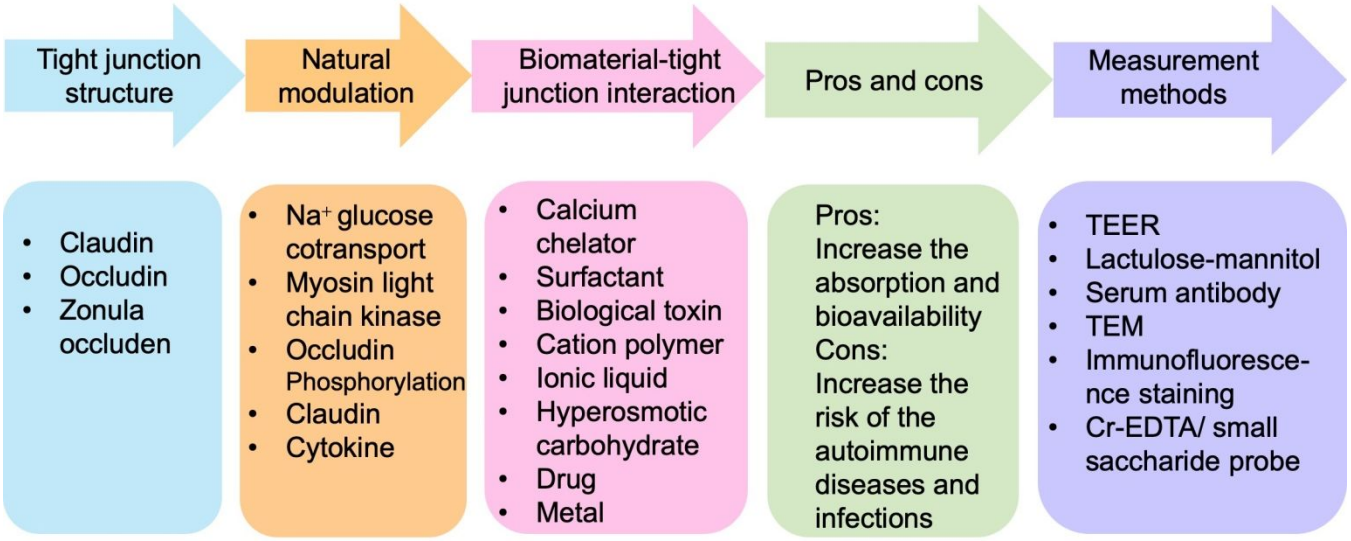

Figure 1. Biomaterial-tight junction (TJ) interactions: Analyses of the TJ structure and natural modulation, interaction mechanism, potential impact and measuring methods.

2. Tight junction structure

Paracellular usually refers to the intercellular junctions which consists of the TJs, adherens junctions and desmosomes from apical to basal direction.[2] (Figure 2) These three junctions consist of a packed connection of actin and myosin surrounding the apical elements and supporting the cortical actin which facilitates the formation of the dense microvillus barrier.[3] TJs are multi-protein complexes consisting of transmembrane proteins, peripheral membrane proteins, and modulatory molecules such as kinases. [4,5] The TJ is a specifically permeable barrier which is the rate-limiting step of paracellular transport. The adherens junction and desmosome play no roles of limiting/controlling paracellular transport, but provide essential adhesive force and mechanical support for the integrity of the entire paracellular junctions, e.g., to maintain the cell contact and TJ assembly. When TJs are disrupted, the adherens junction and demosome will also be likely to be destroyed, which inversely facilitates further disruption of the TJs.

The claudin family is of the most importance among all the transmembrane proteins required for TJ assemble, which affects the TJ permeability for ions. Claudins expression varies on different tissues, and the corresponding organ function can be potentially compromised even with a mutation of single member. Another family of transmembrane proteins, occludins, is associated with the intramembrane strand of the actin filament and modulates the diffusion of hydrophilic molecules. Peripheral membrane proteins, such as zonula occludens 1 (ZO1) and ZO2, are of great importance to TJ assembly and stability, partly because they have interaction domains with other proteins such as occludin, actin and claudins.

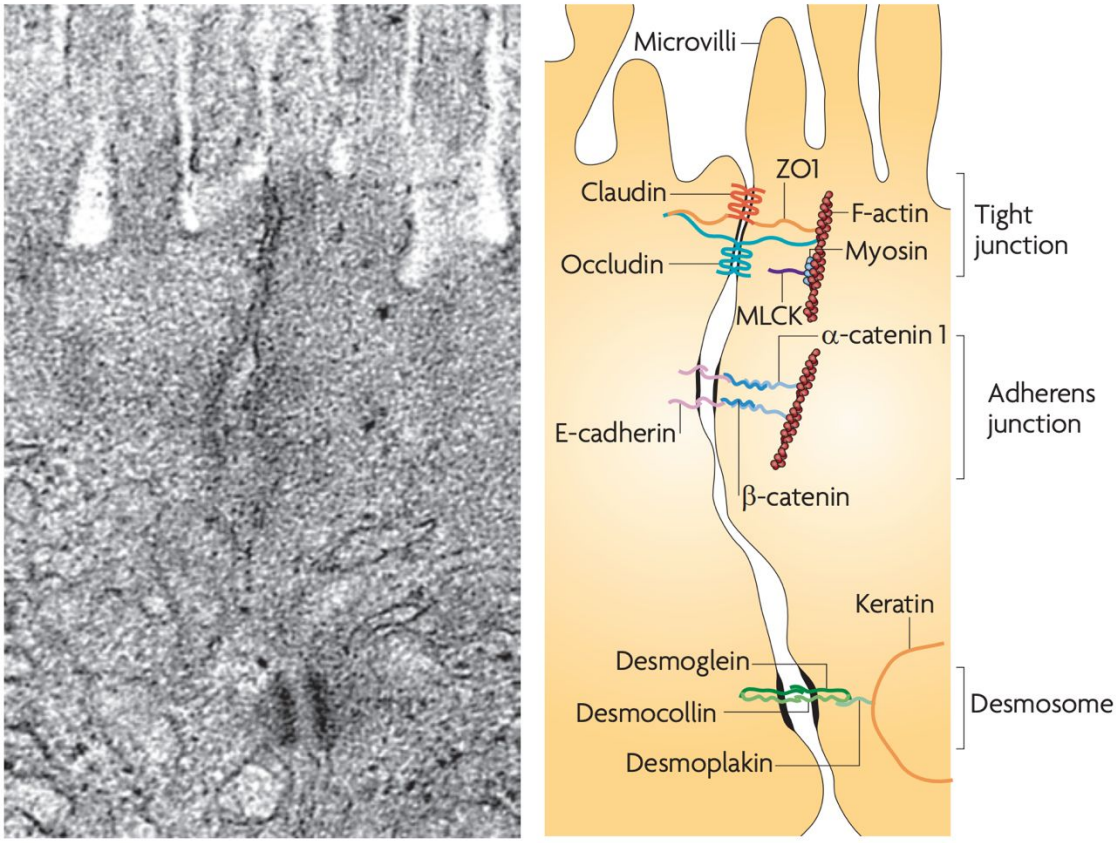

Figure 2. An electron micrograph photo and an artificial diagram for a paracellular junction consisting of tight junction, adherens junction, and desmosome. Reprinted with permission from Ref. [6], 2009 Springer Nature.

Paracellular transportation across the TJs can be achieved through both leak and pore pathways. The leak pathway is solely size-dependent with low capacity whereas the pore pathway is both size and charge-dependent with high capacity. The leak pathway has charge-independent selectivity and enables the transportation of large solutes such as proteins and bacterial lipopolysaccharides. It is mainly controlled by myosin light chain kinase (MLCK) and occludin [7,8,9] Materials whose radius is larger than 10μm cannot get through the channel, but they could be transported in the presence of cytokines like tumor necrosis factor (TNF) and interferon-γ (IFN) overexpression. The pore pathway has both size and charge selectivity, which is attributed to TJ-associated claudin proteins.[10,11] These pores enable various level of permeability of cations and anions across different epithelia, and could exclude molecules larger than 4 Å[12,13]. Therefore, TJs have the ability to exclude compounds based on size and charge and are also modulated by physiological and pathophysiological conditions. When TJs are disrupted, unlimited paracellular transportation of large proteins and molecules can be achieved. In this case, bacteria and viruses may also get through the barrier (e.g., intestine) and cause potential inflammatory disease.[14,15]

3. Physiological modulation of tight junctions

The TJ protein complex is an extremely dynamic system and undergoes continuous physiological modulation and remodeling. Epithelial cells were analyzed using photobleaching and fluorescence recovery methods to evaluate TJ dynamics. It was reported that 76% of claudin was firmly localized at the TJ. Occludin (71 %) diffused fast within the TJ membrane surface, while 69 % of ZO-1 diffused between the membrane and the cytoplasm in an energy-dependent manner.[16] The permeability of the barriers is also modified, as a consequence, and significantly varies in response to natural physiological modulation pathways as discussed below.

3.1 Na^+ glucose cotransport

The most well-documented example of physiological modulation of intestinal barriers is that sodium-glucose cotransport activation increases the paracellular transport of substances. The sodium and glucose cotransportation could activate the epithelial myosin light chain kinase (MLCK) and generate an osmotic gradient across the epithelia. MLCK activation enhances the TJ permeability through the size-selective pore pathway [17] while osmotic gradient enables the increase of the paracellular water influx across the TJs. Since most nutrients are soluble in water and could be transported along with the water, this process could dramatically increase the paracellular transport of nutrients.

3.2 Myosin light chain kinase (MLCK)

Myosin light chain kinase (MLCK) is a calcium/calmodulin-dependent serine/threonine kinase which phosphorylates the regulatory myosin light chains of myosin II. There are two isoforms of MLCK (MLCK1 and MLCK2) expressed in intestinal epithelia with different subcellular localization and functions. MLCK1 is mainly present in the villi and centralized around the junctional actomyosin loop, whereas MLCK2 is predominantly distributed along the crypt villi. MLCK 1 overexpression resulted in the TJ barrier disruption while MLCK 2 upregulation is not well understood.[18] The occludin, peri-junctional actin and ZO-1 reorganization are related with MLCK expression.[19]

3.3 Occludin phosphorylation

Occludin is a basic plasma-membrane protein which is located at the TJs, with the molecular weight around 65kDa. It plays an important role in the TJ's assembly, stability and barrier function. Studies showed that mice with occludin knocked-down had increased intestinal paracellular permeability. Under normal conditions, occludin dramatically gets phosphorylation on the threonine and serine residues, which contributes to the TJ maintenance and assembly.[20] Under circumstance when the occluding tyrosine is phosphorylated, the connection between occludin with ZO-1 is weakened leading to disconnection of the junctional complex. In addition, a protein kinase C, PKC, is involved in the occluding phosphorylation at the threonine residues. When PKC is inhibited, occludin and ZO-1 distribution in the junctional complex is disrupted and thereby the epithelial barrier function is undermined.

3.4 Claudin

Claudin is another important component protein of the TJs and plays a critical role in the barrier function. TJs establish the gate that regulates the molecule flow between the epithelial cells. This is largely based on the strand formation between claudins. The extracellular rings of claudin protein interact with abutting cells; this enables the targeted molecules to bypass barriers or channels in the paracellular pathways.[21] Claudins are known as the predominant element of TJs.[22] Mice with claudin-1 knockdown died within one day, owning to a severe fluid loss along the leaky epithelial barrier. Claudin

belongs to a family of 24 members, and each expression varies from tissues to cell lines.[23] Some of them are phosphorylated and lead to the delocalization and increased permeability.[24]

3.5 Cytokines

Cytokines are glycoproteins or peptides which are secreted by immune cells. They are the most important signaling molecules that modulate cellular activities, inflammation and hemostasis *in vivo*. The cytokines released upon pathophysiological stimuli play an important role in the modulation of the leak and pore pathways. They activate the immune system and cause tissue inflammation, resulting in the opening of TJs. For example, Interleukin 13 (IL-13) could activate the MLCK, increase the claudin-2 expression, and facilitate the pore permeability.[25] In addition, interferon-γ, IL-6, IL-9, and tumor necrosis factor (TNF) are also involved in the TJ modulation process. Moreover, certain growth factors such as epidermal growth factor and transforming growth factor- β are essential for the defense and maintenance of the TJ integrity.[26,27] The impact of cytokines on the TJs and the associated action mechanisms were summarized in Table 1.

Table1. Cytokines and their impact on the permeability and action mechanism

Cytokines	Permeability	Mechanism of action	Reference
Interferon-γ	↑	(1). Tight junction proteins relocation and actin cytoskeleton reorganization. (2). IFN-γ enhances actinmyosin shrinkage through the kinase and tight junctions' internalization	[28]
Tumor Necrosis Factor-α	↑	(1). MLCK expression and MLC phosphorylation and ERK1/2 activation of Elk-1. (2). The involvement of phosphatidyl inositol-3 kinase (PI3K)/Akt signaling.	[29,30,31]
Interleukin-1β	↑	Decrease occluding relocation and expression and increase MLC and MLCK phosphorylation.	[32, 33]
Interleukin-6	↑	Increase claudin-2 and protein kinase(MLK) /extracellular kinase (ERK), and activate the PI3K/Akt pathway	[34]
Interleukin-10	↓	An anti-inflammatory cytokine, which lessens the IL-6, TNF-α, IL-1β expressing level	[35, 36]
Interleukin-17	↑	Increases claudin-1 and -2.	[37]
Interleukin-22	↑	IL-22-dependent claudin-2 overexpression causes diarrhea.	[38]
Epidermal Growth Factor	↓	Activation of EGF receptor-phospholipase-γ-PKCβ1/ε and EGF receptor-MEK/ERK signaling.	[39,26]
Transforming Growth Factor-β	↓	Induce the claudin-1 expressing by the MEK/ERK-dependent signaling.	[27]

4. The mechanism of biomaterials interacting with tight junctions

To further develop desirable protein and peptide delivery systems to cross the TJ barriers, it is necessary to understand the molecular/cellular mechanisms behind the interactions between biomaterials and the TJ proteins and relevant membrane domains. Biomaterials commonly used to open the TJs are categorized below, including calcium chelators, surfactant, toxin, ionic liquid and cation polymer et al. Their interacting mechanisms are discussed in general and also illustrated in Table 2 for specific mechanism of action. For the final formulation to obtain the high bioavailability and targeting efficacy, it requires the biomaterials interacting with the TJs in a proper way enabling efficient drug transport.

4.1 Calcium chelators

Extracellular calcium ions are necessary for the cell-cell interaction and maintenance of TJ structures. Removing them affects the epithelial polarization and changes the ZO-1 distribution. This leads to the recessions of microfilaments and microtubules, and ultimately the TJ rupture. In an *in vitro* study when cells were cultured in calcium-free media, it was observed that ZO-1 redistributed from the epithelial cell surface to the intracellular regions and there was an obvious TJ strands rupture and a transepithelial electrical resistance (TEER) reduction. [40] There are several types of calcium chelators causing the disruption of TJs between epithelial cells, including nitrophenyl egtazic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), bis(2-aminophenoxy) ethane tetraacetic acid (BAPTA) et al.[41,42,43]

4.2 Surfactants

Surfactants typically contain both hydrophilic and hydrophobic domains and form micelles spontaneously at a concentration above their critical micelle concentrations (CMC) through intermolecular interactions. When the concentration is maintained below the CMC, the micelles dissociate and the hydrophobic domains or tails easily insert into the membranes and fluidize them, and/or interact with TJ proteins (note that surfactants are known for binding to and denaturing proteins.[44] This typically causes permeability increase of the epithelia and TJ opening. Short-tailed fatty acid surfactants with higher CMCs are more mobile and can diffuse more easily than their long-tailed counterparts. The commonly used surfactants include fatty acids (oleic acid, lauric acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), γ-linoleic acids, et al.), bile acids, phospholipids, Tween 80, Cremophor EL, Gelucire 44/14, sodium taurocholate, benzalkonium chloride, saponin, cyclodextrins, et al., which disrupt the TJs and increase the permeability.[45,46,47,48,49,50,51]

4.3 Biological toxins

Zonula occludens toxin (Zot) is a toxin secreted by vibrio cholerae which binds to a putative surface receptor, activates the intracellular signaling, and ultimately disassembles the TJs. Recent studies showed that Delta G, which is a 12 kDa active section of Zot, temporarily enhanced the paracellular transportation.[52,53,54] There are side effects for the prokaryote toxins however, because of their expression and purity issues. As an improvement, synthetic peptide AT-1002 was further developed consisting of six amino acids of Delta G to enhance the intestinal permeability and avoid the above mentioned problems.[55,56]

4.4 Cationic polymers

Positively charged cationic polymers extensively interact with cells having negatively charged membrane surface. Chitosan and its derived nanoparticles for example have shown the interaction with the integrin receptors, followed by the activation of integrin clustering, and the initiation of tyrosine kinases phosphorylation. Meanwhile, claudin 4 was transferred to the intracellular domain and the TJ permeability was increased.[57]

4.5 Ionic liquids

Ionic liquid usually refers to a salt in the liquid state at a temperature below 100˚C, which mainly consists of ions and short-term ion pairs. The mechanism for ionic liquid to open the TJs may be due to the extraction and fluidization of the lipids from the bilayers. In particular, the ionic liquid may disperse into the lipid layer, mitigate the interactions between lipid molecules, and break down the bilayers of membrane. The ionic liquid system based on choline and greanate was reported to significantly protect insulin from degradation and enhance the intestinal paracellular transport of insulin.[58,59]

4.6 Hyperosmotic carbohydrates

Certain carbohydrates such as mannitol and starch polymers could form a hyperosmolar environment once exposed to water. This generates a hydrostatic pressure on the treated epithelial cells and induces them to shrink. TJs were destroyed during this process, which enabled a pulsatile delivery of insulin drug across the intestinal epithelium.[60] Most recent finding indicates that fast food consumption could reduce the expression of genes coding for TJ proteins ZO-1 and occludin, resulting in increased intestinal permeability.[61]

4.7 Pharmaceutical drugs

Certain pharmaceutical drugs are known for affecting the intestinal TJ structures and causing the diarrhea. For example, mycophenolic acid (MPA) is an immunosuppressive agent for post-transplantation treatment. It was reported that MPA represented around half of post-transplantation diarrhea, whereas one fifth of MPA complications happened in the gastrointestinal tract.[62] It was reported that MPA could disrupts the TJ structure through Midkine and PI3K genes activation and Claudin-1 epigenetic repression, which leads to the loss of TJ integrity. That is the possible reason why GI disorder like diarrhea happened in the patients who take the MPA after the organ transplantation.[63]

Recently a peptide drug, PIP 640, has shown the function of increasing the TJ permeability specifically for cationic macromolecules.[64] The potential mechanism may be due to the increased phosphorylation of myosin light chain which stimulated a contraction of TJ-associated actomyosin filament. For TJ proteins, PIP 640 seemed only increase the claudin-2 level which was correlated with the observed permeability bias toward cations. *In vitro* and *in vivo* experiments showed that the increased transport of macromolecules could last from minutes to hours, and then the TJ permeability was recovered. Different substances such as salmon calcitonin and exenatide were used to investigate the correlation between substance charge characteristics and permeability enhancement after applying the PIP 640. This study demonstrates a new way to enhance the intestine absorption solely for positive-charged macromolecules.

4.8 Metal nanoparticles

Different types of metal nanoparticles (zinc, iron oxide, alumina oxide and vanadium) have been reported to disrupt the TJs through claudin or occludin downregulation, oxidative stress induction, or inflammatory response. For example, alumina nanoparticles could break down the blood brain barrier

through destabilizing claudin-5 and occluding, changing the cellular reductive status and causing mitochondrial malfunction [65]. It should be noted that alumina nanoparticles induced significant endothelial toxicity possibly due to the alternation of mitochondrial function. Supplying glutathione appeared to prevent the TJ protein alternation and reduce the alumina-related toxicity to the endothelial cells [65].

4.9 Others

Other biomaterials such as thiomers, nitric oxide, cyclodextrin could also interact with the TJs and increase the intestinal permeability through different ways. Inhibition of protein tyrosine phosphatase and increase of the cyclic guanosine monophosphate (cGMP) may be the possible mechanism of opening the TJs.

Table 2. Mechanisms of biomaterials interactions with the tight junction

Category	Biomaterial	Mechanism of action	Reference
Calcium chelators	EDTA	Chelation with extracellular calcium initiates the PKC activation and preventing ZO-1 relocation	66,67,68,69
Surfactants and surfactant-like molecules	Bile acids	Decreasing JAM-1and claudin-1, -3 expressing	70
	Sodium dodecyl sulfate, and sodium dodecylbenzene sulfonate	No change on mRNA expression of major TJ-related proteins. Binding to and denaturing TJ proteins.	71
	Sodium caprate	Fluidizing the cell membrane, resulting in the intracellular calcium increase, and claudin-5 and tricellulin expression changes	46,72,73,74
	Sucrose monoester fatty acids (for food industry)	Induce actin disbandment	75
	Cyclodextrins	Specifically displacing lipids from raft-like membrane domains	76
	Decanoylcarnitine	Increasing the intracellular calcium level	41

	Phospholipids	Changing the detergent-solubility of zonula occludens-protein and occludin.	77
Metal nanoparticles	Zinc Ion	(1). Glycogen synthase kinase (GSK) 3β phosphorylation and the snail transcriptional repressors overexpression (2). Claudin-1downexpression, ZO-1 rings and occludin collapse, and the basolateral F-actin breakdown	78,79
	Vanadium	Induction of oxidative stress	80
	Fe_3O_4 nanoparticles	(1). Decreasing claudin-1, -3, -4, -7, ZO-1, and E-cadherin expression (2). P38 stress-induced protein kinase/Jun-amino-terminal kinase (SAPK/JNK)	81
	Nano-alumina	Disruption of claudin-5 and occludin	65
Ionic liquid	Choline/geranic acid	Enhancing the fluidization within the protein and lipid region	82,83,58
Cationic polymers	Chitosan	(1). Activation of integrin receptors and integrin clustering, FAK and Src tyrosine kinases phosphorylation; (2). CLDN4 relocated from the cell membrane to the intracellular domain	84,85,86,87
Biological toxins	Gliadin	Activation of zonulin signaling	88
	Zonula occludens toxin	Binding to a specific epithelial surface receptor led to a reversible rearrangement of F-actin caused by PKC-a dependent polymerization of actin monomers.	89,90
	Peptides (AT-1002 et al)	Acting via claudin-1 and -5	91
	Cytochalasin B	Inhibiting network formation by actin filaments and MLCK activation.	92,93
Pharmaceutical drug	Mycophenolic acid	Midkine/PI3K Pathway	94
	PIP 640	Increasing phosphorylation of myosin light chain	64

Carbohydrates	Mannitol	Hyperosmolar mannitol shrinks endothelial cells	[95,96]
	Starch Microspheres	Hydration causes a hydrostatic pressure leading to the tight junction's separation	[60]
	high carbohydrate /fat fast food	Reducing the expression of genes coding for tight junction proteins ZO-1 and occludin	[61]
Others	Thiomers	(1). Inhibition of protein tyrosine phosphatase (2). Interacting with receptors like IGFR and EGFR and inducing the expression of downstream protein tyrosine kinases Src through phosphorylation, resulting in the claudin-4 disruption	[97,98]
	Nitric oxide	Increase of cyclic guanosine monophosphate (cGMP) and the formation of peroxynitrite	[99]

5. The impact of opening tight junctions

5.1 Benefits

The major benefit of opening TJs is to facilitate transdermal, oral and brain drug delivery with high drug bioavailability.[100,101,102] Most hydrophilic drugs such as proteins and antibodies cannot cross the epithelial membrane barriers under normal conditions, but with opened or dilated TJs they could diffuse through these barriers through the paracellular pathway and elicit improved pharmacological performance.

An immediate benefit of opening TJs and associated improved outcome could be life-changing in terms of patient compliance (transdermal and oral medications replacing injections and infusions). In diabetes treatment, oral insulin has been the Holy Grail, however, an orally taken insulin can hardly be absorbed because of its relatively big molecular size (5808 Da) and the lack of specific receptors on the intestinal epithelial cells.[103] Numerous attempts have been made to increase oral bioavailability of insulin formulations. Majority of them directly or in-directly opened the TJs, allowing insulin to be transported from the intestine lumen to lamina propria and reach the systemic circulation.[58 104105]

In the field of chemotherapy, potential oral chemo-medicines could provide patients a less invasive treatment choice, compared with common infusion therapies, and less frequent hospital visits.[106,107] Most anticancer drugs are very toxic even at a low dose; hence it is important to improve the bioavailability to reduce the off-target side effect for the gastrointestinal tract (GIT).

Opening TJs potentially leads to a successful targeted drug delivery to treat brain diseases (glioma, Parkinson and Alzheimer's) which has long been impeded due to the blood-brain barriers preventing

majority drugs from entering the brain. Paracellular pathway could be promising to deliver these drugs into the brain and increase therapeutic outcomes.[108,109]

5.2 Drawbacks

Opening the TJs, the natural barriers, could be a double-edged sword. Despite the pharmaceutical benefits, TJ opening may cause irreversible intake, together with APIs, of dietary antigens, or pathogens such as bacteria, virus and lipopolysaccharides from the barrier surface, such as the inner gut, where they permanently or opportunistically occurred or resided (Figure 3). It was reported that an increase of intestinal permeability is related with the high incidence of infection and autoimmune disease such as inflammatory bowel disease. [47,110] In addition, the increased intestinal permeability could contribute to the Type 1 diabetes,[111,112] graft-versus-host disease propagation(GVHD),[113] HIV/AIDS,[114,115] et al. TJ opening by biomaterials could be transient and recoverable after few hours or days.[116] But it is still unknown if the opening of TJs, particularly when periodically repeated, could exceed the body's repairing capacity and cause allergies or autoimmune conditions given many unknown substances in presence on gut surface.

Nevertheless, current clinical trials have involved TJ opening materials, although with patients having gastrointestinal disorders excluded.[105] One notable example was sodium caprate (used in GIPET technology, a known TJ opener) [117], which has obtained food additive status and has been used for oral insulin delivery by Novo Nordisk in Phase 2 trials. Despite of a success in clinical outcome, the company suspended this oral insulin program since the product was not commercially viable due to a low oral absorption efficacy.[105] If any technology obtained approval involving TJ opening materials, post-marketing data will still be required to explain potential common and rare toxicological effects. Additionally, it should be more cautious when these formulations are tested on patients who have celiac disease or inflammatory bowel disease, since their TJs have already been damaged to some extent.

The risk of opening TJs cannot be overlooked even for naturally occurred materials or for materials generally considered to be safe. Bile acids for example are natural compounds in the human body, derived from cholesterol and secreted from the liver to the intestine. Excess bile acids are known to affect TJ structure and their barrier function.[70,45] Naturally most bile acids are reabsorbed in the ileum and returned to the liver via enterohepatic circulation. Other minority of bile acids goes into the colon and manages cell proliferation, immune response, motility, and ion transport.[118] There are many cases (weakened bile acid recycling or overproduction of bile acid) leading to the accumulation of bile acids in the intestine (bile acid malabsorption) that was involved in the pathophysiology of inflammatory bowel disease (IBD).[119,120] It was also reported that the excessive accumulation of bile acids (>3 mmol/L, 1.2mg/ml) in the intestine could cause severe side effects such as epithelial barrier disruption, diarrhea and cancer. [70,121,122] Most current bile acid-based nanoparticle systems for drug delivery have far higher bile acid concentration above this threshold (1.2 mg/ml).[123,124,125] Further study is needed to evaluate potential risk of similar side effects.

In addition, it has been reported that high carbohydrate/calories fast-food consumption could cause type 2 diabetes and the reason behind this is related to increased TJ permeability and relocation of proteases.[61,126] Chronic high carbohydrate/calories diet would affect the expression of ZO-1 and occludin protein which are key structural components for TJs, leading to an increase of the intestinal permeability. Under normal conditions, the pancreatic proteases stay inside the lumen of small intestine and break down most of the macromolecules originating from food. Once the intestine barrier becomes

leaky, these proteases relocate to the systemic circulation and downregulate the insulin receptor on the cell surface, leading to insulin resistant type 2 diabetes.

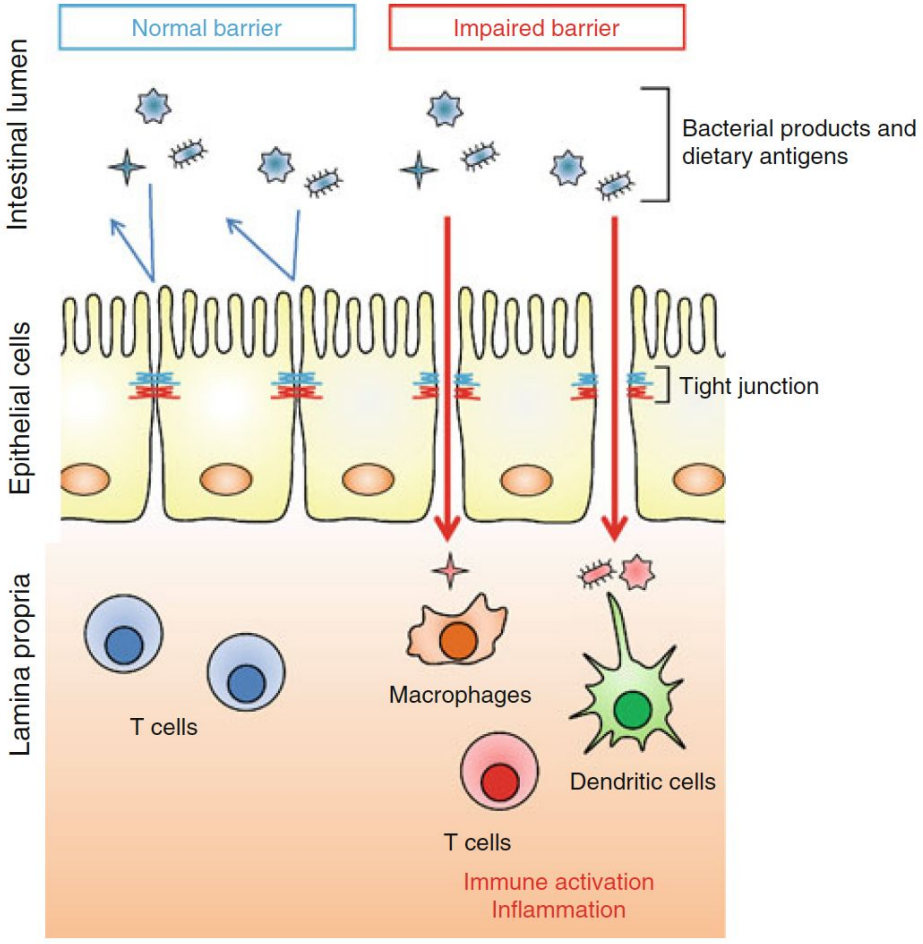

Figure 3. Luminal virulent substances such as bacterial products and dietary antigens could get through the epithelium if the tight junction barrier is disrupted. This leads to the mucosal immune activation and inflammation. Reprinted with permission from Ref. [49], 2012 Springer Nature.

6. Methods to evaluate tight junction permeability

Given the benefits and drawbacks of TJ opening, the capability to evaluate TJ opening and barrier permeability is critical. Several methods have been adopted in both academic and clinical research and were introduced below, including transepithelial/transendothelial electrical resistance, urine lactulose-mannitol ratio, serum antibodies, TEM, immunofluorescence staining, Cr-EDTA, and small saccharide probes. Majority of these methods detect the barrier permeability and some of them (TEM, immunofluorescence staining) directly evaluate the TJ structure and integrity.

6.1 Transepithelial electrical resistance (TEER)

The electrical resistance across a cell monolayer (typically cultured on a transwell) can be measured and the ionic conductance reflects the permeation level of TJs. The advantage of this technique is non-invasive and that all kinds of cells regardless of their growth stages can be tested.[127] The disadvantage is

the measuring variations due to inconsistent parameters such as electrode types, measuring temperature, media, cell culture duration, and cell passages. TEER was widely applied in blood–brain barrier, gastrointestinal tract, pulmonary and alveolar epithelial models. For the intestinal TJ test, monolayer of human colon epithelial cell line was typically used.

6.2 Urine: lactulose-mannitol test

Sugar molecules were widely used for intestinal permeability test. The lactulose-mannitol test requires administering simultaneously two sugars orally: disaccharide (lactulose), and a monosaccharide (mannitol), followed by a quantification of the two sugars in urine at a time point.[128] The basis for this test is that lactulose cannot get through the paracellular pathways of the intestine whereas mannitol could freely travel under normal conditions. The paracellular permeability of the small intestine was evaluated by the ratio of lactulose to mannitol absorbed (or secreted in urine). On drawback of this test is that it only shows the small intestine's permeability rather than the colon's, because colonic bacteria could break down the lactulose and mannitol.[5,129] Another drawback of this test is its low specificity and the possibility of false positives because of the inter-human variation of sugar absorption.

6.3 Serum antibodies of lipopolysaccharides, zonulin-occludin and actin-myosin

An intact intestinal inter-epithelial barrier could prevent the paracellular translocation of lipopolysaccharides (LPS). But once the TJs are disrupted, the LPS could get into the lamina propria and induce relevant antibody production. So intestinal barrier permeability can be evaluated by measuring LPS and anti-LPS IgA, IgG and IgM antibodies levels in serum.[130,131,132] Moreover, zonulin-occludin and actin-myosin are critical for maintaining the tight junction integrity, hence measurement of their IgA or IgM, IgG antibodies in serum could also be clinically valuable for the detection of intestinal permeability.[133]

6.4 Transmission electron microscopy (TEM)

Transmission electron microscopy is based on an electrons beam transmitting through a sample and enables the higher-resolution imaging than optical microscopes.[134,135] The procedure to examine the TJs involves fixing, embedding, sectioning, and staining the barrier tissue samples and observe the TJ structures directly under the TEM. [136] Another method is to examine the signal of lanthanum (La^{3+}), an electron dense element similar to the size of Na^{+}, penetrating TJs under the TEM. La^{3+} could pass through more of junctional complexes and got into the intracellular domain after the opening of TJs. [137]

6.5 Immunofluorescence staining of tight junctions

The TJ proteins' organization or expression could be assessed by the immunofluorescence technique, such as a surface biotinylation method.[23] Different antibodies for various TJ-related proteins can be applied onto tissue slides containing the TJ areas to be analyzed. After the immunofluorescence staining, the tissue sample can be imaged under a confocal fluorescent microscope.[138] The distribution change of these TJ proteins can be analyzed and used to estimate the change of the TJ integrity. In addition, western blot could be used to evaluate the relevant protein expression level which is another parameter for measuring TJ integrity.

6.6 Cr-EDTA/ small saccharide probes

Chromium 51-labeled ethylenediaminetetraacetic acid (^{51}Cr-EDTA) is hydrophilic, safe, chemically inert, and totally excretable through the kidney, and thus has been widely used as a probe for oral administration in clinics. This probe works well for detecting epithelial impairment, tracking intestinal

integrity, determining the permeability speck with intestine enteropathy, and contributing to the small intestinal bacterial overgrowth (SIBO) diagnosis. ^{51}Cr-EDTA in the urine is commonly used to indicate the intestine permeability conditions. Westermarck et al also found that the serum test of the ^{51}Cr-EDTA for dogs have high consistency with the urine results, which could dramatically reduce the complexity of the urine collecting work in animal studies. [139] In addition, FITC-conjugated dextran (10-kDa) and the ethylene glycol probes (of various molecular weights) can also be orally administered and the excretion level in the urine was used as an indicator of the TJ permeability.[140]

7. Conclusions and future perspectives

Advances have been made in understanding the physiological structure of TJs and the cellular mechanisms of biomaterial-tight junction interactions, but this subject area remains exciting and open to new discoveries. Of note, past and current research mostly focused on opening the TJs to increase the proteins/peptides absorption and bioavailability, but rarely considered potential pitfalls of TJ opening as discussed above. This is a particularly important issue for the widespread applications of drug delivery through paracellular pathways, particularly for those hydrophilic drugs with poor membrane permeability. Future research shall focus on potential strategies taking the benefit of TJ opening while overcoming the drawbacks. This requires fundamental understanding on the "extent and duration" of TJ opening induced by a given biomaterial, and its implication for the transport/absorption of desired payloads and unwanted potential presented bacteria, dietary pathogens, viruses or lipopolysaccharides. The way collaborated between biomaterials and APIs should also be considered, such as whether the material and the drug are associated (e.g., conjugated, bound, encapsulated, etc) or not (e.g., physical mixture) during the transport. An associated biomaterial-API might utilize the TJ opening more efficiently for API absorption, while dis-associated biomaterial-API might create opened TJs not occupied by the desired payload transport, increasing the risk for pathogen infections. Furthermore, the natural modulatory biomolecular process could further inspire the development of novel potent biomaterials to interact with TJs, facilitate controllable absorption of APIs and decrease potentially relevant side effects. Different measurement methods introduced here for the paracellular permeability served as tool kits to determine the impact on TJs as precise as possible, in order to balance the pharmaceutical benefits and health risks of TJ opening.

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