Molecular Therapy
Nucleic Acids
Review

Poly(β-amino ester)s-based nanovehicles: Structural regulation and gene delivery

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The first poly(β-amino) esters (PβAEs) were synthesized more than 40 years ago. Since 2000, PβAEs have been found to have excellent biocompatibility and the capability of ferrying gene molecules. Moreover, the synthesis process of PβAEs is simple, the monomers are readily available, and the polymer structure can be tailored to meet different gene delivery needs by adjusting the monomer type, monomer ratio, reaction time, etc. Therefore, PβAEs are a promising class of non-viral gene vector materials. This review paper presents a comprehensive overview of the synthesis and correlated properties of PβAEs and summarizes the progress of each type of PβAE for gene delivery. The review focuses in particular on the rational design of PβAE structures, thoroughly discusses the correlations between intrinsic structure and effect, and then finishes with the applications and perspectives of PβAEs.

INTRODUCTION

Targeting abnormal genes through knockout, editing, correction, or compensation is a promising approach to treating diseases, known as gene therapy.[1] This innovative strategy offers new hope for treating a range of conditions related to human genetic abnormalities, including tumors and genetic disorders. To achieve therapeutic effects, scientists have developed various gene delivery vectors that can effectively introduce genes into abnormal sites in the body.[2,3] The COVID-19 pandemic in 2020 led to the fastest approval of an mRNA vaccine in history, taking only 10 months from concept to market approval.[4] This remarkable achievement was made possible by early research on lipid nanoparticles (LNP) delivery technology.[5] The US government's National Nanotechnology Initiative, launched 20 years ago, aimed to develop targeted drug delivery systems for tumors. However, the success of LNP has led to the fastest approval and listing of the COVID-19 vaccine, propelling nucleic acid therapy into the fast lane. Despite the success of LNP, the low delivery efficiency, weak targeting, and cytotoxicity of LNP itself have become significant challenges in the gene delivery field. Therefore, the development of new delivery systems is crucial to break through the innovative development of the gene delivery field.[6]

PβAEs are a group of biodegradable cationic polymers first prepared by Chiellini et al. in 1983 (Figure 1).[7] In around 2000, Prof. Langer's group reported a pioneering study that used PβAEs as gene transfection reagents. Since then, interest in the investigation of PβAEs has been inspired.[8] PβAEs possess several advantageous characteristics, including biodegradability, hydrophobicity, low cytotoxicity, chemical modification diversity, structural diversity, and derivative diversity, making them highly versatile for a wide range of applications. The polymer's positive charge interacts electrostatically with negatively charged nucleic acids, forming a non-viral vector for gene transfection with high efficiency and no immunogenicity. The chemical structure of PβAEs contributes to their excellent performance as gene vectors (Figure 2). First, thanks to the ester bond in the backbone, PβAEs are hydrolyzable under aqueous conditions. The formed molecules through the degradation of PβAEs, such as bis (β-amino acids) and diol, are negligibly toxic to mammalian cells in physiological environments.[8] Second, a large number of protonatable amino groups were contained in the polymers (e.g., primary and secondary amines on the end groups and tertiary amines on the backbone), making them positively charged easily. In particular, the tertiary amine group on the backbone of PβAEs shows superior pH sensitivity from 5.5 to 7.2, and the protonated tertiary amine allows the PβAEs to be positively charged and hydrophilic. This characteristic thus leads to a "proton sponge effect" that facilitates its efficient escape from the lysosome.[9] The solid, non-protonated form of PβAEs is not soluble at a physiological pH of 7.4; however, when the pH of

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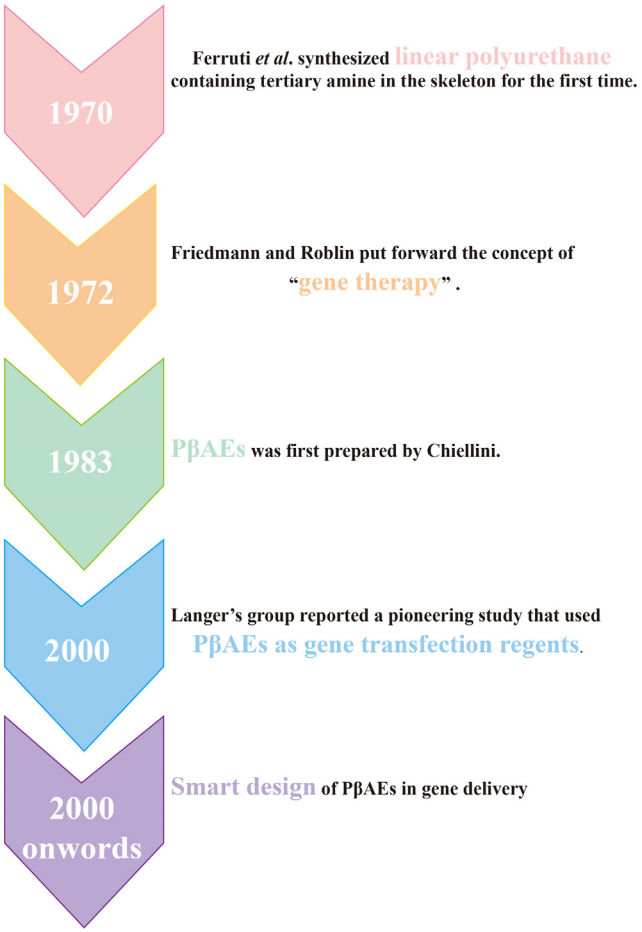

Figure 1. The history of PβAEs in gene delivery

the solution drops below 6.5, it rapidly dissolves in aqueous media. These polymers have potential applications in drug delivery near tumors, as they can transform from solid preparations to dissolved substances in extracellular and lysosomal environments (pH 7.4 and 5.0–6.5, respectively).[10] Aside from being employed as a transfection reagent, the rational structure design of PβAEs allows for their use as delivery vehicles for small compounds,[11] proteins,[12] and imaging agents,[13] as well as scaffold materials for tissue engineering.[14]

This review provides a brief introduction to PβAEs. It first describes the details related to the synthesis of PβAE, including the monomers used for synthesis, reaction temperature, and solvents (Table 1). Then the effects of different structures on delivery efficiency, and cytotoxicity are explored. In this section, readers will learn that the backbone, side chains, end groups, and even the topology of the polymers could affect the outcomes of gene delivery. In addition, several PβAE-based carrier forms will be presented, including core-shell nanoparticles, hydrogels, and layer-by-layer (LBL) self-assembled multilayered films. Finally, the applications of PβAEs will be mentioned and briefly described.

SYNTHESIS

PβAEs can be easily produced through either Michael-addition reaction or ring opening polymerization (ROP) using step-growth polymerization. The Michael reaction involves the addition of a carbanion (or another nucleophile) to an α,β-unsaturated carbonyl compound with an electron-withdrawing functional group (Figure 3A).[15] ROP, on the other hand, involves the interaction of a reaction center at the end of one polymer chain with another cyclic monomer to open its ring system and create a longer polymer chain (Figure 3B).[15] By-products are not produced during these reactions, so further purification of the products is unnecessary. This allows researchers to construct large libraries of PβAEs and screen for suitable polymers for gene transfection.

PβAEs are often used as agents for transferring genes, whereby they can electrostatically interact with the negatively charged nucleic acids to create polymer/nucleic acid complexes, also known as polyplexes.[16] Moreover, the size of the formed polyplexes is generally in the range of tens to hundreds of nanometers, well known as nanoparticles. Therefore, the physicochemical properties of nanoparticles based on PβAEs, such as stability, size, surface potential, surface groups, degradability, stimulus-response properties, etc., can be tailored by modulating the chemical structure of PβAE polymers.[17]

Monomers

The first step in synthesizing PβAEs is the selection of the monomer. This procedure is essential for determining critical physicochemical properties such as the polymer skeleton structure, side-chain structure, topology, and so on.[18,19] First, the choice of monomer is a decisive step for the topology of the polymer. Typically, linear polymers are produced by diacrylate and primary amines or secondary diamines, while branched products are formed when diacrylates and triamines or triacrylates and diamines are applied.[20,21] Moreover, the choice of monomer also affects the reaction rate. A crucial factor determining the rate of Michael-addition depends on the availability of electrons in the amino group to the electron-deficient carbon atom in the acrylate. Therefore, the primary amino group containing monomers possesses a minor steric hindrance, leading to a faster reaction rate. According to the principle, the secondary amino groups have a higher steric hindrance than the primary amines, generally causing a slower reaction rate.[22,23] Figure S1 provides a list of the acrylates and amino monomers utilized in the production of PβAEs. In a typical example, the primary amino group can be reacted covalently to acrylate groups twice, wherein the second Michael-addition reaction on this amine is severely constrained. The secondary amino group on a ring, in contrast, exhibits strong reactivity and is likely to expose the lone pair of the electron cloud. The piperazine- and piperidine-based monomers are the most prevalent example.[22,23] The selection of the monomers' reaction equivalent ratios is another critical step. The synthesized PβAEs can be designed to be terminated by amine groups or acrylate groups, depending on the feeding ratios of monomers. Notably, the subsequent derivatization of PβAEs and functionalization of PβAEs-based nanoparticles significantly rely on those end groups.[24–29]

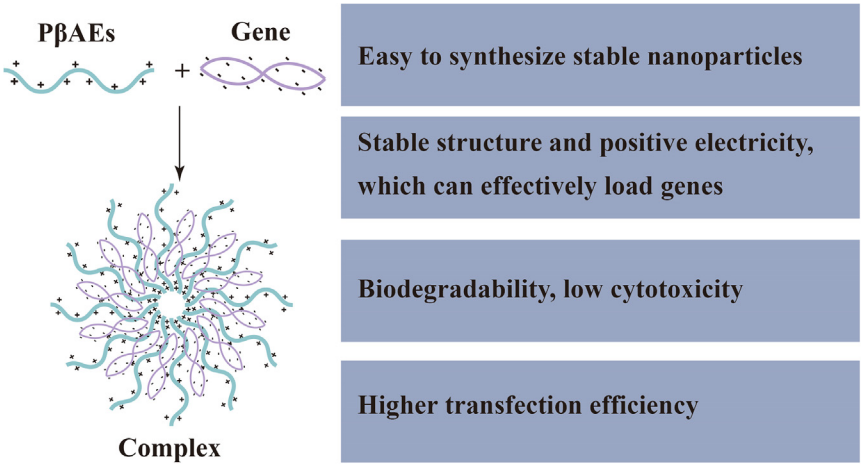

Figure 2. Main characteristics of PβAEs suitable for gene delivery

Reaction temperature

The second step is selecting the reaction temperature. The addition reaction proceeds more quickly at higher reaction temperatures. Higher temperatures can dramatically reduce the reaction time and elevate the molecular weight of the finished polymer in the reaction of diacrylate monomer with primary amines or secondary diamines.[15] The choice of temperature is related to the solvent. Solvent-free procedures are usually performed at higher temperatures and for shorter periods of time than procedures with solvents. Karimi et al. synthesized PβAEs using 1,6-Hexanediol diacrylate and 1, 4-aminobutanol under the solvent-free condition at 90°C for 24 h. The reaction in other research taken dichloro-methane (DCM) as solvent was performed at 50°C for 48 h.[30,31] In addition, the reaction temperature between diacrylates and triamine monomers is crucial in controlling the structure of the resulting polymers. According to a study, the chemical system between 2-aminoethyl piperazine and diacrylate has a critical transition temperature of 45°C.[32] Reactions below this temperature produce linear polymers, whereas reactions above this temperature yield hyperbranched polymers.[32]

Table 1. The factors that influence synthesis of the PβAE

Factors		Attentions
Synthesis		
Monomers	branched polymers	diacrylates and triamines or triacrylates and diamines
	linear polymers	diacrylate and primary amines or secondary diamines
Reaction Temperature	high temperature	diacrylate and primary amines or secondary diamines
		no solvent
	low temperature	with solvent, need more time
Reaction Solvent	no solvent	high temperature and short time
		linear polymers with high molecular weight
	with solvent	require lower temperatures and more time
		high polarity, higher molecular weight and reaction rate

Reaction solvent

Without a solvent, the Michael-addition polymerization may still occur.[8] There are several benefits to solvent-free polymerization. (1) A guarantee can be made for the highest monomer concentration.[33] (2) Without the solvent's boiling point as the temperature limit for polymerization, the reaction temperature can be increased, favoring the reaction rate.[33] (3) Higher molecular weight polymers can be gained.[23,33] (4) The purifying procedure can be avoided.[23,33] However, there are also significant restrictions on solvent-free polymerization. Solvent-free reactions are suitable for synthesizing linear polymers while branching polymers cannot be created in this synthetic manner because of the strong cross-linkability of the monomers in these reactions. Besides, there are other limitations to solvent-free polymerization, even for synthesizing linear polymers, including the melting points of monomers and obtained products, as well as difficulties in controlling dose ratios due to monomers vaporizing at high temperatures.

Instead, we can use solution polymerization, by which the reaction can be performed in a wide range of solvents. However, solvents containing hydroxyl groups should be avoided, mainly because of hydrolysis and ammonolysis during the synthesis process. Besides, many studies have pointed out that the solvent's polarity greatly influences the reaction rate and the molecular weight of the final product.[11,34] Generally, solvents with high polarity produce higher molecular weights and reaction rates. When using solvents-involved polymerization, two issues should be kept in mind: (1) the reactions require lower temperatures and more time than solvent-free polymerization strategies, and (2) the solubility of the monomer and polymer in the solvent, and if necessary, a solvent mixture is required.

STRUCTURE

The balance between cytotoxicity and gene transfection effectiveness is one of the central dilemmas of polycations as gene carriers. PβAEs are typically considerably less cytotoxic than other commercially available cationic polymers such as polyethyleneimine (PEI),[33,35] poly(L-lysine) (PLL),[33] and poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA).[36] The outperformance bioavailability is brought on by the reduced amino density, the backbone's hydrophobicity, and the ester bond's biodegradability. Concurrently optimizing

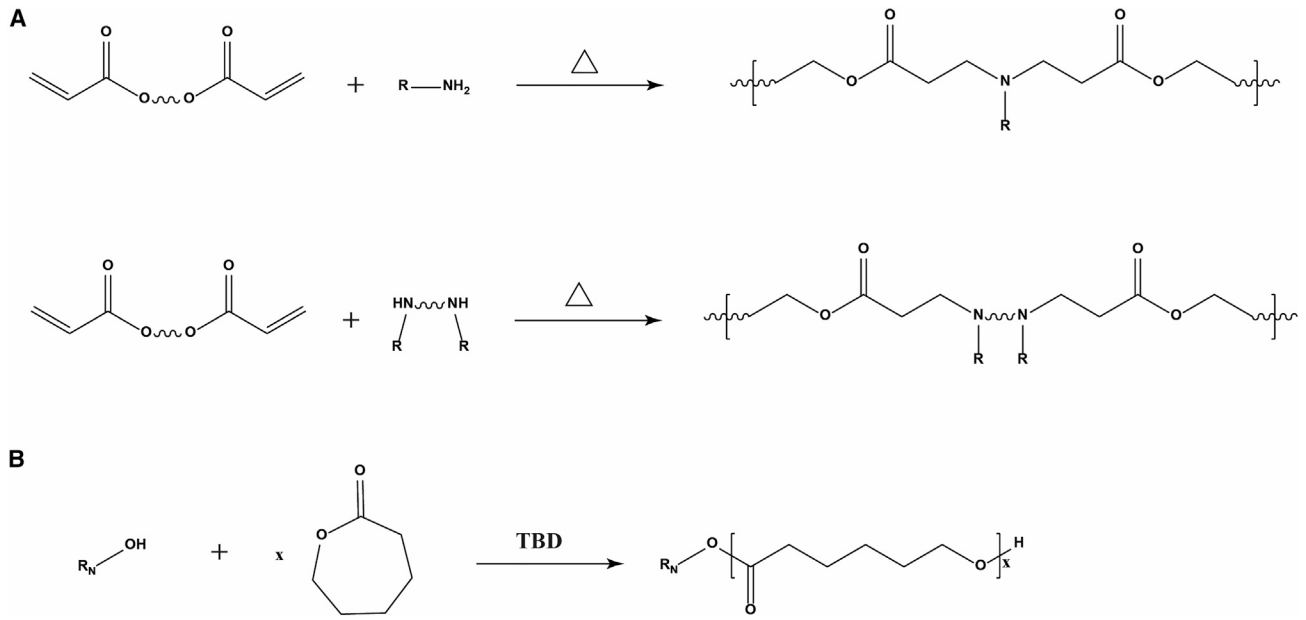

Figure 3. Synthesis of PβAEs
(A) Michael-addition reaction.[8] (B) Ring opening polymerization (ROP).[8]

cytotoxicity and gene transfection effectiveness is still a significant challenge for PβAEs.

Backbone of PβAEs

The feature of backbone and side-chain in polymers has a noticeable relationship with cytotoxicity and transfection efficiency. As a result, investigators have paid substantial attention to modulating the backbone structure of PβAEs to achieve optimal transfection efficiency.

When building polyplex nanoparticles with DNA or RNA, PβAEs with more carbon atoms in their backbone generally display enhanced gene delivery efficacy under physiological conditions. These formed nanoparticles outperform in terms of stability and size.[37] In 2005, Anderson and co-workers created a library of PβAEs and used it for gene transfection in COS-7 cells (Figure S1).[22,23] The findings indicated that the three outperforming PβAEs were C28, C32, and JJ28, in which the only difference between their chemical structures was a single additional carbon in the backbone. The diacrylate monomers of these polymers, C, JJ, and E, have respective carbon chain lengths of four, five, and six carbons. Similar to this, 20, 28, and 32 of the amine monomers are simply carbon chains with three, four, and five carbons, respectively. These most efficient polymers have nanoparticle sizes under 150 nm and positively charged surfaces. The two most efficient PβAEs had the smallest particle diameters, reporting 71 nm (C32) and 79 nm (JJ28), and a positive surface charge of greater than 10 mV.[23] So, if we increase the carbon chain length of amine monomers, i.e., C32 (five carbon atoms) > C28 (four carbon atoms) > C20 (three carbon atoms), PβAEs prepared with the same diacrylate monomer (C) will endow an increased gene transfection efficiency of the formed polyplex nanoparticles with a smaller size.[38] This scenario is well understood because other weak interactions for nanoparticle stability need to be considered in addition to the electrostatic interactions between polycations and DNA inside the polyplexes. One of these is the adjustment of carbon chain length mentioned above, which also imparts higher hydrophobicity to the core of nanoparticles, improving their stability in the aqueous phase.

Modifying diacrylate monomers with particular bonds, such as those containing disulfide linkages, acetals, nitrobenzene, etc., will dramatically alter the backbone properties of PβAEs. Reduction responsiveness and better gene transfection performance can be added to the vector by using diacrylate monomers with disulfide link structures, which are employed to create reducible PβAEs.[39] Duan et al. synthesized light-responsive PβAEs using NPBMDA, which refers to the UV-responsive monomer 2-nitro-1,3-phenylene bis(methylene) diacrylate.[40] When exposed to UV light, the polymer-containing 2-nitrobenzene groups underwent fast cleavage and degradation. A unique light-controlled gene delivery resulted from this approach, which encouraged the release of embedded genes and decreased the toxicity of the polymer. Therefore, the polymer demonstrated much greater DNA/small interfering RNA (siRNA) transfection efficiency and decreased cytotoxicity in diverse mammalian cells under light circumstances than PEI 25 kDa and Lipofectamine 2000.

Side chains of PβAEs

Based on the PβAEs synthesized by Prof. Langer's team in 2003 and Anderson et al. in 2005, the researchers discovered that polymers containing hydroxyl groups in their side chains performed remarkably well.[22,23] One of the most exemplary performing polymers, for instance, is a PβAE based on 1,4-butanediol diacrylate and

5-amino-1-pentanol, marked C32.[41] In the presence of serum, C32-based polyplexes considerably outperform jetPEI and Lipofectamine 2000 in transfecting HUVEC cells. The polyhydroxy surface of the nanoparticles, which can offer improved solubility for the nanoparticles in the aqueous phase and hence facilitate the stability of the polyplex nanoparticles in water, is thought to be responsible for this phenomenon. Additionally, the polyhydroxylated surface lowers the excessively high positive charge on the nanoparticle surface, assisting in the reduction of cytotoxicity.

End groups of PβAEs

Small molecules end-capped PβAEs

The characteristics of the gene delivery system, including its biological and physicochemical properties and ultimate efficacy of gene transfection, are tremendously impacted by alterations in the terminal structure of PβAEs.[22,23] Changing the stoichiometric ratio of diacrylate/amine is the first step in altering the terminal groups of PβAEs and makes it simple to create acrylate- or amine-terminated PβAEs.[22,23] Subsequently, modification with small compounds can result in functionalized PβAE homopolymers,[42] and we will obtain block polymers with large molecules as terminations. The multifunctionalization of PβAEs is highly convenient because most conjugation processes toward the end of PβAEs involve moderate reactions such as Michael-addition reactions, amidation reactions, esterification reactions, etc.

In a study comparing two different PβAEs, C28 was prepared from 1,4-butanediol diacrylate and 4-amino-1-butanol, and E28 was prepared from 1,6-hexanediol diacrylate and 4-amino-1-butanol.[23] The study's findings showed that polymers that ended with amino groups could transfect cells more effectively than those terminated with acrylates and that none of the polymers made with PβAEs terminated with acrylates could give successful gene transfection results.

The investigators then concentrated on the gene delivery capabilities of several amine-capped PβAEs. Among these PβAEs, C32, the most effective transfection PβAE, was chosen for various terminal alterations.[43] Compared with pristine C32, PEI (25,000 g/mole) and Lipofectamine 2000, C32 with amine end-capping (C32-103 and C32-117) could transfer DNA efficiently, roughly two orders of magnitude higher. The amino groups on the surface of the nanoparticles may be responsible for the 5-fold increased cellular absorption of modified C32 compared with unmodified C32.

Zugates et al. produced PβAEs modified with various terminal groups to learn more about the impact of terminal structures on gene transfection efficacy.[44] In general, transfection efficiencies were better for PβAEs having amine or hydroxyl groups as terminal groups. The transfection effectiveness of PβAEs terminated with an aromatic ring or alkyl chain was much limited. The level of hydrophobicity and the distance between the amine groups were found to affect the cytotoxicity of polymers that are terminated with two primary amine molecules. Generally, cytotoxicity rises with the amine's alkyl chain length or the terminal group's hydrophobicity. The positive charges and hydrophobicity of the polyplex nanoparticles improve their ability to interact with cell membranes. Nanoparticles between 85 and 130 nm are formed when PβAEs are terminated with diamines, but bigger particles between 150 and 220 nm are created when PβAEs are terminated with monoamine molecules. Additionally, this illustrates how terminal alteration affects DNA binding strength from the side. The ideal polymer/DNA ratio, also known as the N/P ratio, provides the strongest direct evidence for PβAEs binding to DNA. When compared with monoamine-capped polymers, diamine end-capped polymers yield high levels of DNA at lower polymer/DNA ratios.[43]

Oligopeptide is the other predominant end-modification category that can provide PβAEs with different functionalities. To evaluate the impact of PβAE C32's terminal alteration on gene transfection, the researchers used a variety of oligopeptides serving as terminal groups. The oligopeptides employed cysteine-terminated oligopeptides with essential amino acids such as arginine, lysine, histidine, and 2-methyl-1,5-pentane diamine.[45] The findings demonstrated that PβAEs with arginine or lysine-containing oligopeptides had higher transfection effectiveness than those with histidine- or diamine-containing oligopeptides or even those made with the GeneJuice commercial gene transfection reagent. Additionally, various levels of gene transfection were seen, depending on the cell lines. High gene transfection levels were seen in human immortalized keratinocytes (HaCaT) and normal human skin fibroblasts (NHDF) for polyplexes based on arginine-modified PβAEs. Using polyplexes with histidine-terminated PβAE bases, high transfection levels were achieved in A549 cells. When a combination of PβAE polymers modified with arginine and histidine (in a 1:1 ratio) was utilized, HeLa cells demonstrated significant transfection expression.[46] These findings imply that gene transfection in various cell lines is impacted by minor variations in the structure of PβAEs.

End-capping agents with various structural variations, such as mannose,[47,48] guanidine,[49] phosphonate,[50] quaternary amino,[51] and folic acid,[52] might give PβAEs extra functionality like biocompatibility, cellular uptake, or tumor targeting. Jones et al. assessed a collection of mannosylated PβAEs,[48] exhibiting effective gene delivery for stimulating antigen-presenting cells and subsequent immune response. Mannosylated polyplexes display elevated standardized antibody titers in the ovalbumin (OVA) mice vaccination paradigm than the OVA protein plus adjuvant control. This finding shows that mannosylated complexes can be used for genetic immunization at lower antigen levels without needing an adjuvant.

PβAEs were modified with lactate and folate to specifically interact with sialoglycoprotein receptors and folate receptors, which are frequently overexpressed in cancer cells.[53] Pullulan is a ligand for the sialoglycoprotein receptor, which is increased in hepatocellular carcinoma cells, hence functionalizing PβAE-based polyplexes with pullulan improved hepatocellular carcinoma targeted gene delivery.[54–57] These polyplexes appear to enter cells primarily through receptor-mediated endocytosis. Upon analysis with fluorescence

microscopy, it was observed that the rate of cellular internalization was more rapid in HepG2 cells, which are hepatocellular carcinoma cells that express the sialoglycoprotein receptor, than in A549 cells, which do not express this receptor and are derived from lung tissue.

Polyethylene glycol (PEG) end-capped PβAEs

PEGylation of PβAE to construct block copolymers is an excellent strategy for overcoming the various physiological and pathological barriers that polyplexnanoparticles face *in vivo*. The "PEG dilemma" exists but can be resolved by combining PEG's merits with the stimuli-responsibility of PβAE to increase biostability and intracellular drug release simultaneously.[58] As a result, PEGylation of nanoparticles can preserve a compact size, substantially improving their stability, and their transfection efficiency.

In a typical exemplary study, Prof. Suk's team created PEG-b-PβAE-b-PEG to transport plasmid DNA (pDNA) in the lung parenchyma of mice.[59] PEG-b-PβAE-b-PEG-based nanoparticles were administered intratracheally, penetrating the porous, extremely viscous mucus barrier. These PβAE-based mucus-penetrating DNA nanoparticles (PβAE-MPPs), which outperformed several industry-recognized gene delivery technologies, offered homogeneous and high levels of gene transfection throughout the mouse lung. PβAE-MPPs successfully transfected genes for at least 4 months following a single dose, and repeated dosing did not result in a reduction in transfection efficiency, underscoring their therapeutic importance. Importantly, PβAE-MPPs displayed a favorable safety profile after intratracheal injection without noticeable toxicity.

PEG-b-PβAE-b-PEG block copolymers combined with PβAE were employed in a project by Prof. Hanes and Prof. Suk to deliver genes to specific brain areas.[60] These novel formulations combine PEG5000-b-PβAE4000-b-PEG5000 and PβAE6000 polymers in various ratios. The greatest performing nanosystems include the physicochemical characteristics necessary for fast diffusion inside the brain parenchyma, including a hydrodynamic diameter of roughly 56 nm and a nearly neutral surface charge. The results show that, following cerebral perfusion via conventional improved delivery, these nanosystems undergo widespread, uniform, and high levels of gene transfection throughout the rat brain parenchyma both *in vitro* and *in vivo*.

The researchers created PβAE at various amine/acrylate ratios, which were then end-capped by PEG diacrylate and aminopropyl triethoxysilane in order to study the impact of PEG on the structure of PβAE and the effectiveness of gene delivery.[61] *In vitro* experiments conducted with 293T and HeLa cells revealed that both transfection efficiency and cell viability increased as the amine/acrylate ratio was increased from 0.7/1 to 6/1. An interesting observation was made regarding the consistency of transfection activity in culture media with and without serum. The most favorable PβAE, synthesized at a molecular weight of 16,000 g/mol and an amine/acrylate ratio ranging from 6/1 to 16/1, exhibited excellent gene transfer efficiency and low cytotoxicity when compared with PEI (25,000 g/mol) and Lipofectamine 2000. Despite using low PβAE/DNA weight ratios (as low as 10/1), these polymers maintained strong DNA condensation capabilities and produced stable particles that measured 133 nm.

Polycations end-capped PβAEs

Although PEGylation has traditionally been the preferred method for creating PβAE-based copolymers, many alternative copolymers have recently drawn attention. In order to co-deliver siRNA (Snail siRNA and Twist siRNA) and paclitaxel (PTX), PEI was first utilized to alter the ends of PβAE to improve the positive charge density.[62] In a 4T1 lung metastasis mouse model, siRNAs and PTX were substantially accumulated and maintained in the tumor area following intravenous injection of the nanosystem.[63] Additionally, after 21 days, the mixture of PTX, Snail, and Twist siRNAs suppressed tumor development and metastasis.[63]

A triblock copolymer of PDMAEMA end-capped PβAEs for pDNA delivery was recently created by a team.[64] Compared with bPEI25000-based polymers and the commercial transfection reagent TurboFectTM, PDMAEMA-b-PβAEs-b-PDMAEMA showed greater transfection efficacy and reduced cytotoxicity. The researchers created three PDMAEMA-b-PβAEs-b-PDMAEMA block copolymers with various molecular weights of PβAE to assess the impact of the PβAE segment's size on the biological activity.[65] The study results indicated that polyplexes, created using copolymers containing PβAEs with higher molecular weights (PDMAEMA3000-b-PβAEs12000-b-PDMAEMA3000), displayed the greatest biological activity in both HeLa and COS-7 cells. The most effective formulation showed a transfection efficacy 40–60 times higher compared with those obtained with bPEI25000 and TurboFectTM when using the PDMAEMA3000-b-PβAEs12000-b-PDMAEMA3000-based copolymer at an N/P ratio of 100/1 in both cell lines.

Topology of PβAEs

Since 2002, synthetic hyperbranched PβAEs have been reported, with the structure taken as a model from the PEI that serves as the gold standard for polycations.[66] In two separate investigations published in 2005, they were initially described as non-viral gene vectors for the delivery of pDNA.[67] These hyperbranched PβAEs have several physicochemical characteristics that make them suitable as gene vectors.

Among these, hyperbranched PβAEs were created by mixing two different diacrylates (ethylene glycol diacrylate and 1,4-butanediol diacrylate) with various multi-amines, including N-methylethylenediamine, 1-(2-aminoethyl) piperazine, and 4-aminomethyl piperidine.[20,21,23,68] With an estimated branching degree of 0.30, these polymers possess several favorable characteristics that make them suitable for use as gene carriers. These include water solubility, physiological degradation, elevated buffering capacity in the pH range of 5.1–7.4, and the ability to compactly condense pDNA into nanoparticles with positive surface charges. Studies carried out *in vitro* on COS-7 cell lines, even in the presence of serum, revealed considerable transfection activity and no (or minimal) cytotoxicity.[69] Transfection levels were highest

in polymers made from 1,6-hexanediol diacrylate and 1-(2-aminoethyl) piperazine.[69] The values were greater than or at least on par with polyplexes built based on bPEI25000 or linear PDMAEMA. In another investigation, 1-(2-aminoethyl) piperazine and trimethylolpropane triacrylate were used to create hyperbranched PβAEs. A primary amine is located outside the molecular structure, and secondary and tertiary amines are located at the center, similar to PEI. A stable polyplex nanoparticle is created when the polymer is condensed with pDNA. Human embryonic kidney (HEK 293) and COS-7 cells were used for *in vitro* gene transfection experiments. The results showed equivalent transfection effectiveness and less cytotoxicity compared with polyplexes made using bPEI25000.[70] Following this, the study was broadened to investigate how the pDNA delivery was affected by the terminal amine type using hyperbranched PβAE vectors. Interestingly, it was observed that the amino-terminal groups had no discernable influence on DNA transfection efficacy, toxicity, or the capacity for DNA condensation mediated by hyperbranched PβAEs.

Due to the limited selection of monomers and the complexity of the synthesis stages, the above ABn monomer synthesis technique of creating hyperbranched PβAEs has had difficulty controlling the branched structures throughout the polymerization reaction. Researchers are also struggling with the polymerization process' tendency to gel, which is an issue. Prof. Wang's team developed a brand-new "A2 + B3 + C2" Michael-addition polymerization technique.[32] The center branching monomer is an acrylate with numerous identical reactive sites, such as trimethylolpropane triacrylate (TMPTA). Triacrylate, diacrylate, and primary amine (A2-type monomer, B3-type monomer, and C2-type monomer) are then copolymerized in a single pot and ended with amines (E). This method improves the characteristics and capabilities of PβAEs as gene carriers while lowering the risk of gelling by reducing the concentration of cross-linked monomers. The method improves the characteristics and capabilities of PβAEs as gene carriers while lowering the concentration of cross-linked monomers, which reduces the possibility of gelling. Numerous works have demonstrated that this strategy enables adjustable manipulation of the chemical component ratios within the esterified polymers, improving the efficacy of gene transport.[71–73] Additionally, the presence of terminal groups offers a distinct benefit for further polymer modification. The findings support the hypothesis that the terminal functionalization of amine and guanidine groups enhances the transfection activity of polyplex nanoparticles based on hyperbranched PβAEs. This higher activity can be attributed to the polyplex nanoparticles' improved cellular binding and internalization, which is caused by the interaction of the guanidine groups with phosphate or other anionic molecules on the cell membrane surface.

The degree of branching or hydrophobicity of hbPβAEs is two minor structural changes that significantly impact gene transfection efficacy.[58,74] In comparison to polyplexes based on counterparts or other hbPβAEs, bPEI25000, or SuperFect with a higher degree of branching, it was found that those with a lower degree of branching (10%) had a higher gene transfection rate (24%, 43%, and 73%). Compared with polyplexes based on linear equivalents, or even bPEI25000 or Lipofectamine 2000, the addition of hydrophobic alkyl chains in hbPβAEs led to greater gene transfection activity. Additionally, it was discovered that these hbPβAE-based polyplexes are hydrophobic and can transfect even when employing low DNA doses or high serum ratios (30% and 50% FBS) (120 and 60 ng).

The development of a new biodegradable polymer, highly branched PβAEs (HPAESS), has opened up new possibilities for gene therapy (Figure S2). This polymer has been modified through the introduction of disulfide bonds and specific ligands, resulting in two versions of HPAESS: HPAESS-FA, which is functionalized with folic acid, and HPAESS-Lac, which is functionalized with lactic acid.[53] These modifications allow the polymer to target specific receptors found in certain types of cancer cells. The results of studies on these vectors have shown that they have superior transfection capacity compared with other currently available methods. Furthermore, they do not cause significant harm to non-target cells, making them a promising candidate for gene therapy.

FORMULATIONS OF PβAE

PβAEs are frequently prepared into diverse formulations based on their physical, chemical, and mechanical properties in order to satisfy various needs (Figure 4). For instance, core-shell nanoparticles, which have a hydrophobic core that serves as a drug reservoir and a hydrophilic shell that prevents core micelles from aggregating, considerably increase delivery efficiency.[75] To address the local and sustained release of siRNA, PβAE-based hydrogel systems were employed to take advantage of their injectability, tunability, and degradability.[76] To cover scaffolds and planes for local administration of therapeutic siRNA, PβAEs were prepared into multilayer films.[77]

Core-shell nanoparticles

The electrostatic interaction is the primary force between PβAEs with DNA or RNA, leading to the formation of polyplex nanoparticles. Using block copolymers, like PEG-PβAEs, will likely prepare micellar nanoparticles with a polyplex core and a PEG shell.

Utilizing hybrid nanocarriers is another method of producing micellar nanoparticles. Lipid-involved polyplexes created with PEG-lipid and PβAEs are the most widely used hybrid nanocarriers. For instance, mRNA and C14-PEG2000 PEG-lipid (5% mol PEG-lipid) were combined with a PβAE terpolymer made from bisphenol A glycerol diacrylate (DD), 2-morpholinoethylamine, and dodecylamine (C12). With a high transfection efficiency (about 75%) on the lung endothelium, this formulation was demonstrated to be a lung-targeting formulation when supplied intravenously.[78]

Recently, Yu et al. employed PβAEs with acid-cleavable ketone bonds to bind Bcl-2 siRNA, then co-precipitated with epirubicin (EPI) to create a core and then coated the core-shell nanosystem with PEG-liposomes.[75] The nanosystem efficiently reduced cell growth *in vitro*, and cotransfection dramatically reduced P-glycoprotein (P-gp)

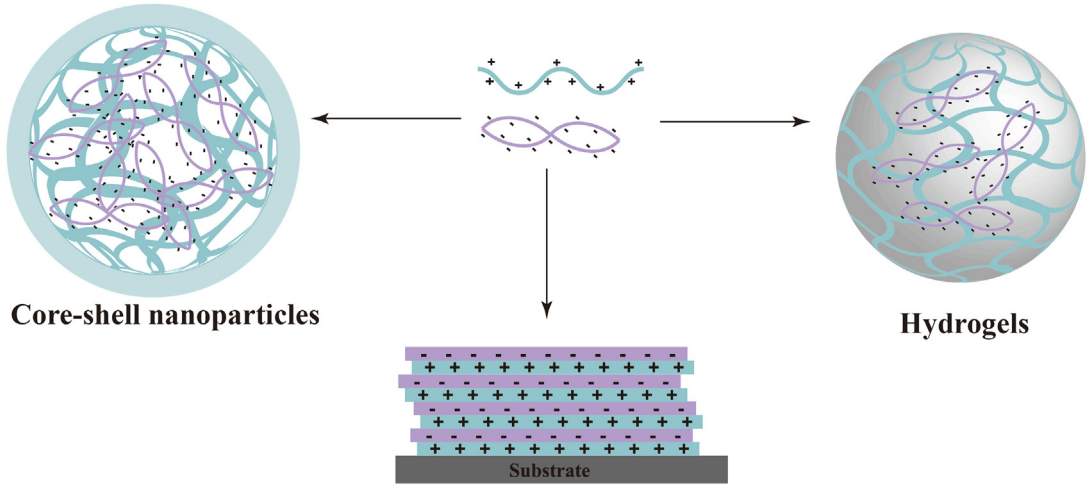

Figure 4. The different formulations of PβAE

expression, suggesting that it may be a possible treatment for tumor cells resistant to drugs.

Hydrogels

Recent studies have demonstrated that local and sustained administration of nucleic acids using hydrogels can resolve the issue of low nucleic acid stability, improve anticancer efficacy, and decrease systemic toxicity.[79] The hydrogels made employing PβAEs in the main chain directly, however, are vulnerable to fast hydrolysis and deterioration. Because of this, scientists are now using hydrogels with PβAEs-based polyplexes embedded in them. Nucleic acid medications with good, controlled release qualities and biocompatibility can be successfully encapsulated by the developed delivery technology. For instance, using PAMAM/dextran hydrogel scaffolds and arginine-capped PβAE nanoparticles, Segovia et al. reported a unique siRNA local sustained delivery platform.[80] With 96% cell survival, this delivery system has good cytocompatibility, and *in vitro* silencing efficacy was greater than that of the commercial transfection agents Lipofectamine and INTERFERIN. The mouse breast cancer model, in particular, showed a more substantial persistent silencing effect (55% *in vitro* and 70% after 6 days *in vivo*). According to this study, PβAEs may be helpful for hydrogel gene delivery.

Furthermore, our team has created various series of nano-hydrogel systems employing PβAEs with acrylate bonds capping as the cross-linking agent of the macromolecules, even though PβAE polymers cannot be used in the main chain in the creation of hydrogels.[81–83] We take advantage of the structural characteristics of PβAEs, especially hyperbranched PβAEs, in these systems to improve the hydrophobic drug loading efficiency and lysosomal escape capability.

LBL self-assembled multilayered films

The first degradable LBL self-assembled multilayer film (PEM) based on electrostatic interactions was created in 2002 by Prof. Hammond's research team using PβAEs.[84] A PEM of PβAEs/pDNA for *in situ* pDNA controlled release and distribution was created by Lynn et al. in 2004.[85] On the surface of planar silicon and quartz substrates with thicknesses up to 100 nm, pDNA encoding enhanced green fluorescent protein (eGFP) and PβAEs were constructed into PEMs using an LBL deposition technique. The pDNA was fully liberated after 30 h in phosphate-buffered saline (PBS). Preliminary transfection tests performed on COS-7 cell lines also showed that the released pDNA was transcriptionally viable and encouraged eGFP expression using Lipofectamine 2000.

Jewell et al. observed COS-7 cells grown in serum-containing medium were exposed to film-coated slides, gene expression was observed in the cells located beneath the film-coated area (Figure S3).[86] Subsequently, they investigated the use of these PEMs for stainless steel surface modification of intravascular stents using their findings on the use of PEMs for pDNA delivery.[86,87] The stents were uniformly coated with thin PEM films with a thickness of about 120 nm (eight layers of polymer1 and pDNA). PEMs were durable in the presence of external mechanical forces. For 4 days at 37°C, pDNA was released *in vitro* in PBS. In a rat model of sphere-induced artery damage, additional *in vivo* studies were carried out using inflatable embolic catheter balloons with changed surfaces. Ultrathin PEMs based on polymer 1 and pDNA encoding eGFP or β-galactosidase were employed to facilitate local tissue transfection. The outcomes demonstrated that polymer 1/pDNA films transported DNA well. The outcomes demonstrated that functional pDNA was effectively transferred to the vessel wall using polymer 1/pDNA films. Investigations into *in vivo* transfection in pigs and rabbits were also conducted.

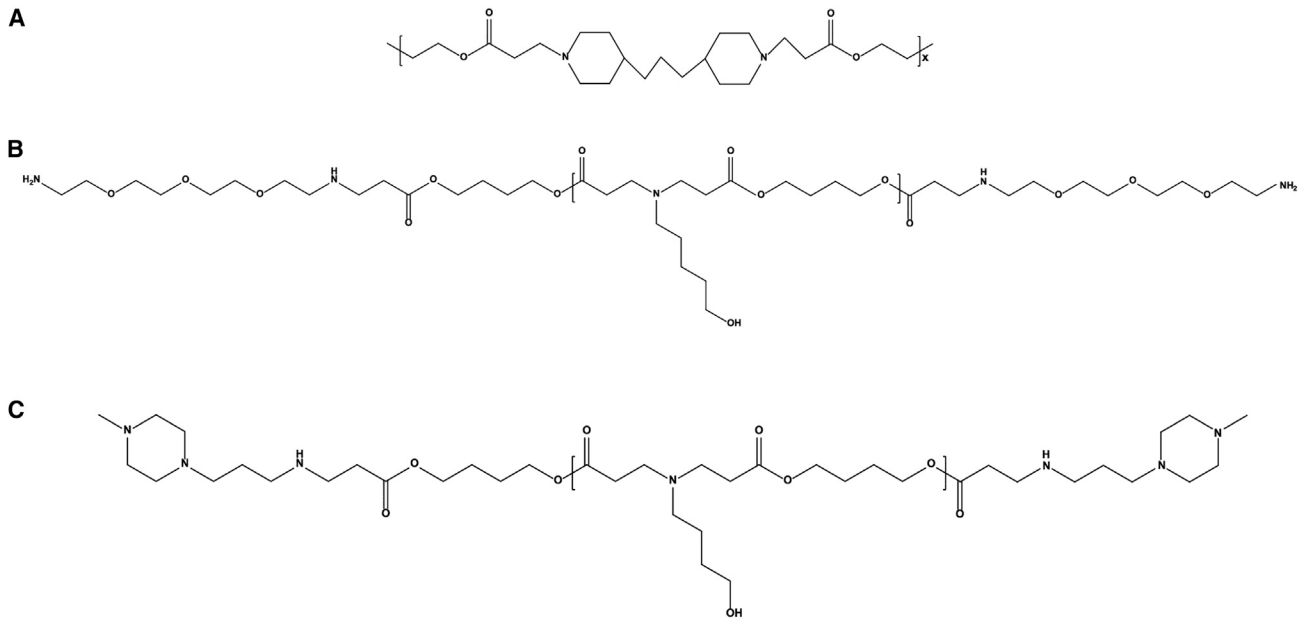

Figure 5. The structure of PβAE polymers in vaccines

(A) PβAE with degradability and pH responsiveness was used in non-viral genetic vaccines.[89,90] (B) PβAE was contained in a mRNA-based vaccine.[91] (C) Lipid-modified PβAE was used in COVID-19 vaccines.[92]

Transgene expression in scaffold tissue was verified in rabbits and pigs 2 days after implantation. The transfection in the subendothelial tissue of the pigs' arteries was also uniform.

Nanocarriers made by LBL assembly processes are used in another method. Prof. Green's research group created a hybrid nanosystem to transport numerous genes.[88] Before coating the top layer with PβAE B2, they first created a therapeutic nanoplatform of LBL made of a gold nanoparticle (AuNP) core combined with PEI. 1-(3-aminopropyl)-4-ethylpiperazine, a gene carrier for previously investigated and confirmed glioblastoma multiforme cell lines, was used to modify the PβAE B2. Earlier studies have also reported the delivery of pDNA and siRNA by PβAEs in human primary brain cancer cells. The end result of this study is a nanotherapeutic platform that can induce siRNA-mediated gene knockdown and transgene expression, with a significantly stronger knockdown effect compared with the standard control Lipofectamine 2000 transfection reagent.

THE APPLICATION OF PβAEs

Vaccines

In recent years, nucleic acid vaccines have emerged as a promising solution for preventing and treating infectious illnesses and cancer. Compared with traditional immunizations, they are highly effective and low cost, making them a valuable tool in the fight against diseases. Professor Langer's team reported in 2004 that microparticles containing PβAEs improve the activity of non-viral genetic vaccinations (Figure 5A).[89] They found that hybrid PLGA/PβAE microparticles successfully stimulate dendritic cells *in vitro* and considerably increase DNA transfection efficiency by 3–5 orders of magnitude. *In vivo* administration of these microparticle formulations as vaccines resulted in the specific elimination of transplanted syngeneic tumor cells, which is not feasible with conventional formulations.

PβAEs with lipids were performed to deliver mRNA by Su and colleagues.[90] Lipids make PβAEs more biocompatible and stable, whereas PβAEs are pH-responsive and biodegradable, which helps transported mRNA escape from endosomes and reduce cytotoxicity. Shen and colleagues developed a platform based on PβAEs that transports mRNA effectively (Figure 5B).[91] An envelope of lipid surrounding the PβAEs-mRNA complex is primarily made up of 1,2-dioleoyl-sn-glycero-3-ethyl phosphocholine (EDOPC)/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-amin (DSPE-PEG2k). This study showed that core-shell structured mRNA vaccine has a significantly higher transfection efficiency than bare PβAEs-mRNA. Additionally, the mRNA vaccine activated the TLR7/8 signaling pathway, which led to the production of cytokines by dendritic cells (DCs) and consequently slowed tumor growth.

Recently, Li et al. created a "particle-in-particle" (PNP) nanostructure for gene delivery.[92] Using enzyme-catalyzed esterification, researchers developed a new type of lipid-modified polymeric PβAEs (L-PβAEs) that were further used to form L-PβAEs in combination with poly(d,l-lactic acid-ethylene glycol)-b-poly (ethylene glycol) (PLGA-PEG), resulting in the self-assembly of L-PβAEs into PNPs (depicted in Figure 5C). Out of the 24 potential PNPs, the best-performing PNP/C12-PβAE nanoparticle was able to deliver both DNA and mRNA both *in vitro* and *in vivo*, exhibiting improved transfection efficacy, sustained gene release, and excellent

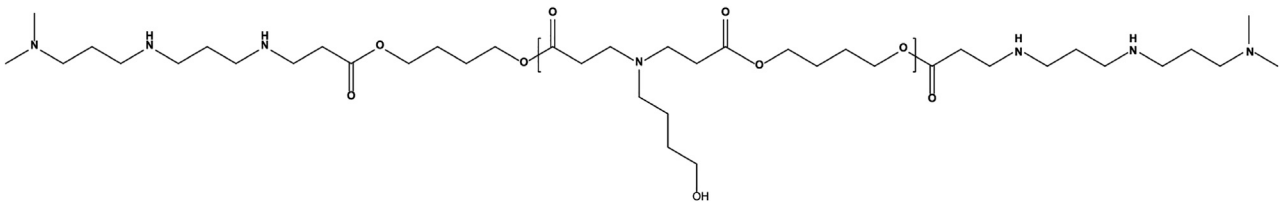

Figure 6. The structure of PβAE polymers in vascular formation
The polymer 44E49 was used in vascular formation.[93]

stability even after lyophilization and storage at −20°C for up to 12 months without any impact on its transfection effect. Furthermore, when mice were vaccinated using a COVID-19 vaccine containing plasmid DNA and spike-expressed mRNA, the resulting product generated spike-specific antibodies and Th1-biased T cell immune responses.

Vascular formation

Apart from the potential applications in disease treatment, PβAE-based nanobiotechnology also holds immense promise as a strategy for generating blood vessels that could be used in tissue engineering and various clinical applications. The formation of stable blood vessels is not only a challenge for *in vitro* human tissue engineering but is also valuable for clinical applications such as peripheral arterial disease. Nanocomplexes formed by self-assembly of PβAE polymers (Figure 6) with HIF-1α plasmid DNA were able to induce three-dimensional vascularization of human adipose-derived stem cells (hASCs).[93] The study showed that cells subjected to transfection exhibited 106 times higher expression of HIF-1α and around a 2-fold increase in secreted VEGF levels. The resultant complexes led to the development of vascular tubules compared with un-transfected control cells. These findings strongly imply that PβAE-based nanobiotechnology holds immense potential as a strategy for generating blood vessels, which can be useful for both tissue engineering and various clinical applications.

Genital tract infections

When it comes to treating genital tract infections, topical vaginal administration is a highly effective method that offers several benefits. Not only does it maximize therapeutic effectiveness, but it also minimizes off-target local negative effects. In fact, two innovative gel delivery techniques have been developed by Niu et al. to further enhance the efficacy of this approach. These two formulations are based on modified montmorillonite (mMMT) and vermiculite gels, which serve as the foundation for PβAE-plasmid multimeric nanoparticles (NPs) (HTT) (Figure 7). By using the CRISPR-Cas9 system to target porcine endogenous retroviruses (PERVs), the viral copy number can be significantly reduced.[94] One of the key advantages of this approach is that the PβAE-based mMMT gels allow for the successful delivery of the CRISPR-Cas9 system into vaginal epithelial cells. This targeted delivery method ensures that the treatment is highly effective while minimizing any potential negative side effects.

Furthermore, locally injected CRISPR-Cas9 system is relatively harmless and non-toxic, as it only expresses in the vagina/cervical region and does not disseminate to other surrounding organs. This makes it a safe and effective treatment option for genital tract infections. Overall, the use of topical vaginal administration and innovative gel delivery techniques offer a promising approach for treating genital tract infections. With further research and development, this approach could potentially revolutionize the way we treat these types of infections in the future.

CONCLUDING REMARKS AND PERSPECTIVES

The purpose of gene delivery is to allow exogenous nucleic acids to enter cells. Some nucleic acids encode proteins with therapeutic effects, such as pDNA, mRNA, or can affect the expression of target proteins, such as siRNA. Theoretically, nucleic acids can treat many diseases, including cancer, infectious diseases, rare diseases, etc. However, nucleic acids are hydrophilic and negatively charged, while cell membranes are hydrophobic and negatively charged. Therefore, nucleic acids need to be wrapped by vectors before being delivered into cells. There are two types of gene delivery vectors: viral vectors and non-viral vectors. Viral vectors, such as adenoviruses, are more efficient in delivery but challenging to prepare, yield little, and are prone to inflammatory responses. On the other hand, non-viral vectors play an essential role in nucleic acid transfection due to their abundant material sources, controllable chemical structures, low immunogenicity, and straightforward large-scale preparation. Although lipid nanoparticle platforms have demonstrated potent applications in gene transfer, their patents are held by a few corporations, and their necessity to be transported and stored in ultra-low temperature settings makes them unsuitable for general use.

Cationic polymers do not have strict storage and transportation constraints. A common feature of their structure is the presence of many amino groups in the molecule, which are protonated into positively charged polymers capable of being used for gene delivery. It is widely believed that the higher the molecular weight of a cationic polymer, the greater its ability to condense nucleic acids and be taken up by cells, and the less cell viability and nucleic acid release. On the other hand, lower molecular weight polymers have a reduced ability to coagulate nucleic acids and be taken up by cells but are better in terms of cytotoxicity and nucleic acid release. PβAEs balance these factors. They are biodegradable, biocompatible, and pH-responsive. The polymers form a backbone with ester bonds and are readily degraded by hydrolysis

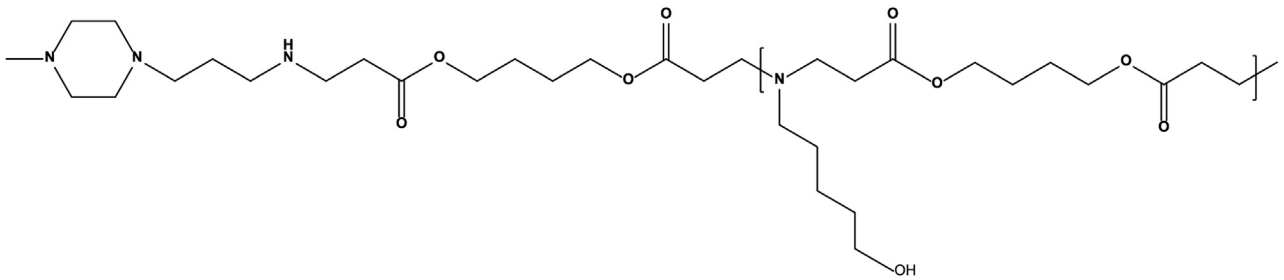

Figure 7. The structure of PβAE polymers in genital tract infections
PβAE was used in vaginal gene therapy.[94]

reactions under physiological conditions. When hydrolyzed, the polymers become small molecules such as bis(β-amino acids), diols, etc., which are considered harmless to mammalian cells. Compared with other cationic polymers, PβAEs have a lower surface charge density, resulting in weaker binding strength to nucleic acids. Therefore, the amount of PβAEs required for nucleic acid binding should be significantly higher than PEI, PPI, and other cationic polymers. Even so, the transfection efficiency with PβAEs as a vector is higher due to its reduced cytotoxicity. Endcaps containing secondary amines or other amine-containing groups have been designed to improve the binding capacity and overall transfection efficiency of PβAEs. In addition, PβAEs can be converted from hydrophobic to hydrophilic with changes in pH values. This property can be used to prepare pH stimulation responsive carriers. Therefore, PβAEs are uniquely suited as an alternative to lipid nanoparticle platforms among cationic polymers.

The degradation of PβAEs is an important process that affects its stability and release of the payload. *In vitro* studies have shown that the hydrolysis of ester bonds in PβAEs plays a dominant role in PβAEs' degradation, depending on the polymer's chemical structure and ambient pH, temperature, etc. Hydrophobic PβAEs tend to hydrolytically degrade more slowly than hydrophilic ones.[95] But a study found that PβAEs containing pendant primary amine demonstrated an unusual PβAE degradation behavior, where the relatively most hydrophobic PβAEs degraded fastest. The half-lives of this PβAE were 48 h, 14 days, and over 65 days at pH values of 7.4, 6.0, and 5.0, respectively.[19] This unique behavior is attributed to abundant free primary amines in PβAEs with pendant primary amines that facilitate the intra- or inter-molecular nucleophilic attack on ester bonds, forming amide as the main factor influencing degradation rate.[19,95–97] Tertiary amines are less reactive than primary amines, so they play an insignificant role in degradation. Therefore, the hydrophilicity of PβAEs determines the degradation rate.[95–97] *In vivo* studies of PβAE hydrolysis are limited, but some studies have shown that PβAEs can be rapidly degraded in the presence of esterases and other enzymes in the body. The rate of hydrolysis can also be affected by the location of the polymer, with PβAEs implanted in tissues showing slower degradation than those injected into the bloodstream. Overall, the kinetics of PβAE hydrolysis is complex and depends on various factors. Further research is needed to fully understand the mechanisms of PβAE hydrolysis *in vitro* and *in vivo*.

Although some features of PβAEs can cause instability of nanoparticles and low transfection efficiency in physiological environments, these polymers can be easily modified chemically and mixed with other materials in a non-covalent manner, making them customizable to overcome various delivery barriers for specific applications. A recent investigation utilized PβAEs to encapsulate mRNA, which was then orally administered and delivered to the stomach using the self-orienting millimeter-scale applicator device.[98] The nucleic acid nanoparticle formulation was contained in an oral pill that could be injected into the gastric mucosa with a microneedle, allowing for absorption in the stomach and throughout the body. The study demonstrated that using PβAE polymer-encapsulated mRNA molecules can enhance the transfection efficiency of oral delivery compared with other types of nanoparticles. This research presents a promising solution for delivering nucleic acid-based therapies/vaccines orally while avoiding degradation by enzymes and physical barriers in the gastrointestinal tract.

It is important to note that PβAEs can disrupt the stability of negatively charged cell membranes. The effect of the small molecules generated by the degradation of PβAEs on the local microenvironment is not fully understood. Additionally, the release of amino groups during degradation may affect the local pH and cause inflammation or other issues. Therefore, caution should be exercised when using PβAEs as delivery reagents, and these factors should be carefully considered.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.04.019.

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AUTHOR CONTRIBUTIONS

J.Z. and X.C. grafted and edited the manuscript. R.D., C.G., and J.T. collected information. Y.H. revised and verified the manuscript. H.C. and J.C. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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