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ε-Polylysine and next-generation dendrigraft poly-L-lysine: chemistry, activity, and applications in biopharmaceuticals

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Polylysine is an important class of polyamino acids with a broad spectrum of applications in biomedical research and development. It can be divided into two classes, α-polylysine and ε-polylysine, the former is synthesized by artificial chemical synthesis and has limited applications due to its high toxicity, and the latter is produced by microbial synthesis as a class of natural polymers and is widely used in various food, medicinal, and electronics products. Another major class of synthetic polymers is dendrimers (after linear, cross-linked, and branched polymers). Dendrigraft poly-L-lysine (DGL) has the favorable properties of polylysine and dendrimers, with a broad spectrum of applications in drug discovery and development, including drug delivery, gene carriers, diagnostic imaging, diagnostics, biosensors, and special cancer therapies (such as boron neutron capture therapy and photodynamic therapy). As there are still some problems with the development of DGL, further research is warranted for its broad applications.

Keywords: biomedical application; dendrimers; poly-L-lysine; drug delivery system; gene carrier

1. Introduction

As the applications of polymers are expanding and increasing in various fields, there is a significant interest in the discovery and development of novel polymers with various structures and characteristics.[1–3] Polyamino acids are a class of polymers in which multiple amino acids of the same type are connected through amide bonds, they exhibit excellent biocompatibility and other properties that are important for a biomaterial to be used in various industries, especially in the biomedical and biopharmaceutical fields.[4,5] For instance, polylysine, formed by the polymerization of the lysine monomer, is one of the most investigated polyamino acids.[6] Polylysine can be divided into two classes, α-polylysine and ε-polylysine, the former is artificially synthesized, with 50 lysine residues being linked between the α-carboxyl and the α-amino groups, and has limited practical applications due to its high toxicity.[7,8] The naturally occurring ε-polylysine has better chemical and biological properties with a much better safety profile,[9] attracting more attention from researchers in various fields.

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Among various polymeric architectures (such as linear, cross-linked, and branched polymers), dendritic polymers [10] have attracted a substantial attention, due to their novel three dimensional, hyperbranched globular nanopolymeric architectures.[11–13] Various dendrimers have been reported, including dendrimers (dendrimer), hyperbranched polymers (hyperbranched polymer) and high branched polymers (dendrigraft).[11,12] Although the dendrimers have the perfect tree structure, their lengthy synthesis route, low yield, and slow growth of molecular weight have limited the development of dendrimers.[14,15] Compared with the dendrimers, the synthetic method for hyperbranched polymerization is simple, but also has certain drawbacks, such as high defect rate and broad molecular weight distribution. However, the high branched polymers, a new member of the dendrimer family, not only have many similar features of dendrimers (such as structural symmetry control, repeated iteratively growth, and geometrically delivery increase), but also present characteristics of a hyperbranched polymer branched random distribution, and narrow molecular weight distribution.[16]

Dendrigraft poly-L-lysine (DGL) polymers, a recently discovered subset of biodegradable high branched polymers, have favorable properties of both polyllysine and dendrimers, which has gained a huge interest in the field. DGL polymers exhibit similar physicochemical properties of dendrimers, but turn out to be non-immunogenic.[17] Moreover, they are able to carry molecular agent within their dendritic framework due to its less crowded structure. Hence, the DGL present numerous potential applications in health, biopharmaceutical and life sciences. Recent reports have shown that DGL are used for gene therapy, drug delivery carries and bio-imaging.[18]

Although there are a large number of review articles on dendrimers, hyperbranched polymer and dendrigraft, quite few reviews focus on DGL, and the applications of DGL in biopharmaceuticals. This review article provides an overview of the structure, properties and application of ε-poly–L-lysine in biopharmaceuticals and an in-depth discussion on the structure, properties and application of DGL.

2. ε-Polylysine
2.1. Structure and properties
Historically, ε-polylysine was accidentally discovered by Shima and Sakai as an extracellular material produced by a filamentous fungus – *Streptomyces albulus* 346 (now known as *S. albulus* NBRC14147) in soil.[19,20] It consists of 25–35 lysine residues with linkages between the α-carboxyl and ε-amino groups and is a Dragendorff-positive substance, i.e. an alkaloid or quaternary nitrogen compound.[6,21]

The structure and properties of ε-polylysine determine its applications. It is water soluble and biodegradable and has antibacterial effects against a wide spectrum of bacterial pathogens, including both Gram-positive and Gram-negative bacteria.[22] In addition, it has antimicrobial effects against yeast, fungi, and phage.[23] Its antimicrobial activity is linked to the electrostatic adsorption of ε-polylysine to the cell surface, resulting in stripping of the outer membrane and abnormal distribution of the cytoplasm, and ultimately leading to physiological cell damage. It has been demonstrated that it does not cause mutations in treated bacteria or other pathogens.[22–24] The safety of ε-polylysine has been determined using various parameters for reproduction, neural function, fetal growth, and development in rats.[6,25] Pharmacokinetic studies have indicated that ε-polylysine is not accumulated significantly in certain organs or tissues analyzed. Moreover, it is almost completely excreted within 168 h after
administration, indicating that it may be safe for humans.[26] Furthermore, ε-polylysine does not pollute the environment.[9] Based on these observations, it is believed that ε-polylysine and its derivatives may have a variety of applications in various industries such as food, electronic products, medicine, and pharmaceuticals.

2.2. Applications of ε-polylysine in biopharmaceuticals

In the last decade, there have been extensive studies aiming at the development and application of ε-polylysine in various industries. In the following sections, we focus on the use of ε-polylysine in drug discovery and development for various diseases.

2.2.1. Antimicrobial agent

ε-Polylysine has been used as a natural antimicrobial agent in food production.[26,27] Due to its strong antimicrobial activity, the effective dose is relatively low.[22,27] The addition of ε-polylysine does not affect the taste of food.[26] Although it can be used alone, ε-polylysine is often combined with other food additives, such as glycine, ethanol, and acetic acid, which may reduce the amount of each agent needed in food production.[26] In addition, ε-polylysine can be conjugated with dextran as a dual-functional food additive, with emulsifying and antibacterial properties.[6,25,26]

2.2.2. Anti-obesity agent

Obesity is a serious health problem in modern society, leading to many chronic diseases, such as diabetes.[28] Pancreatic lipase plays a crucial role in the absorption of fat from the small intestine [29]; it is possible that inhibition of lipase activity would reduce the absorption of fat substances.[30] Houghton et al. [30] have found that ε-polylysine at doses of 10–100 mg L\(^{-1}\) inhibits pancreatic lipase activity and that ε-polylysine still maintains its activity in an emulsion containing bile salts, phosphatidylcholine, and digestive enzymes. Therefore, ε-polylysine can be used to control obesity.[31,32]

2.2.3. Drug delivery and nonviral gene delivery

It is reported that ε-polylysine can be used as a drug delivery tool to increase cellular drug uptake.[9] Its surface has cationic amino groups, which can facilitate drug transport. For example, methotrexate, an anti-folate agent, can be conjugated with ε-polylysine for the treatment of leukemia, sarcomas, and other neoplastic diseases.[27] ε-Polylysine also has been suggested as a useful non-viral gene delivery approach.[33,34] Since ε-polylysine is not only water soluble and biodegradable but also can prevent conjugated DNA from nuclease degradation and increase the transport efficiency of DNA, it has been shown to be an effective gene vector, e.g. the lactose–polyethylene glycol–polylysine (Lac-PEG-PL) carrier.[35–37]

2.2.4. Boron neutron capture therapy

Boron neutron capture therapy, as a novel approach for cancer treatment, relies on neutron capture reactions and fission reactions. It destroys cancer cells by the nuclear reaction.[38,39] \(^{10}\)B is a natural nonradioactive element that reacts with a thermal neutron with low energy, generating a high-energy α particle (4He) and \(^{7}\)Li. The reactions are illustrated in Scheme 1.
When boron reaches tumor cells, irradiation with thermal neutron radiation releases highly lethal rays. Such radiation does not cause damage to the human body, but the neutron and boron entering into the cancer cells can cause a strong nuclear reaction. These rays have a very short range and selectively kill cancer cells without damaging the surrounding normal tissues.[40] To achieve this effect, it is critical to transport boron to the tumor cells, and the concentration must reach at least 20 μg g⁻¹ of tumor cells or 10⁹ atoms/tumor cell, in addition to the absorption of adequate thermal neutrons of the reaction.[40] For normal antibodies against a tumor, such a large amount of boron may affect their solubility and targeting, requiring a new delivery system. Umano et al. [41] have conjugated ε-poly-lysines-based polyamines with boron clusters and this polymeric boron carriers can deliver safely and efficiently into tumor tissues. Nowadays, researchers are constantly using a more specific tumor targeting antibody to reduce the accumulation in normal tissues such as the liver and spleen.[42]

Additionally, ε-polylysine can be used to prevent periodontal disease and to treat oral bacterial toxin infection.[22,37] In addition, ε-polylysine is used as a component of the solution in disposable wipes for sanitation.[27] It is also widely used in agriculture and electronics applications.[36]

3. Dendrigraft poly-L-lysine

3.1. Structures and synthesis

The branched polymer as shown in Figure 1 is the earliest studied polymers, and is a prototype of modern high-branched polymers. The brush-type polymerization is also referred as ‘cylindrical brush’. Their characteristics are short chain branching [43–45] and varied by grafting density-flexible molecular skeleton and side chains. Dendrimer is highly regular, the rules are derived from a collateral center,[46,47] and dendrimers can also be viewed as a combination of comb binding molecule and dendrimer molecules.

However, the hyperbranched polymers and dendrimers have shown slight differences in structure; the former exhibits less regular structure than the latter. The repeating structural units of dendrimer are also small molecule monomers.[48]

Different from dendrimer and hyperbranched polymers, the high branches polymers (dendrigraft) are represented by different side chains through random access branches. And like the hyperbranched polymers, the high branched polymers can precisely control the amount and composition of the side chains.[49,50] In addition, the type and quantity of grafted side chains of each generation can be designed. Moreover, the morphology of high branched polymer can be grafted with different monomers in one-step reaction, thereby forming a dense graft.

DGL has been developed as a new generation of high branched polymers (Figure 2). The DGL is polymerized by Ne-N-trifluoroacetyl lysine N-carboxyanhydride (Ne-TFA-Lys-NCA) in aqueous solution. This synthesis uses a series of protection–de-protection protocols, each cycle production is called a generation (Gn) and is used as
The initiator of the next generation synthesis.[51,52] The first step of the synthesis consists of the polymerization of $N_\varepsilon$-TFA-Lys-NCA without any initiator, leading to an insoluble product. In the second step, the insoluble product is collected and the $N_\varepsilon$-TFA protecting group is removed, leading to the production of the first generation of soluble DGL (DGL-G1). In the third step, DGL-G1 is used as the initiator to repeat the synthesis of step one. In the fourth step, the TFA protecting group is removed to obtain the second generation of soluble DGL (DGL-G2). And the same sequence is used to produce the next generations of DGL (such as DGL-G3–DGL-G5).[53] Scheme 2 shows the formation details of an aminooxy-functionalized poly-lysine G1, G2L, and G3.[54]

Scheme 2. Synthesis of aminooxy-functionalized poly-lysine G1L, G2L and G3L.
### 3.2. Properties

Nanoscale DGL have a monodisperse, accurate three-dimensional, and symmetrical multi-branch tree structure, with the presence of a large number of surface reactive groups and a hydrophobic internal cavity.[53,55–58] Their molecular weight and structure can be well controlled.[59,60] Different from ordinary polymers, dendritic polymers have favorable properties such as low viscosity, high solubility, miscibility, and high reactivity.[52] In addition, DGL is softer and more flexible than other dendrimers, moreover it is biodegradable and nonpolluting to the environment. It has good biocompatibility, easily crosses through the cell membrane, and has no immunogenicity.[61–66] In vitro testing indicates that it is safe, and in vivo testing has not yet found any severe adverse effects.[17] Due to these favorable properties (Table 1), there is an increasing interest in exploring its applications in various fields.[53,67–73] Its potential use for drug delivery, as a gene vector, and for medical diagnostics has been gradually recognized.[17,18,71,73,74]

### 3.3. Applications of DGL in biopharmaceuticals

Recently, there is an increasing interest in expanding the use of DGL in various industries. In the following section, we focus on its implication in drug discovery, development, and drug delivery, especially in the biopharmaceutical field.

#### 3.3.1. Drug delivery

DGL can be used as a drug delivery system.[51,75,76] Since it has a controllable molecular size and structure, DGL can satisfy different drug delivery requirements.[12,13,52] DGL has a plurality of reactive groups on its surface and a hydrophobic cavity inside, with good water solubility. Therefore, it can transport hydrophobic drugs via its hydrophobic internal cavity and also increase the solubility of a drug through electrostatic interactions and/or chemical bonding with its surface active groups. Dendrimers can extend the residual time of a drug in blood circulation, control the release rate of a drug, and protect a drug from degradation.[67] Additionally, some modified DGLs cannot only bring a drug to its target organs or tissues but also can reduce the adverse effects to other organs or tissues.[67,77] Hence, it would be a good choice for sustained release and molecular targeting. For example, Kaminskas and colleagues [78] have conjugated methotrexate with PEGylated DGL, injected the ³H-labeled conjugate intravenously to rats bearing breast cancer xenografts, and evaluated the drug disposition. Their results indicate that DGL is an effective carrier for the delivery of hydrophobic tumor-targeting drugs.[79–85]

#### Table 1. Properties of DGL.

<table>
<thead>
<tr>
<th>Properties</th>
<th>References</th>
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<tbody>
<tr>
<td>1    Nanoscale molecule</td>
<td>[51,55,59]</td>
</tr>
<tr>
<td>2    Accurate 3-D structure</td>
<td>[56,57,59]</td>
</tr>
<tr>
<td>3    Numerous surface-reactive groups, a hydrophobic internal cavity</td>
<td>[58,61]</td>
</tr>
<tr>
<td>4    Biocompatible and biodegradable</td>
<td>[61–66]</td>
</tr>
<tr>
<td>5    Pharmacokinetics</td>
<td>[75,76]</td>
</tr>
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DGL also shows the potential for protein drug delivery.[86] There are many challenges in the oral administration of polypeptide or protein drugs such as insulin, as protein drugs can be hydrolyzed by proteolytic enzymes in the gut and undergo a dramatic change in the harsh pH environment.[79,85] In addition, they have low permeability crossing intestinal epithelial cells, resulting in an extremely low absorption rate. Compared with the current route of administration of insulin (subcutaneous injection), an oral route would be an attractive choice due to its convenience, low cost, and noninvasiveness, which can also effectively avoid local infection at the injection site and improve patient compliance. Therefore, it is desirable to develop an oral delivery system for insulin, which can deliver insulin to its active sites without affecting the properties of insulin. Sideratou et al. [87] have reported a DGL derivative (arginine guanidinylation) as a second generation DGL for insulin delivery. The DGL was mixed with insulin via electrostatic interactions with a low molar ratio (1:10) at room temperature and pH 7.4, reaching a combined ratio greater than 99%. The complex substance can protect insulin from α-chymotrypsin hydrolysis. This reaction is reversible, and the structure and properties of redissolved insulin are not changed. The complex reaches a release rate of about 70% in the absence of any enzyme of the simulated small intestinal fluid. The high guanidine DGL derivative releases insulin with a slow and uniform rate within 6 h in the small intestine. The release rate of insulin correlates with the number of arginine residues on the end of the DGL, therefore, the release rate of insulin is regulated by controlling the amount of guanidine used. However, this reported in vitro study was only conducted in a simulated environment, its application in vivo needs to be studied further.

3.3.2. Nonviral gene delivery

DGL has been suggested as a safe nonviral gene vector.[18,88,89] The surface of DGL has numerous cationic amino groups that can be used to form complexes with the anion groups of the nucleic acid.[18,88] The complexes of DGL-nucleic acids can enter cells by endocytosis, after entering the cell, the complex can be released from the endosomes into the cytoplasm and reach the destination of the nucleotides. In addition, this system protects DNA from being hydrolyzed by nuclease, facilitating the delivery of a therapeutic gene to its destination.[18,67,77,88] Many efforts have been made to improve the efficacy and performance of the DGL-mediated gene delivery system.[18] For example, Huang and colleagues [90] have successfully used PEGylated DGL to carry plasmid DNA (pDNA) into human embryonic kidney cells (HEK293 cells). The efficiency of the complex of DGL and pDNA is maximum at a weight ratio of 10:1. Cytotoxicity testing of the complex based on the cell survival rate has demonstrated that DGL at concentrations of 200 μg/mL or less has no significant cytotoxicity. The in vitro transfection efficiency was detected quantitatively using the commercial transfection reagent Lipofectamine 2000, but the in vivo efficacy has not been determined and requires further research. Kodama et al. [18] have reported the efficiency of in vitro transfection of various complexes in B16-F10 cells (a mouse melanoma cell line), including the complexes formed by a pDNA that had the luciferase DNA, polyglutamic acid (γ-PGA), and DGL (DGL/γ-PGA/pDNA) inserted, or the luciferase DNA, DGL (DGL/pDNA), and complexes formed by pDNA that were inserted with luciferase DNA and PLL (PLL/pDNA). The transfection efficiency was determined by the expression of luciferase under a fluorescence microscope. Their results indicated that the transfection efficiency of DGL complexes is 100 times greater than that of
PLL complexes. In animal studies with intravenous injection into mice, DGL/γ-PGA complexes showed better tissue selectivity than DGL complexes, and DGL complexes had gene expression in the liver, spleen, and lung. However, the DGL/γ-PGA complex only caused significant gene expression in the spleen, indicating that DGL can be used to produce gene vaccines against specific diseases.

3.3.3. Drug delivery crossing the human blood–brain barrier (BBB)

The human BBB is an important protective barrier for many diseases and infections, but it also hinders diagnostic and therapeutic drugs or genes from reaching the central nervous system. It has been suggested that DGL can be used as a carrier of diagnostic or therapeutic drugs or genes to enter the central nervous system.[91] For example, PEGylated DGL and specific ligand-modified DGL could be carriers with better biocompatibility and tissue selectivity.[91] Diagnostic or therapeutic drugs or genes could enter the central nervous system by specific ligands of the carriers connected with specific receptors in the BBB. For instance, leptin is a peptide secreted by fat cells and Leptin 30 can be combined with the specific leptin receptor in the brain and then enter into the brain. Liu et al. [86] have synthesized a PEGylated DGL–Leptin 30 complex (DGL-PEG-Leptin 30) and demonstrated that it can enter BV-2 microglia cells, which have leptin receptors like other cells in the brain. In mice, DGL-PEG-Leptin 30/DNA was detected in the central nervous system, suggesting that it may provide a safe and noninvasive approach for the delivery of genes across the BBB.

3.3.4. Photodynamic therapy

Photodynamic therapy has been used for the treatment of cancers.[92,93] The key components of this treatment approach are photosensitizers, light-sensitive molecules such as porphyrins, and the application of light at a specific wavelength. After oral or intravenous administration, the photosensitizers accumulate in the target tumor and are activated after applying the specific wavelength of light.[92,93] The photodynamic reaction using singlet molecular oxygen can induce apoptosis, necrosis, vascular ischemia, and other chemical pathways to damage tumor tissues, achieving the purpose of cancer treatment.[94] It is reported that DGL can mediate the delivery of photosensitizers to specific tumor cells to improve the efficacy and safety of photodynamic therapy.[95]

3.3.5. Medical imaging

DGL can also be applied to medical imaging.[74] Molecular imaging of nuclear medicine is highly sensitive, but it has some limitations, such as very short half-lives of the tracers, expensive testing equipment, and complicated operation procedures. Optical imaging is highly sensitive and economical, and invasive endoscopy can also be used in the treatment. However, its penetration is very low, deeply located tumors are not as easily detected as with the use of computed tomography or magnetic resonance imaging. It has been reported that DGL combined with protons or tracers can detect tumors earlier.[96] For example, gadolinium-benzyl-diethylenetriamine pentaacetic acid-DGL (Gd-BzDTPA-lysine dendri-graft) has been investigated as a magnetic resonance imaging agent in vitro and in vivo.[97,98] In addition, it has an improved half-life in vivo. It is promptly excreted through the kidneys and does not have a long-term
impact on the body.[97,98] Moreover, it may be combined with other therapies (e.g. hyperthermia, radiation, or photodynamic therapy) to achieve the goal of treating cancer under imaging.[77,96]

3.3.6. Immune diagnosis

It has been demonstrated that DGL has no immunogenicity, no significant side effects, and no influence on the functions of its combined drugs or genes.[99] Therefore, it is assumed that the antibody produced by DGL binding with antigen or hapten can be used for immune diagnosis and therapy.[99] Romestand and colleagues [17] have found that, in animal experiments, DGL coupled with an antigen promotes the production of antibodies, which can be further used to produce vaccines.

3.3.7. Sterilization

DGL also can be used in sterilization applications.[100] VivaGel™ (SPL7013 Gel) is the product of nanotechnology. The active ingredient is SPL7013, a dendrimer that is designed specifically with antiviral activity against human immunodeficiency virus and herpes simplex virus and human safety in mind.[101] Its effectiveness has been demonstrated in animal experiments, and a phase I clinical trial has proven its safety in humans.[102]

3.3.8. Biosensor

DGL also can be used as a biosensor, carrying specific ligands to specific receptors on tumors to detect tumor markers or tumor cells.[103]

3.3.9. Applications in orthopedics

In bone diseases, bone defects and cartilage injury are present. Current treatment methods mainly focus on biological material filling, joint replacement, or cartilage.[104–106] However, there are some problems, such as biocompatibility, immunogenicity, possible changes of the materials in vivo, and patient satisfaction.[107] There is an increasing interest in promoting bone regeneration. We speculate that DGL can carry the human bone morphogenetic protein gene into mesenchymal stem cells and further induce mesenchymal stem cells to differentiate into bone cells. Alternatively, DGL can carry other genes, such as transforming growth factor-β, bone morphogenetic protein, insulin-like growth factor, vascular endothelial growth factor, and nerve growth factor, into the lesion to promote bone formation.[108] Additionally, DGL derivatives can be used as a biological dimensional scaffold carrying bone growth factors according to the characteristics of the structure and the properties (molecular weight, containing a hydrophobic cavity, having biodegradability, etc.), providing both support and space for bone formation.[109,110] In bone tumors, DGL can be applied not only to carry drugs or genes for tumor therapy but also in boron neutron capture therapy and photodynamic therapy. Moreover, DGL has a role in the prevention of tumor recurrence,[111] thereby improving the quality of life of patients. Amputation for patients is a major impediment, therefore, there is an urgent need for developing effective methods to preserve the limbs.[112,113]
4. Conclusion

Due to the non-toxic, biodegradable and hydrophilic properties of ε-polylysine, it becomes potential tool in many applications such as medicine, biopharmaceutical, and food. However, even ε-polylysine can be industrially produced by microbes, the yield of polymer is still low.

Dendrimers are a major class of synthetic polymers that have attracted much attention due to their unique structure, favorable biological properties, and wide spectrum of potential applications, including drug delivery, gene vectors, special cancer treatments, imaging contrast agents, immunodiagnostic agents, antiseptics, and biosensors. DGL has emerged as a next-generation dendrimer. The nanoscale dendrimer has a monodisperse and accurate three-dimensional structure, with numerous surface reactive groups and a hydrophobic internal cavity. It is biodegradable, biocompatible, and nonpolluting to the environment; moreover, it has no significant immunogenicity. Although DGL has unmatched merits in pharmaceutical applications, most available results are from in vitro experiments or limited animal studies. Therefore, many problems still need to be solved before a safe and effective application can be realized in humans.

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