ARTICLE IN PRESS

Advanced Drug Delivery Reviews xxx (xxxx) xxx

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/adr

Highly branched poly(β-amino ester)s for gene delivery in hereditary skin diseases

Ming Zeng [a,1], Qian Xu [b,1], Dezhong Zhou [c,*], A Sigen [b], Fatma Alshehri [d], Irene Lara-Sáez [b], Yue Zheng [e], Ming Li [f,*], Wenxin Wang [b,*]

[a] Department of Dermatology, the First Affiliated Hospital of Jinan University, Guangzhou Overseas Chinese Hospital, Guangzhou 510630, China
[b] Charles Institute of Dermatology, University College Dublin, Dublin 4, Ireland
[c] School of Chemical Engineering and Technology (SCET), Xi'an Jiaotong University, Xi'an, Shaanxi 710049, China
[d] Princess Nourah Bint Abdulrahman University, Riyadh 11671, Saudi Arabia
[e] Department of Dermatology, the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, China
[f] Department of Dermatology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

ARTICLE INFO

Article history:
Received 15 April 2021
Revised 28 May 2021
Accepted 15 June 2021
Available online xxxx

Keywords:
Cutaneous gene delivery
Polymeric vectors
Highly branched poly(β-amino ester)s
Hereditary skin diseases
Recessive dystrophic epidermolysis bullosa

ABSTRACT

Non-viral gene therapy for hereditary skin diseases is an attractive prospect. However, research efforts dedicated to this area are rare. Taking advantage of the branched structural possibilities of polymeric vectors, we have developed a gene delivery platform for the treatment of an incurable monogenic skin disease – recessive dystrophic epidermolysis bullosa (RDEB) – based on highly branched poly(β-amino ester)s (HPAEs). The screening of HPAEs and optimization of therapeutic gene constructs, together with evaluation of the combined system for gene transfection, were comprehensively reviewed. The successful restoration of type VII collagen (C7) expression both *in vitro* and *in vivo* highlights HPAEs as a promising generation of polymeric vectors for RDEB gene therapy into the clinic. Considering that the treatment of patients with genetic cutaneous disorders, such as other subtypes of epidermolysis bullosa, pachyonychia congenita, ichthyosis and Netherton syndrome, remains challenging, the success of HPAEs in RDEB treatment indicates that the development of viable polymeric gene delivery vectors could potentially expedite the translation of gene therapy for these diseases from bench to bedside.

Contents

* Corresponding authors.
E-mail addresses: dezhong.zhou@xjtu.edu.cn (D. Zhou), liming01@xinhuamed.com.cn (M. Li), wenxin.wang@ucd.ie (W. Wang).
[1] Ming Zeng and Qian Xu contribute equally to this work.

https://doi.org/10.1016/j.addr.2021.113842
0169-409X/© 2021 The Author(s). Published by Elsevier B.V.

Please cite this article as: M. Zeng, Q. Xu, D. Zhou et al., Highly branched poly(β-amino ester)s for gene delivery in hereditary skin diseases, Advanced Drug Delivery Reviews, https://doi.org/10.1016/j.addr.2021.113842

1. Introduction

The human genome project has accelerated the discovery of the genetic basis of an increasing number of diseases and corresponding interventions for treatment. Following almost three decades of continuous investigations, gene therapy is starting to take center stage as a state-of-the-art biotherapy with obvious clinical benefits [1]. Gene-based therapies are broadly defined as the intentional modulation of gene expression in target human cells for treating a pathological condition. More specifically, some researchers restrict the definition of gene therapy to introduce exogenous nucleic acids that can achieve gene addition, gene correction/alteration and gene knockdown [2,3]. This modulation is achieved by exploiting the exogenous nucleic acids such as DNA, mRNA, small interfering RNA (siRNA), microRNA (miRNA) or antisense oligonucleotides (AONs) [3]. Based on the nature of the vector, gene therapy is broadly classified into viral and non-viral approaches. Compared with viral counterparts, polymeric vectors have multiple advantages: (i) they do not integrate into the host genome and thus exhibit a high biosafety profile with minimal immunogenicity and pathogenicity [4]; (ii) they have a greater capacity for delivering large gene segments than viruses [5]; (iii) their synthesized nature and design flexibility enable a good balance in efficiency and safety [6]; and (iv) they are convenient for scalable manufacturing and thus favored for the bench-to-bedside translational applications in a clinical setting. As a major category of non-viral candidates, polymeric vectors such as poly(ethylene imine) (PEI), poly(L-lysine) (PLL), poly(β-amino esters) (PAEs) and poly [2-(dimethylamino)ethyl methacrylate] (PDMAEMA) have shown great promise for delivering therapeutic gene constructs in a host of disease conditions [3,5,7]. In particular, PEI and its variants have been widely tested in clinical trials with the main focus on oncological studies [3].

The skin is an appealing organ for gene therapy for several reasons: (i) it comprises the largest organ of the human body, maintains homeostasis, and shields the body from the external environment; (ii) it is easily accessible for treatment with topical/intradermal/subcutaneous/electroporation applications for convenient gene transfer; and (iii) the ready accessibility of genetically engineered skin to clinical observations and macroscopic, microscopic, cellular and molecular analyses. Clinically, genetic skin diseases, chronic wounds, skin cancers and vaccine development are among the most suitable and ideal targets for gene transfer technologies through different mechanisms [8-11]. Design and development of safe and efficient vectors for skin gene delivery is of great significance. However, currently the majority of gene delivery vectors in the treatment of genetic skin diseases are viral. By combining the structural advantages of polymeric vectors and the versatile properties of poly(β-amino ester)s (PAEs) based systems [12,13], our research group developed highly branched poly (β-amino ester)s (HPAEs) for the treatment of a representative monogenic skin disease – recessive dystrophic epidermolysis bullosa (RDEB). This review first introduces an overview of RDEB and the main treatment methods, and the development of HPAEs for RDEB treatment are then reviewed. Finally, the challenges, prospects and favorable delivery routes of polymeric vectors for targeting hereditary skin diseases are discussed. We believe the rapidly developing polymeric gene delivery systems will facilitate gene therapy for hereditary skin disorders.

2. Development of HPAEs for RDEB therapy

2.1. RDEB overview

First described with subtype classification in 1962, epidermolysis bullosa (EB) has drawn increasing attention from researchers for more than half a century [14]. It represents a heterogeneous group of skin fragility diseases characterized with blisters, erosions, and chronic ulcers of cutaneous and mucosal surfaces [15]. EB is caused by mutations in various genes encoding structurally or functionally related proteins. Generally, EB is divided into four major subtypes depending on the level of blister formation in the skin structure, known as EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB) and Kindler syndrome (KS) [16,17]. To date, among more than 30 subtypes of EB which have been reported, RDEB is considered one of the most devastating [18]. Clinically, RDEB is more severe than the dominant DEB (DDEB) [17]. Genetically, RDEB is caused by the biallelic loss-of-function mutations within the *COL7A1* gene which results in deficient or absent expression of type VII collagen (C7) [18]. Fig. 1a shows the schematic illustration of pathogenesis of RDEB. It is well known that both human keratinocytes and dermal fibroblasts can secrete full-length 290 kDa C7 [19], the main component of anchoring fibrils (AFs), which offer the main structural connection between the skin basement membrane zone (BMZ) and papillary dermis, exerting the role of a "biological Velcro" at the dermal-epidermal junction (DEJ) [20]. The compromised skin integrity in EB is attributed to the absence or diminished amounts of C7. In addition to the chronic and recurrent wounds, tissue fibrosis, severe pain, and frequent failure to thrive, there is a very high risk for RDEB patients to develop aggressive squamous cell carcinoma due to the chronic remodeling and increased cell proliferation occurring at lesion sites [21]. This risk increases to 67.8%, 80.2% and 90.1% by the age of 35, 45 and 55, respectively [22]. Affecting about one in one million newborns in the United States, RDEB often shows symptoms from birth with a life expectancy of 30 years in severe patients and a median survival of 55–65 years in mild phenotypes, respectively [23,24]. Economically, EB imposes a heavy burden on global healthcare, and the treatment cost in a severely affected case can approach $300,000 per year in the USA [17].

2.2. Treatment limitations of RDEB

Currently, there is no cure for RDEB. The clinical management of RDEB is limited to symptomatic treatment and skin care, and aims to promote would healing, control infection, relieve pain and itch, and prevent disease complications [18]. Novel and emerging therapies toward RDEB mainly consist of suppressing inflammation and fibrosis, restoring C7 and accelerating wound healing through various mechanisms, some of which have already been tested for

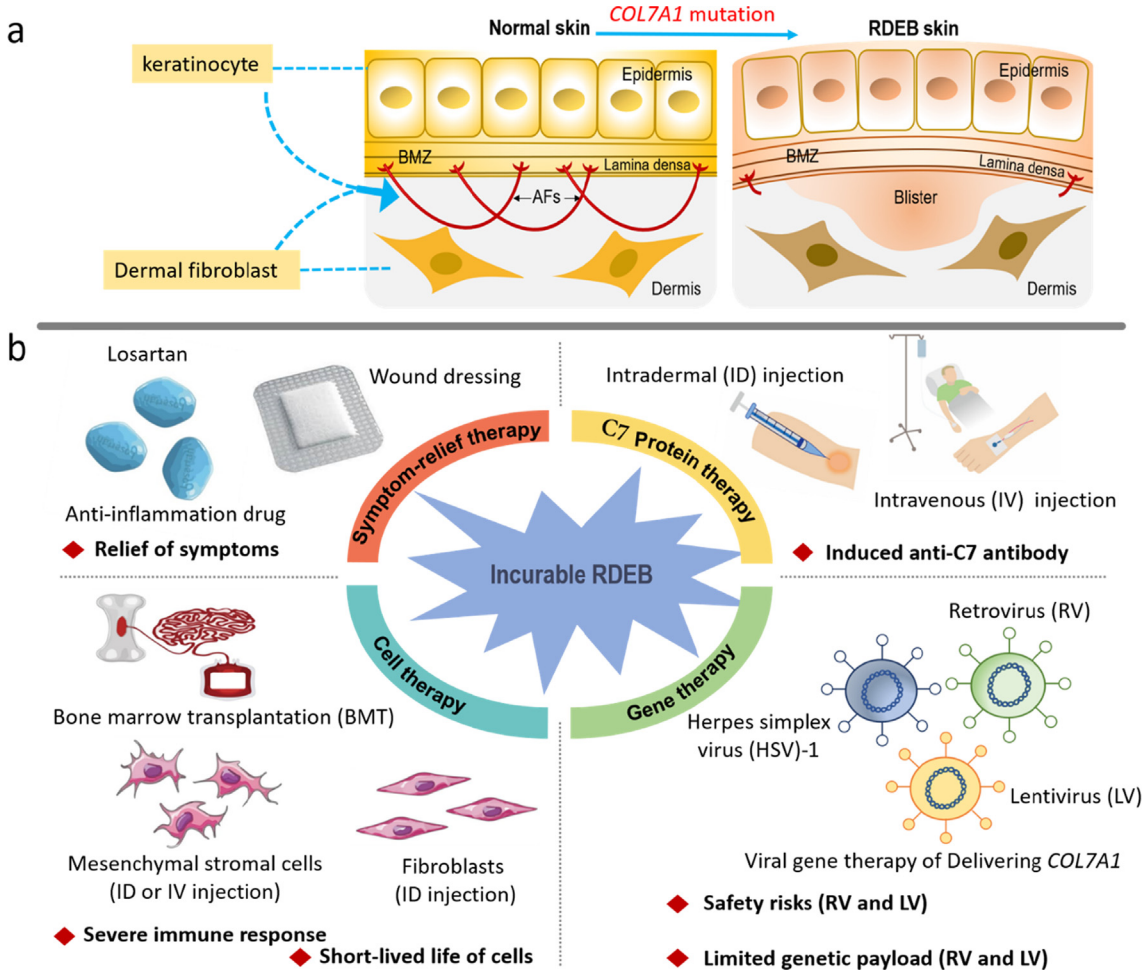

Fig. 1. (a) RDEB is caused by *COL7A1* mutations that lead to blistering beneath the lamina densa in BMZ. Keratinocytes and fibroblasts are main sources to secrete C7 which aggregates into AFs; and (b) the majority of treatment approaches and their main characteristics towards incurable RDEB in clinical applications.

their translational applications in clinical trials. However, none of these therapies are consistently effective, and some might have severe adverse effects and even cause high mortality. The main treatment approaches in clinical development for RDEB are summarized in Fig. 1b.

Although drug therapies targeted towards relief of symptoms have shown some benefits, novel topical drugs containing betulin-rich triterpene extract and allantoin failed to show their superiority to the meticulous daily care [25]. An anti-inflammation drug, Losartan, has been shown to inhibit excessive transforming growth factor-β (TGF-β) signaling to reduce systemic inflammation and fibrosis progression in RDEB mice [26]. A phase I/II clinical trial (EudraCT number: 2015-003670-32) was proposed to assess safety, tolerability and efficiency of Losartan in children with RDEB.

Allogeneic cell therapy is also being clinically evaluated for the treatment of RDEB. Intradermal injection of allogeneic fibroblasts [27,28], bone marrow transplantation (BMT) [29], and intradermal [30] and intravenous [31] administrations of bone marrow-derived mesenchymal stromal cells (BM-MSCs) may hold some promise for re-epithelization of chronic erosions in RDEB patients. However, the benefits of intradermal fibroblast injection are still controversial, since a phase II randomized vehicle-controlled trial has found that both the vehicle group (injected with suspension solution containing 2% albumin) and the fibroblast therapy group showed similar improvement [28]. In addition, a critical side effect of high mortality was observed in the BMT study: two out of seven patients died due to either the immunosuppression before transplantation or graft rejection and infection [29]. MSCs are favorable for skin regeneration and wound closure. However, systemic MSC infusion approach cannot carry adequate cell numbers required for an efficacious treatment [32]. The optimal dosing, administration route and consequences of multiple repeated treatment with MSCs have yet to be fully evaluated in RDEB [18]. For a better understanding of the role of intravenous allogeneic MSCs in RDEB patients, a phase I/II trial is planning to investigate the therapeutic responses and their impact on reducing disease morbidity and severity (NCT02323789).

C7 is soluble in neutral buffers and blood, unlike most collagens, and protein replacement therapy to restore the full-length functional C7 is therefore an option [33]. Using RDEB mouse models, purified human recombinant C7 (hrC7) can be administrated intradermally [34], topically [35], and intravenously [36]. All of these studies have confirmed that this functional protein can be incorporated into DEJ to improve the epidermal-dermal adherence and extend the survival of mice. A phase I/II clinical trial using systemic protein therapy with hrC7 in RDEB has completed its recruitment (NCT03752905). and the regimen of intravenous hrC7 is set at 3 mg/kg given weekly for 4 doses, followed by bi-weekly for 7 doses in a phase II trial (NCT04599881). Although these studies have provided a proof-of-concept for protein therapies, the likely requirement for repeated injections, high therapeutic costs and the possibility of anti-C7 antibody formation may hamper their translational applications [37].

30% of RDEB patients harbor nonsense mutations which introduce premature termination codons (PTCs) and mRNA decay. Gentamicin, an aminoglycoside antibiotic that can induce PTC readthrough, has been applied topically and intradermally for efficient C7 restoration in a pilot trial (NCT02698735) [38]. Based on these findings, one phase I/II clinical trial is recruiting RDEB patients for evaluating the safety and efficacy of intravenous gentamicin (NCT03392909). Nevertheless, this class of antibiotics has been associated with non-negligible cytotoxicity and nephrotoxicity, and this treatment is limited to RDEB patients with restrictive nonsense mutations. Gene editing for RDEB preclinical studies is mainly based on the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease systems and the transcription activator-like effector nucleases (TALENs) [39,40]. However, possible off-target adverse effects are still an important safety concern for these gene editing tools, especially when using the CRISPR/Cas9 system *in vivo*.

Restoration of the expression of full-length *COL7A1* by gene therapy is considered to be one of the most promising approaches for the treatment of RDEB. Viral systems have dominated the area of RDEB therapy, however, only one publication reporting the clinical outcomes is available [41]. In this study, autologous patient keratinocytes were transduced with retrovirus carrying full-length *COL7A1* and then assembled into epidermal grafts for patients. Results showed that the treatment efficiency generally declined over one year and the response was variable among patients and grafted sites. Other investigations to explore the possible treatment methods are currently under clinical evaluation. Using a self-inactivating lentiviral (SIN-LV) vector, two trials are focused on intradermal injections of *COL7A1* gene-modified autologous fibroblasts (NCT02493816 and NCT02810951). Through an *ex-vivo* strategy, autologous skin cells are transduced with RV vectors to form engineered skin sheets for treatment of wound (NCT02984085, NCT04186650 and NCT04227106). Herpes simplex virus (HSV)-1 is an episomal viral vector which has very strong gene capacity even >100 kb, the disadvantage is that it exhibits high inflammatory potential [42]. Another topical gel with HSV-1/*COL7A1* is employed for DEB therapy (NCT03536143) in a phase II trial. Despite the high efficiency of viral vectors, concerns associated with the large *COL7A1* gene size, tumorigenesis and immunogenicity remain, and a long follow-up period is needed to validate the therapy outcomes.

AONs are synthetic, single-stranded oligodeoxynucleotides that can influence RNA processing and modulate protein expression through several distinct mechanisms [43]. Hovnanian and co-workers have demonstrated that AONs can mediate mutated exon skipping and restore C7 expression *in vivo* in a xenograft model of RDEB [44]. A first-in-human non-viral clinical trial is currently active, using a topical approach for RDEB/DDEB patients who harbor one or more pathogenic mutations in exon 73 of *COL7A1* (NCT03605069). The drug is named QR313, which is a 21-nucleotide AON designed to hybridize to a specific sequence in the *COL7A1* pre-mRNA. Given the merits of non-viral vectors mentioned above, non-viral gene therapies, especially synthesized chemical vectors, are actively being pursued.

2.3. LPAEs as polymeric gene delivery vectors

2.3.1. LPAE synthesis and screening

It is widely accepted that PEI is non-degradable, and thus induces substantial cytotoxicity in various cell types [45]. As an alternative to PEI, LPAEs have been identified as high-performing candidate polymeric vectors. LPAEs were first developed as gene delivery vectors by Langer and co-workers in 2000 [46]. In general, LPAEs can be synthesized through a one-pot "A2 + B2" type Michael addition of amines to diacrylates (Fig. 2a). LPAEs can effectively condense DNA to formulate LPAE/DNA polyplexes with diameters in the range of 50–250 nm. In aqueous conditions, LPAEs can degrade into non-toxic small molecules. Due to the broad availability of inexpensive commercial diamines and diacrylates, and convenient one-pot synthesis without the production of any by-products, large libraries of structurally unique LPAEs were synthesized and screened for gene delivery soon after their potential was first discovered. In 2003, Langer and co-workers conducted the first high-throughput and semi-automated LPAE synthesis and screening [47] (Fig. 2b). 50 high-performing LPAEs were identified from over 2350 candidates and it was found that LPAEs with mono- or dialcohol side groups and linear bis(secondary amine) backbone were more favorable for gene transfections. In-depth mechanistic studies showed the DNA condensation ability of LPAEs, polyplex size, zeta potential, cellular uptake efficiency and endosomal escape ability dictate the ultimate gene transfection efficiency [48]. Three important factors, namely molecular weight (M_w), polymer terminal groups and polymer/DNA weight/weight (w/w) ratio were systematically investigated and optimized [49]. Results showed that LPAE with a M_w of 13 kDa and terminated with amine groups were favorable for gene transfection at the LPAE/DNA weight ratio (w/w) ranging from 30:1 to 150:1 [49]. After these initial investigations, a new polymer library of >500 LPAEs was screened to deliver suicide gene therapy for cancer [50]. Among them, C32 polymers (Fig. 2c) that contain amines with alcohol groups and acrylates with hydrophobic spacers were identified to be the top-performing candidates which exhibited high levels of intra-tumor gene transfection efficiency without inducing obvious cytotoxicity. Based on the findings mentioned above, using acrylate and amino monomers in Fig. 2d, 486 s-generation LPAEs were synthesized and screened by Langer and co-workers [51]. They found that the three most effective LPAEs (C32, JJ 28, and C28) share a common structure but differ by only one carbon in the amino alcohols. Once again, C32 with M_w of approximately 10 kDa was confirmed to be the optimal candidate.

2.3.2. Optimization of LPAE by end modification

In 2007, an end modification strategy was developed to further improve the gene transfection efficiency of LPAEs [52]. In this strategy, as end-capping agents, a second amine was used to conjugate with the vinyl groups of acrylate-terminated LPAE base polymers (Fig. 3a), and the combined effects of LPAE backbone, side group and terminal structure on gene transfection were systematically assessed [52-54]. It was found that after end modification with primary diamine groups, both the *in vitro* and *in vivo* gene transfection performances of C32 were improved significantly [52]. In this study, C32 was end-modified with four diamines (103, 116, 117 and 122, Fig. 3b). Results showed that the four modified C32 polymers yielded higher gene expression than unmodified C32 and jet-PEI after intraperitoneal administration in whole-body scans. It was believed that the terminal diamine groups effectively decreased the size of LPAE/DNA polyplexes and thus increased their cellular uptake efficiency [54]. In particular, alteration of the terminal group from alcohols to primary amines facilitated the formation of nano-sized LPAE/DNA polyplexes by increasing the positive charge density and thus DNA binding affinity of LPAEs [53]. Encouragingly, the lead end-modified C32 polymer exhibited high transfection efficiency, with its performance even comparable to adenovirus in stem cells. From 2009 to 2011, amine end-capped LPAEs, especially those using C32 as the base polymer, demonstrated great promise for several clinically relevant applications by delivering functional genes to ovarian cancer [55], mouse gland mammary epithelial cells [56] and glioblastoma cells [57]. Later, Anderson and co-workers investigated the influence of M_w of end-modified C32 polymers on transfection efficiency and toxicity [58]. Results showed that LPAEs of intermediate M_w (~5–8 kDa)

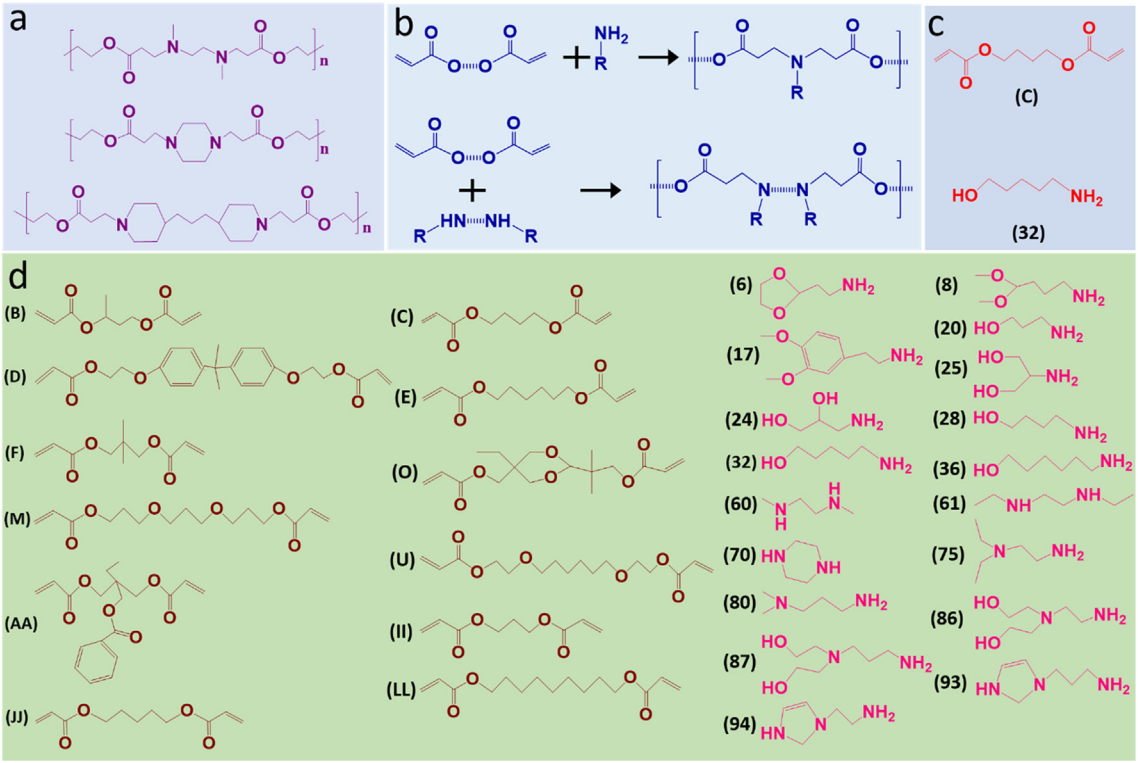

Fig. 2. Development of LPAEs as gene delivery vectors. (a) LPAEs developed for self-assembly with plasmid DNA in 2000. LPAEs were synthesized via the addition of N,N′-dimethylethylenediamine, piperazine, and 4,4′-trimethylenedipiperidine to 1,4-butanediol diacrylate, respectively. (b) Screening of LPAEs synthesized by the conjugate addition of primary or bis(secondary amines) to diacrylates. (c) C32 polymers were identified as one of the most effective LPAEs. (d) Acrylate and amino monomers used to synthesize a library of 486 s-generation LPAEs.

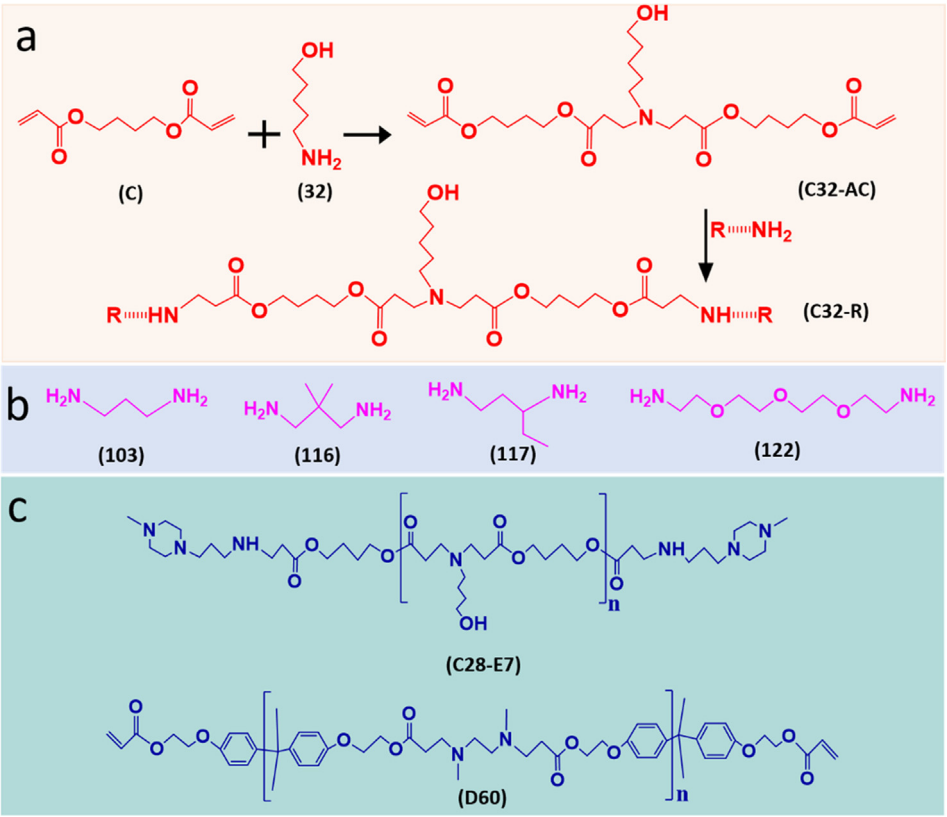

Fig. 3. (a) Synthesis of amine-terminated C32 polymers. Acrylate-terminated C32 base polymer (C32-Ac) was synthesized first and then end-capped with different primary amine molecules. "R" is referred to the rest chemical groups. (b) Four diamine molecules used as C32 end-capping agents. (c) Chemical structures of the representative C28-E7 and the D60 base polymers.

generally mediated optimal DNA transfection efficiency in human cervical cancer cell (HeLa) cells. All LPAEs induced low cytotoxicity over a wide range of M_w (2.4–16.4 kDa), highlighting the high biocompatibility of these polymers. Other studies were carried out with C32-based LPAEs included, such as the quantification of plasmid number within the polyplexes [59] and consideration of the mechanisms involved in cellular uptake and transfection efficiency [60]. Using a nanoparticle tracking analysis (NTA)-based quantification, LPAE/DNA polyplexes effective at co-transfecting cells were shown to contain ~ 100 plasmids per particle in aqueous solution. Structure-function relationship revealed that LPAEs spontaneously formed positively charged (+21–29 mV of zeta potential) polyplexes with a 130–150 nm diameter, buffered 1.4–4.6 mmol H^+/g with half-lives ranging from 1.5 h to over 6 h, and mediated a maximum of 95% cellular uptake and 93% transfection efficiency, respectively.

Besides the C32 polymers, other top-performing LPAEs have also been explored to achieve successful treatment, such as the C28-E7 and D60. Chemical structures of C28-E7 and D60 base polymers are shown in Fig. 3c. C28-E7 was investigated for the treatment of malignant glioma [61,62]. In addition, C28-E7/DNA polyplexes can be fabricated, lyophilized, and stored for at least two years without losing gene transfection efficacy [61]. This polymer was used to effectively transfer a suicide gene of HSV-derived enzyme thymidine kinase (HSVtk) to a malignant glioma model, and this treatment extended brain tumor survival when combined with the prodrug ganciclovir (GCV) [62]. After end-capping with the same set of diamines, D60 based polymer showed gene transfection potency which was comparable with the best-performing C32 polymers [53]. Additionally, the diacrylate (D) also plays a critical role in contributing to the high levels of gene transfection efficiency in LPAE terpolymers with alkyl side chains [63]. All these studies reveal that the structure of LPAEs has a significant effect on gene transfection outcomes, and any subtle alterations to the M_w or the monomer combination strategy can lead to remarkable enhancement or reduction in gene transfection efficiency. These findings have enlightened the way of developing a new generation of PAEs with a branched topological structure for RDEB therapy in our work.

2.4. Development of HPAE for gene therapy of RDEB

2.4.1. Development of HPAEs and gene transfection evaluation

It has been repeatedly demonstrated that the topological structure of polymeric gene delivery vectors plays a critical role in dictating their gene transfection performance. Branched polymers have a three-dimensional (3D) structure and multiple terminal units. Branched PLL [64], PDMAEMA [65], and glycopolymers [66] have all exhibited superior gene transfection ability compared to their corresponding linear counterparts. Therefore, it is conceivable that HPAEs could exceed LPAEs in gene transfection studies. To verify this hypothesis, studies have sought to synthesize HPAEs as gene carriers [67,68]. However, these polymers are either limited to specialized monomers in which the functional groups are of unequal reactivity or synthesized in a harsh condition where reduced pressure, high temperature (140 °C) and a special catalyst are required. The restricted starting monomer selection and tedious synthesis process would hamper the application of HPAEs in practice, especially when they are applied in a large-scale screening process. Most importantly, these synthesized HPAEs are not as efficient as the 25 kDa PEI in gene transfection work [68]. In 2015, our group introduced trimethylolpropane triacrylate (TMPTA) as the branching monomer to synthesize HPAEs through an "A2 + B3 + C2" type Michael addition strategy (Fig. 4a) [69]. The facile "A2 + B3 + C2" strategy not only enables HPAEs to maintain the same functional components derived from LPAEs that had already proven efficient in gene transfections, but also confers branched topological structures on these polymers. Utilization of 28, TMPTA and D as the A2, B3 and C2 monomer, respectively, HD28 base polymer was first synthesized and then end-capped with 3-morpholinopropylamine (91) to produce HD28-91 (Fig. 4b) [69]. All these HD28-91 polymers have exhibited excellent transfection performances. Quantified with a reporter Gaussia luciferase (Gluc) assay, HD28-91 mediated up to 22.3-fold enhancement of transfection efficiency compared to commercially available transfection reagents PEI and SuperFect in both RDEB keratinocyte (RDEBK) and normal human keratinocyte (NHK) cell lines. This efficiency, combined with over 80% cell viability, demonstrated that HPAEs hold great potential in keratinocyte transfections [69]. Soon after, HD28-91 was shown to generate much higher transfection efficiency than the lead linear polymer of C32-103 in RDEBK, NHK and HeLa cells. HD28-91 was further investigated to show that high transfection efficiency and low cytotoxicity can be achieved by increasing the M_w of HD28-91 [70]. In this work, among HD28-91 polymers with the M_w ranging from 5.4 kDa to 21.0 kDa, the 21.0-kDa HD28-91 was the best-performing polymer in HeLa and SHSY-5Y astrocyte transfection. To verify the broad applications of HPAEs in transfection studies, 12 structurally diverse HPAEs were synthesized in gene transfection evaluations with HeLa, rat adipose derived stem cells (rADSC) and SHSY-5Y astrocyte cells. Results showed that all HPAEs mediated much higher gene transfection efficiency than their linear counterparts [71]. In this work, the top-performing HD28-91 achieved excellent transfection efficiency across 12 cell types including keratinocytes, fibroblasts, cancer cells and stem cells. Under the optimized transfection parameters, HD28-91 displayed up to 8521-fold enhancement of gene transfection efficiency compared to that mediated by the corresponding LPAE counterpart, PEI, SuperFect and Lipofectamine 2000. Most importantly, the optimized HD28-91 polymers can effectively deliver *COL7A1* to RDEBK cells to restore the recombinant C7 expression.

2.4.2. Design and synthesis flexibility of HPAE in gene transfection studies

Design and synthesis of HPAEs can be very flexible for achieving a required efficiency in transfection studies. Another representative HPAE developed in our group is directly derived from the top-performing C32 candidate, which is termed as branched C32 (HC32) [72-75]. As illustrated in Fig. 5, there are two ways to synthesized HC32 polymers. Firstly, the general synthesis strategy of HC32 was to polymerize A2, B3 and C2 monomers simultaneously to generate the base polymer. Alternatively, linear C32 oligomers were prepared first and then combined by the branching unit (TMPTA) to generate a novel linear-branched hybrid poly(β-amino ester) (LBPAE). The topological structure of these two polymers is quite similar, however, LBPAE displays a more uniform distribution of branching unit among the linear segments, and length of the linear segments in the obtained LBPAEs would be predetermined and thus can be tailored easily. Gene transfection studies showed that HC32 polymers displayed far superior efficiency than the C32, PEI and SuperFect in RDEBK, rADSC and HeLa cells [72]. It was also found in this work that HC32 polymers terminated with the diamine molecules (103 and 122) were more favorable for gene transfection. The representative HC32-122 with a proper branched structure can be further guanidinylated to exhibit broad applicability in gene transfection across a variety of cell types such as astrocytes and stem cells. Additionally, more than 60% GFP expression efficiency was observed in both NHK and RDEBK cells, and over 90% cell viability was preserved. Another study showed that HC32-103 polymers with low branching ratio (10%) mediated higher gene transfection efficiency in RDEBK cells [73]. Further-

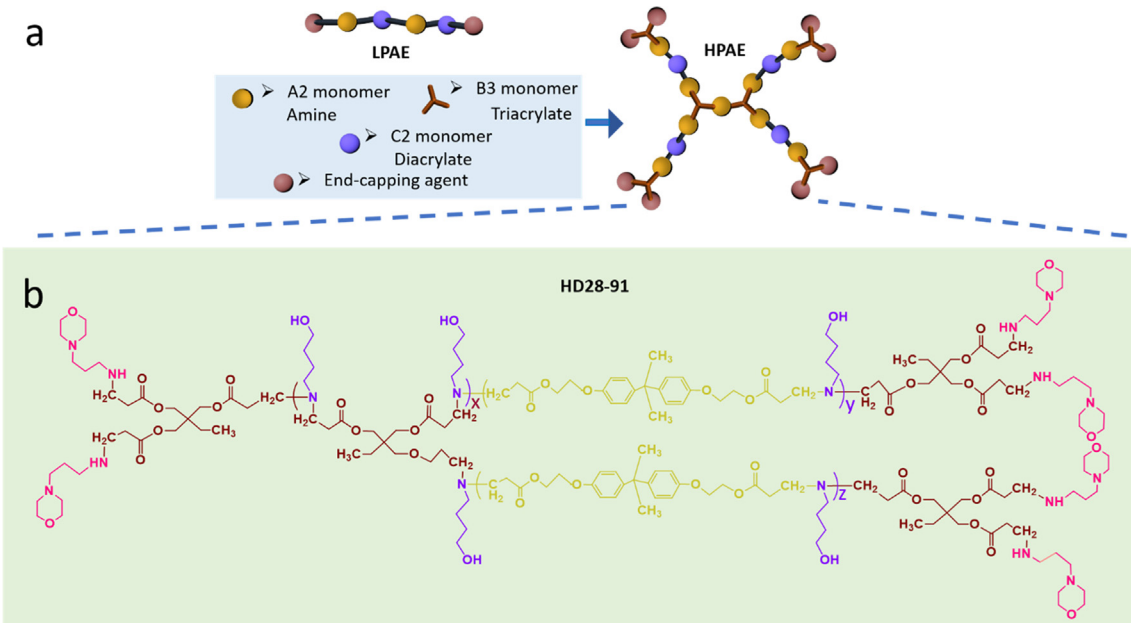

Fig. 4. (a) Illustration of branching strategy to synthesize HPAEs. Typically, HPAEs are synthesized with an "A2 + B3 + C2" strategy (A2: amines; B3: triacrylates; and C2: diacrylates). (b) Chemical structure of HD28-91.

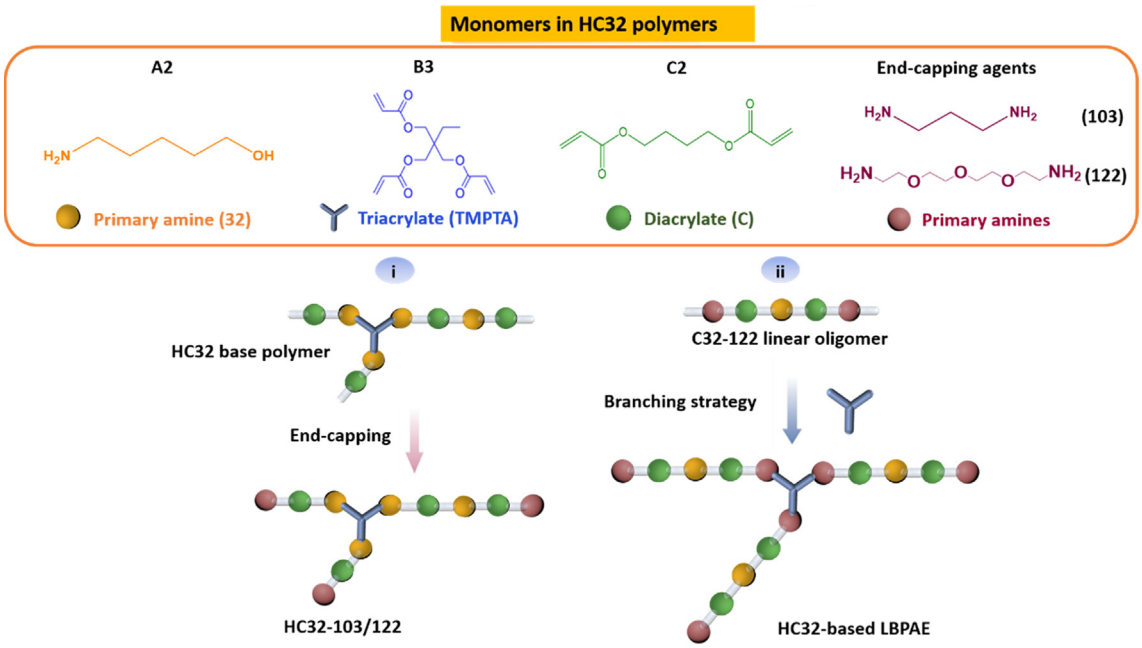

Fig. 5. Synthesis of HC32-122 and LBPAE with "A2 + B3 + C2" Michael addition. (i) The conventional synthesis of HC32-122; and (ii) HC32-based LBPAE was synthesized with a linear oligomer combination approach.

more, formulation study showed that in the presence of sucrose, lyophilized HC32-122/DNA polyplexes maintain most of their transfection potency. Direct frozen polyplexes can retain the full gene transfection efficiency after one year in storage [75].

Human primary dermal fibroblast (HPDF) is the most attractive cell type that secretes C7. After genetic engineering by transfection, fibroblasts can be adapted to be more suitable for gene therapy in RDEB. However, HPDFs are considered difficult to transfect by non-viral vehicles [76]. Only 27% and 44% of enhanced GFP (EGFP) expression efficiency were achieved by electroporation systems [77,78]. To overcome this challenge, a novel LBPAE was synthesized for better gene transfection performance in HPDF cells [74]. In the study, LBPAE maintained the same set of monomers as in HC32-122 while the synthesis was conducted through the oligomer combination approach. Transfection results showed that LBPAE mediated over 90% gene transfection efficiency in both HPDFs and mouse embryo fibroblasts (3T3s), and exhibited excellent *in-vitro* biocompatibility. Overall, the above findings indicate that branching strategy is beneficial to improve the gene transfection performances and HPAEs have broad utility and potency to transfect diverse cell types efficiently. Recently, researchers introduced a trifunctional amine (N-methyl 1,3 diaminopropane) as the branching unit to synthesize HPAEs for better *in vitro* transcribed (IVT) mRNA delivery to lung epithelium [79].

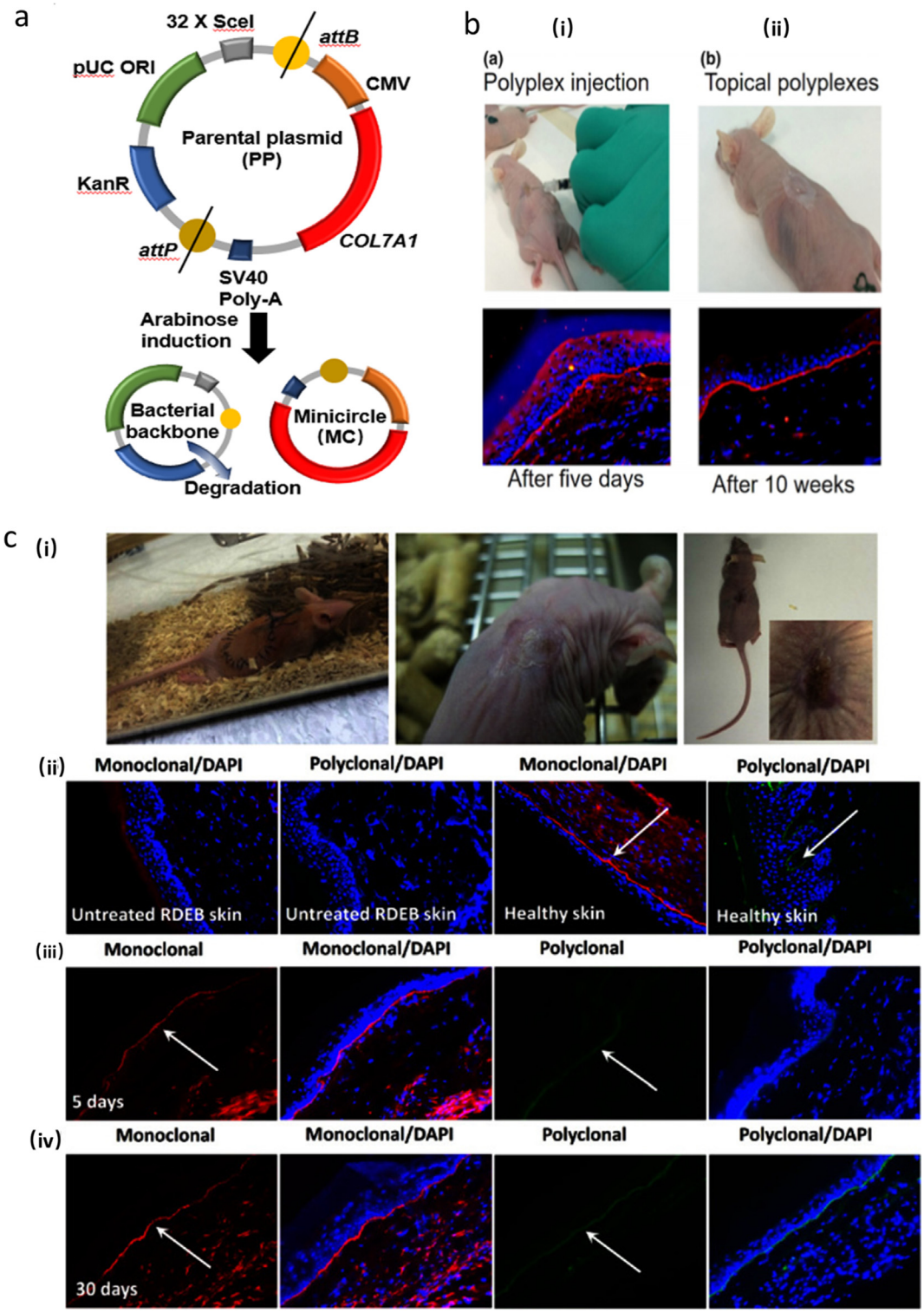

Fig. 6. (a) Production of minicircle DNA with the PhiC31 integrase/SceI system. The *COL7A1* transgene sequence is inserted in the MN511A-1 cassette to form the parental plasmid, which is induced by arabinose to produce the MCC7 and excise the bacterial backbone. Reused with permission, Copyright © 2019 American Chemical Society. (b) HD28-91/MCC7 polyplexes for C7 restoration in a human graft mouse model. Recombinant C7 was detected along the BMZ by intradermal injection or topical application. (Copyright © 2016 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd. (c) HD28-91/pcDNA3.1COL7A1 mediated long-term expression of C7 in a human graft mouse model (Reused with permission, Copyright © 2016 AAAS). (i) 0 day (left), 21 days (middle) and 60 days (right) after surgery in human RDEB grafts. (ii) C7 band was shown along the BMZ (indicated by arrows) in the "healthy" skin graft. DAPI, 4',6-diamidino-2-phenylindole. (iii) Significant level of C7 expression 5 days after the last injection of the HD28-91/*COL7A1* polyplexes. (iv) Persistent C7 expression after 30 days.

Additionally, the structure of HPAE can be tailored very flexibly by incorporating functional monomers. Adipose derived stem cells (ADSCs) and astrocytes are good resources for neural tissue regeneration but difficult to transfect [57,80-82]. To achieve high gene transfection efficiency in ADSCs and astrocytes, HPAEs containing biodegradable disulfide units in the backbone (HPAESS) and guani-

dine moieties (HPAESG) at the extremities were synthesized to deliver minicircle DNA to these cells [83]. This work demonstrated that 77% and 52% of transfection efficiency was achieved by the optimized polymers in human ADSCs and primary astrocytes, whilst 90% cell viability was preserved. In particular, the top-performing polymer mediated high levels of nerve growth factor (NGF) secretion in astrocytes, promoting neurite outgrowth in a model PC12 cell line, and indicating the possible application of HPAEs in gene therapy for neural degenerative diseases such as Parkinson's disease.

2.4.3. Minicircle COL7A1 (MCC7) construction and in vitro studies

Since HPAEs have been developed and optimized to show high gene transfection efficiency, to further improve the treatment efficacy for RDEB, the gene construct of *COL7A1* was also optimized. Bacterial plasmid DNA (pDNA) is a promising genetic material for gene therapy because of its non-integrative, safe and stable nature [84]. Plasmids contain a bacterial backbone consisting of a bacterial origin of replication (ORI) and antibiotic resistance genes (e.g., kanamycin) to allow for easy propagation and purification [85]. However, the bacterial backbone is not desirable for therapeutic gene expression. Minicircle DNA is a new form of supercoiled DNA molecule which is devoid of the ORI and antibiotic marker [86]. It is derived from a parental plasmid (PP) by removing the bacterial backbone. Minicircle DNA can be obtained in *Escherichia coli* (*E. coli*) via site-specific recombination, mediated by the recombinase of phage λ integrase, which excises the unwanted plasmid sequences [87]. The Streptomyces bacteriophage PhiC31 integrase can mediate unidirectional recombination events at its *attP*/*attB* sites [88]. The full-length PP construct is grown in a special *E. coli* strain (e.g., ZYCY10P3S2T) that harbours an arabinose-inducible system to produce the PhiC31 integrase and the SceI endonuclease simultaneously. As shown in Fig. 6a, after the arabinose induction, PhiC31 integrase mediates site-specific recombination between the *attP* and *attB* sites. After the recombination, a minicircle DNA is produced carrying only the recombinant *attR* site and the transcription unit containing a eukaryotic promoter (e.g., cytomegalovirus, CMV), the transgene (e.g., *COL7A1*) or any other gene of interests (GOI) and a polyadenylation (polyA) sequence. The bacterial backbone which is engineered to contain 32 copies of SceI sites, is processed to be digested by the SceI endonuclease. The minicircle DNA remains intact due to the lack of the SceI restriction sites in its construct. This PhiC31 integrase allows for the production of pure minicircle DNA. The therapeutic efficiency and biosafety of minicircle vectors are improved due to the relatively small size and the removed bacterial gene sequences. Furthermore, minicircle DNA vectors can mediate sustaining gene expression for months with 10–1000 fold higher of therapeutic efficiency than that of their standard plasmid counterparts [89-91]. Due to the above advantages, minicircle DNA has been extensively used in diabetic wound healing [92,93], DNA vaccine development [94], peripheral arterial disease [95], rheumatoid arthritis [96] and cancer treatment [97].

It was expected that the structurally optimized MCC7 would benefit gene therapy of RDEB, and thus MCC7 was constructed and its performance was evaluated in our work [98]. The biosynthesis of MCC7 involved insertion of the full-length *COL7A1* complementary DNA (cDNA) into a commercial plasmid construct (e.g., MN511A-1 cassette, System Biosciences) which is based on the PhiC31 integrase/SceI system outlined above. This was followed by *E. coli* culture, arabinose induction and final DNA purification. Results demonstrated that HD28-91/MCC7 system achieved a level of recombinant C7 expression comparable to that produced by primary keratinocytes, indicating the robustness of this system in achieving required therapeutic efficacy *in vitro*. Further studies showed that HC32-122 with a M_w around 10-kDa was more favourable for RDEBK transfection, and HC32-122/MCC7 polyplexes outperformed PEI counterparts significantly, and achieved 96.4% cellular uptake efficiency, 4019-fold *COL7A1* mRNA enhancement and robust recombinant C7 expression [75]. Besides, HC32-122 based LBPAE mediated over 40% of C7 upregulation in HPDF transfection [74]. These data support that HPAE can efficiently restore C7 for reversing the disease phenotype, showing the potential of HPAE/MCC7 system for polymeric gene therapy in RDEB.

2.4.4. Structure-function relationship of HPAEs and HPAE/DNA polyplexes

To decipher the mechanism behind the high performance of HPAEs in gene delivery to skin cells, structure–function relationships of HPAEs were explored. Generally, a Mark-Houwink (MH) plot α value below 0.5 suggests that the polymer molecules manifest a branched topological structure [69,99]. In these studies [69-71,100,101], HD28-91 polymers were synthesized by varying the triacrylate/diacrylate (T/D) feed ratio. No gelation was observed from these polymerizations. All polymers have an α value < 0.5. In general, the MH α value decreases with increasing T/D ratio, suggesting an increased branching degree (BD). The favorable T/D feed ratio is found to be between 0.5 and 1.2, and the preferred M_w is around 10–21.0 kDa with a polydispersity index (PDI) from 2.2 to 3.82. These results indicate that "A2 + B3 + C2" Michael addition strategy offers excellent controllability and great flexibility for HPAE synthesis, and the branched structure of HPAEs can be easily adjusted by simply varying the T/D feed ratio.

Tremendous efforts have been made to explore some key physicochemical properties of polymeric vectors and their formulated nanoparticles, which would dictate the ultimate gene transfection performance of gene expression and cytotoxicity. Chemical structure, particle surface charge, size, morphology, aspect ratio, and surface coating are crucial parameters to affect the multiple mechanistic steps involved in gene transfections [5]. By meticulously selecting the monomer combination and end-capping agent, HPAEs with multiple primary, secondary and tertiary amines can be produced. These amines would provide HPAE with good DNA binding affinity and high proton buffering capacity. The hydrolytic ester groups on the backbone would endow HPAEs with biodegradability to reduce cytotoxicity after transfection. Moreover, due to the 3D structure, there are multiple terminal groups which provide large chemical space for further functionalization. When the w/w ratio ranges from 10:1 to 50:1, HD28-91/DNA particles have sizes between 47 and 230 nm with zeta potential of +8–24 mV [69-71,100,101]. A previous study has illustrated that LPAE/DNA particles are generally below 250 nm in size and efficient for high levels of cellular uptake [48]. The size range of HPAE/DNA particles is in accordance with that reported in LPAE studies. Cationic polymers are conducive to condensing the plasmid DNA into nanosized particles with a net positive charge, which facilitates the particles to attach to the negatively charged cellular membrane through energetically favorable electrostatic interactions [102]. Polyplexes manifest diverse morphologies such as spherical, toroidal or doughnut-shaped [3,6], which have a significant impact on cellular uptake, intracellular trafficking and transgene expression [103]. In our study, the HD28-91/DNA particles show a uniform spherical morphology, which is preferential for gene transfection. In addition, the HD28-91/DNA nanoparticles display strong DNA binding affinity [70,71], good DNA condensation [71,100,101] and excellent protection against the endonuclease digestion by DNase enzyme [100]. The strong endosomal escape ability of cationic polymers is mainly attributed to the protonable secondary and tertiary amine groups that exhibit a pK_a close to the endosomal/lysosomal pH, which can induce osmotic lysis of the endosome through a "proton sponge effect" [104]. For HD28-91, in addition to the tertiary amine groups in the backbone, the termi-

nated morpholino groups also offer tertiary and secondary amine groups, and thus it exhibits stronger proton buffering capacity than the linear counterpart [71]. Compared with non-degradable polymers, biodegradable polymers are believed to increase the biocompatibility and facilitate the DNA release. The half-life of the biodegradable HD28-91 is around 5 h [100]. In a LPAE study, the authors concluded that modestly long (>2 h) half-lives are needed to protect the DNA all the way to the nucleus [60].

In another study [72], the structure–function relationship of HC32 polymer was investigated in detail. The T/D feed ratio has been set to generate HC32 base polymers with a BD of 5%, 10% and 15%, respectively. After end-capping, HC32-10%-122 was produced with M_w, PDI and MH α values of 13.8 kDa, 3.6 and 0.32 respectively. Compared with the linear C32-122 analogue, HC32-10%-122 particles display around 100 nm decrease in size and 5 mV increase in zeta potential, indicating a more condensed particle morphology. The multiple terminal groups of the HC32-10%-122 were further conjugated with guanidine moieties, which promoted the cellular uptake efficiency of the nanoparticles. Moreover, HC32-122 can effectively condense MCC7 to form small, uniform, compact and positively-charged spherical nanoparticles with high flexibility of DNA release [75]. The HC32-122 based LBPAE has strong MCC7 condensation ability and binding affinity, which results in uniform spherical nanoparticles of 60–250 nm diameter with cellular uptake efficiency of nearly 100% [74]. Besides, the HC32-122 based LBPAE exhibits strong proton buffering capacity and biodegradability with efficient DNA release ability, which are very beneficial for promoting endosomal escape of nanoparticles and gene expression after the nucleus import. Collectively, the strong DNA binding affinity, small particle size, high zeta potential and efficient proton buffering capacity of HPAEs lead to higher cellular uptake and endosomal escape efficiency. The branching strategy facilitates these delivery steps and ultimately enhances the transfection efficiency.

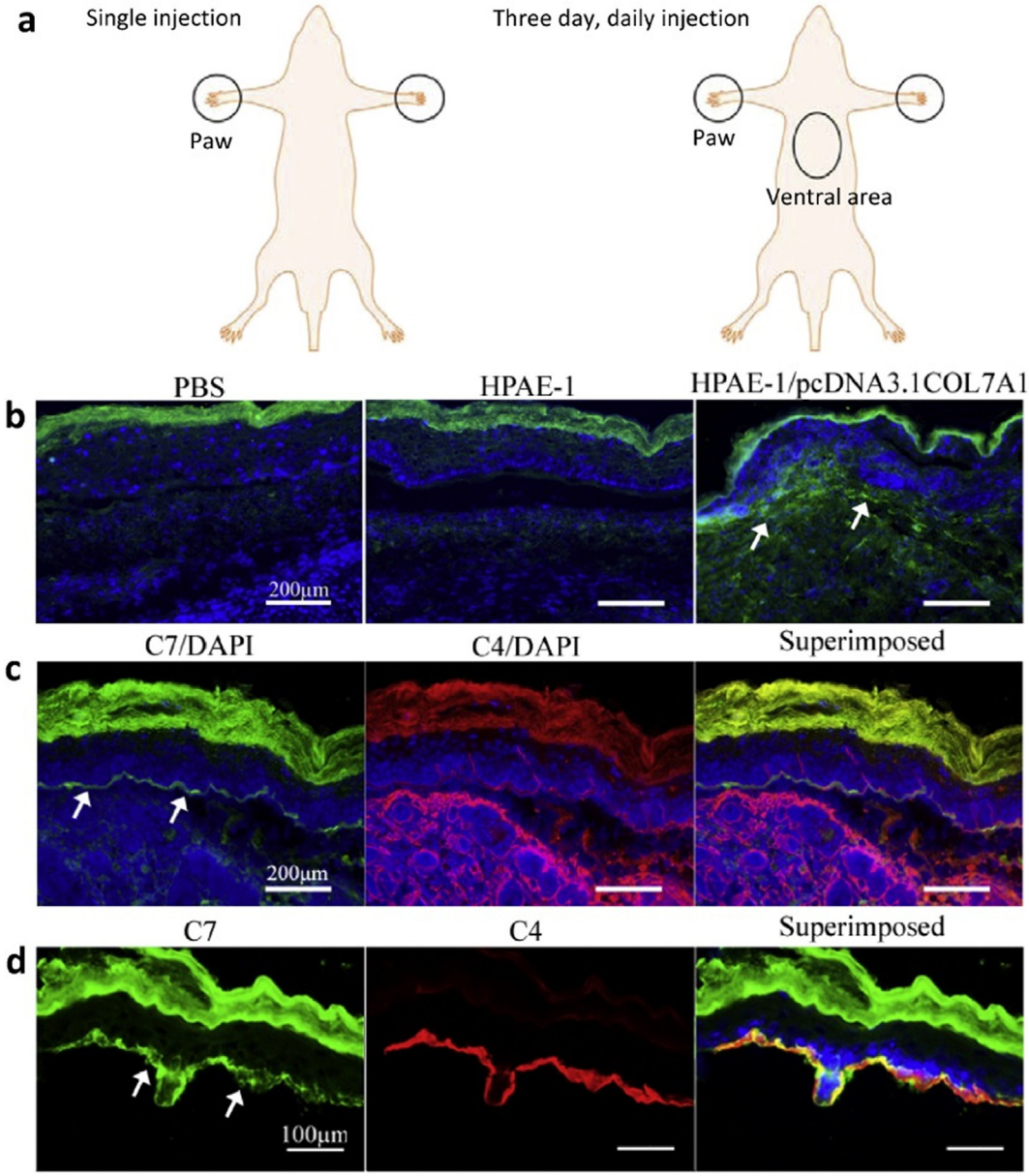

Fig. 7. HD28-91 (HPAE-1) was evaluated in a C7 null RDEB knockout (−/−) mouse model. Reused with permission, Copyright © 2016 Elsevier B.V. (a) Treatment methods with intradermal injections; (b) HD28-1/*COL7A1* mediated human C7 restoration (arrows) while phosphate buffer saline (PBS) buffer and polymer alone exhibited negative results 24 h after injection; and in mouse paws (c) and ventral areas (d), human C7 restoration (arrows) appeared 72 h after injections with HD28-91/*COL7A1* polyplexes while mouse collagen IV (C4) remained unaffected (red).

2.4.5. HPAE for COL7A1 delivery in animal studies

Beyond the high-performance gene delivery *in vitro*, to validate the treatment potency of HPAE in the relevant clinical diseases, a murine graft model of RDEB was employed for therapeutic evaluations in our studies [71,98]. Typically, this animal model is established by preparing C7-deficient primary skin cells obtained from patients into bioengineered skin equivalent which is then grafted onto hairless immunodeficient mice [105,106]. *In vivo* gene therapy is conducted by intradermal injection or topical application of vector/*COL7A1* complexes to the transplantation site. After three intradermal injections with HD28-91/MCC7 polyplexes, human recombinant C7 was produced along the BMZ throughout a month of observation [98]. In this pilot study, the topical administration allowed for recombinant C7 expression 5 days after a single topical application and 10 weeks after three applications, indicating that topical administration is effective, and preferable to intradermal injection as it causes less trauma and discomfort during the treatment. Fig. 6b shows C7 production along the BMZ after intradermal or topical delivery [98]. In addition, HD28-91 showed a high performance in delivering pcDNA3.1COL7A1 plasmid DNA in this mouse graft model [71]. The dorsal area was chosen for transplantation and the growth process of skin equivalent in mice prior to treatment was shown in Fig. 6c. The intradermal injections were carried out three times, once every other day. 5 days and 30 days after the last injection, significant levels of human recombinant C7 were produced.

In another *in vivo* study, RDEB (−/−) mice were used due to their remarkable similarity to the human phenotype with blister and wound formation [107,108], allowing for an evaluation much closer to a real situation in preclinical studies. Results showed that HD28-91 can effectively deliver therapeutic pcDNA3.1COL7A1 DNA for C7 restoration in a C7 RDEB knockout (−/−) mouse model [101]. Fig. 7a showed the areas and regimes of intradermal injections with HD28-91/COL7A1 polyplexes. 24 h after a single delivery, human C7 was restored at the mouse BMZ in the injection site of the paw (Fig. 7b). After daily administration for three days, remarkable amounts of human C7 were produced in mouse paw (Fig. 7c) and ventral region (Fig. 7d). Collectively, the best-performing HPAEs can mediate C7 restoration both *in vitro* and *in vivo* with high efficiency. Especially, by taking advantage of the robust polymer and the miniaturized gene construct, HPAE/MCC7 system provides a powerful tactic for the development of effective and biocompatible nonviral gene therapy system towards RDEB.

3. Prospects of polymeric vectors for hereditary skin diseases

3.1. The skin barrier

Topical and transdermal gene delivery allows for convenient and pain-free self-administration for patients and thus increases compliance, however, delivery of gene-containing drugs through the skin has always been an attractive as well as challenging area. Briefly, skin is divided into epidermis, dermis and junction areas of BMZ. The stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS) and the underlying stratum basale (SB) constitute the epidermal layer. The epidermis is a self-renewing tissue with proliferation of keratinocytes from SB, while keratinocyte terminal differentiation and skin barrier formation take place in the suprabasal layers. The dermis is a supporting collagenous stroma within which are embedded fibroblasts, blood vessels and epidermal appendages [109]. Currently, cutaneous gene transfer methods include microneedles [110], electroporation [111], ultrasound [112], topical administration [113], routine intradermal injections of genetically corrected cells [114] or nanoparticles [98] and epidermal grafts [41]. Fig. 8 illustrates the barriers at organ level for polymeric particles in the skin tissue. When formulated drugs or prodrugs with polymeric nanoparticles are applied onto the skin, several routes or pathways are needed for these particles to achieve a better penetration of the skin, including the transcellular, intercellular, follicular and appendageal routes. It is believed that nanoparticles preferentially make use of the follicular penetration pathway, and they do have a promising future in dermal drug delivery if smart nanoparticle systems are developed with the ability to release their drugs at specific times and locations within the hair follicle [115]. SC is well known as the first obstacle for skin gene delivery, because its 10–20 μm thick structure confers protection against external factors and limits the entry and diffusion of most molecules, especially those hydrophilic molecules and larger compounds with M_w above 500 Da [116]. Under some specific conditions, tape-stripping, sonophoresis, iontophoresis, electroporation and thermal/suction/laser ablation are employed for disrupting the SC barrier and enhancing drug penetration [117]. The brick-and-mortar structure with the hydrophobic lipids and the respective hydrophilic intracellular and hydrophilic intercellular micro-environments set up different challenges and requirements for the transport of delivered drugs, determining their rate and efficiency via the transcellular route and intercellular pathways [5]. For percutaneous delivery, moderate lipophilicity and low M_w are thought to be beneficial physicochemical characteristics of drugs, however, a large number of pharmaceutical reagents fail to meet these criteria [118]. In practice, the pursuit of more hydrophobic properties can be achieved by tailoring the structure of polymers, through copolymerization of the base polymer with some specific molecules, such as alkyl groups in side chains or PEG spacer monomers at the chain end in PAE synthesis [53,63]. To lessen skin irritation, development of skin permeation enhancers and emergence of novel techniques would widen the transdermal application for hydrophilic molecules and compounds. Enhancement strategies with skin hydration, integration with existing lipids and creation of a disrupted micro-environment are options for the improvement of skin permeability [5,119,120].

BMZ serves as the junction area for the connection of the epidermis to dermis. It is comprised of collagen IV, laminin, nidogen, perlecan and other glycol-proteins [121]. Any BMZ gene mutations or acquired skin diseases with damage to this area would induce cracking or separation of the skin, manifesting as fragile skin with blisters and erosion in the skin and membrane, predisposition to skin cancer, and other complications. BMZ disposes a filtration barrier for drug molecules but this role has not yet been clarified. A research has revealed that charge-selective permeability of the skin BMZ is not so selective for macromolecules compared with that of the glomerular basement membrane (GBM) [122]. In the dermis, follicular and sweat glands can serve as routes for absorption of delivered gene products. Notably, skin stem cells can be found in the bulge of the hair follicle and function as the differentiation source of keratinocytes. They are responsible for ensuring the maintenance of adult skin homeostasis and hair regeneration, and participating in the repair of the epidermis after injuries [123]. Epidermal stem cells can be genetically engineered by vectors for the treatment of hereditary skin diseases [124]. Fibroblasts are an important and robust cell type in the dermis for therapeutic protein expression in gene therapy research [125]. Other target skin cells in gene transfer may include Langerhans cells, dendritic cells and melanocytes for different therapeutic indications.

3.2. Systemic and cellular barriers

In terms of pharmacokinetics, gene delivery across the skin not only reduces systemic toxicity, but also eliminates plasma level peaks and valleys associated with oral administration and injec-

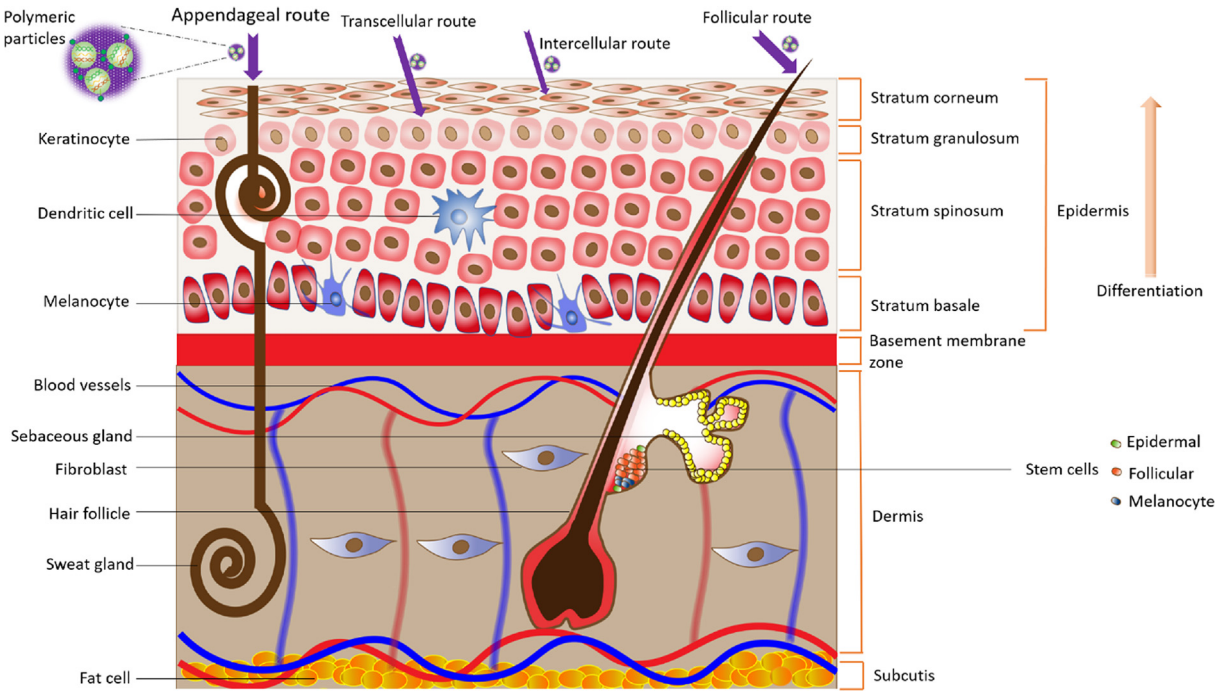

Fig. 8. Schematic illustration of skin barriers for polymeric particles.

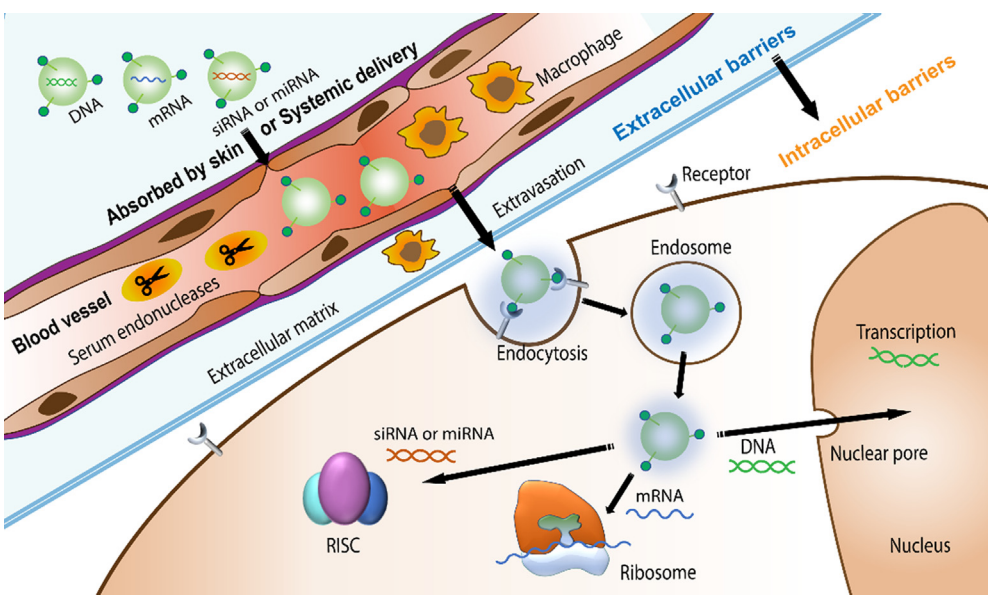

Fig. 9. Systemic and cellular barriers of polymeric particles in delivery of nucleic acids.

tions to maintain a constant drug concentration. Delivery through the skin is a particular advantage for a drug with a short half-life. When transdermal polymeric particles are absorbed by the skin tissue or complexes are given systemically, processed through the circulatory system and redistributed to the skin, these polymer-loaded drugs must overcome multiple systemic and cellular barriers (Fig. 9). Versatile polymers must have the capability to bind to and condense nucleic acid into nanosized particles through electrostatic interactions between the negatively charged nucleic acid phosphate and the positively charged vector groups, and thus provide protection of the nucleic acid. Blood circulation half-life, biodistribution and capacity of nanoparticles to cross extracellular and intracellular barriers will be dictated by their composition, size, charge, shape and surface functionality [126,127].

Besides the local transfection performance, in the circulatory system, non-viral vectors are required to avoid degradation by serum endonucleases and evade detection, phagocytosis and destruction by macrophages and their immune responses. Furthermore, for long-circulating nanoparticles, in order to have prolonged retention time, they must avoid plasma protein interaction, hepatic clearance and renal clearance which are the primary routes of particle excretion. Coating the particle surface groups with polyethylene glycols (PEG), known as "PEGylation", is one of the most widely used approaches for improving particle pharmacokinetic behavior, which can shield the surface from

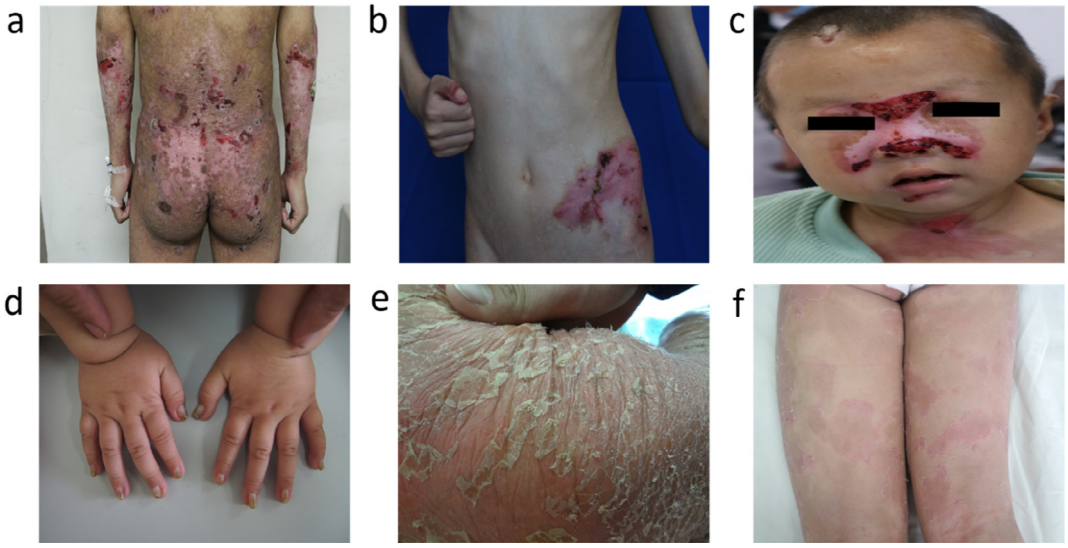

Fig. 10. (a) Extensive drying blisters, erosions, thick scabs, reddish scars and reticular pigmented patches appear all over the body in a RDEB patient. (b) A JEB patient has erythema, erosions and scabs on the left waist with fingertip fibrosis and onychatrophia of some fingers. (c) A butterfly-like skin lesion of shallow ulcers and hypopigmentation on his face with reddish scars at the cervicothoracic junction areas in a JEB patient. (d) A PC patient has thickened fingernails with a cocked end. (e) Abnormal skin scaling over the whole body was seen in ARCI. (f) Ichthyosis linearis circumflexa lesions in NS.

Table 1
Gene therapy towards hereditary skin diseases in clinical evaluations.

Disease	vector	Gene	Drug name (target cell)	Administration route	Trial phase	Ref or trial number	Recruitment status
RDEB	RV	*COL7A1*	keratinocytes	*Ex-vivo*	–	41	–
RDEB	SIN-LV	*COL7A1*	Fibroblasts	Intradermal injections	I	NCT02493816	Completed
RDEB	SIN-LV	*COL7A1*	FCX-007 (fibroblasts)	Intradermal injections	I/II	NCT02810951	Active
RDEB	Gamma-RV	*COL7A1*	Hologene 7 (Skin stem cells)	*Ex-vivo*	I/II	NCT02984085	Active
RDEB	SIN-RV	*COL7A1*	EBGraft (keratinocytes and fibroblasts)	*Ex-vivo*	I/II	NCT04186650	Enrolling
RDEB	RV	*COL7A1*	EB-101(keratinocyte□	*Ex-vivo*	III	NCT04227106	Recruiting
DEB	HSV-1	*COL7A1*	KB103	Topical gel	II	NCT03536143	Active
DEB	AON	*COL7A1*	QR-313	Topical	I/II	NCT03605069	Recruiting
JEB	RV	*LAMB3*	Skin stem cells	*Ex-vivo*	–	135	–
JEB	Gamma-RV	*COL17A1*	Hologene 17 (Skin stem cells)	*Ex-vivo*	I/II	NCT03490331	Recruiting
JEB	MLV- RV	*LAMB3*	Skin stem cells	*Ex-vivo*		136–138	–
PC	Naked siRNA	*K6a*	TD101	Intralesional	I	NCT00716014	Completed
ARCI	HSV-1	*TGM1*	KB105	Topical gel	I/II	NCT04047732	Recruiting
NS	LV	*SPINK5*	Skin stem cells	*Ex-vivo*	I	NCT01545323	Unknown

aggregation, opsonization and phagocytosis, reduce non-specific interactions with serum components and prolong systemic circulation time, and thus promote efficient drug and gene delivery [128,129].

After extravasation from the blood vessel, nanoparticles need to reach the target tissue of interest (i.e. skin) and prevent the therapeutic nucleic acid from endonuclease digestion in the extracellular matrix and bind to the target cells. They are also required to overcome major intracellular barriers such as cellular uptake and endosomal escape. For receptor-specific endocytosis, ligands such as arginylglycylaspartic acid (RGD) peptide [130] and epidermal growth factor (EGF) [131] have been utilized to improve the cell specificity and overall uptake *in vitro* and *in vivo*. After these two intracellular transport steps, DNA must be transported to the nucleus and then start the transcriptional process [6]. siRNA and miRNA mimics have to be loaded into the RNA-induced silencing complex (RISC) to exert its activity of gene silencing [132]. mRNA should be transported to ribosomes, allowing for the translation to protein [133]. The nucleic acids must be released from the vector at some point in time before they are processed in gene silencing or protein synthesis machinery. Any therapeutic agent that failed to pass through these organ-level, systemic and cellular barriers would not achieve successful delivery and would therefore be ineffective.

3.3. Potential target of hereditary skin diseases

Currently, although there are more than 400 known types of genodermatosis [37], few of them have been clinically investigated for gene therapy applications, suggesting that there is a huge space for other hereditary skin diseases which could be further targeted, and more polymeric delivery systems could be developed. Apart from RDEB, JEB, pachyonychia congenita (PC), autosomal recessive congenital ichthyosis (ARCI) and Netherton syndrome (NS) are representative hereditary skin diseases which require more and superior gene delivery platform for the possibility of better treatment outcomes. Clinical manifestations of these life-long diseases are shown in Fig. 10, and Table 1 summarizes different gene therapy approaches for these diseases at various stages of clinical development, the majority of which are still viral vector based.

Among the EB family, RDEB gene therapy attracts the most attention of researchers and clinicians. JEB encompasses all sub-

types of EB having blisters developed within the mid portion or junction, the so-called lamina lucida in the skin BMZ. It can be caused by mutations in a variety of genes including *LAMA3, LAMB3, LAMC2, COL17A1, ITGA6, ITGB4* and *PLEC1* [134]. For patients with *LAMB3* mutations, one study has utilized an *ex vivo* strategy to introduce wild-type cDNA into the autologous stem cells using a retroviral vector, which were then prepared as epithelial sheets for grafting [135]. Results showed that synthesis and proper assembly of normal levels of functional protein were achieved, together with the development of a firmly adherent epidermis that remained stable for the duration of a 1-year period of follow-up without blisters, infections, inflammation or immune response. Using the same strategy, an active clinical trial employs a patient's own skin-derived cultured epidermal grafts containing epidermal stem cells genetically modified with a gamma-retroviral (RV)/ *COL17A1* cDNA system for epidermis restoration. (NCT03490331). Using Murine leukemia virus (MLV)-based RV vector, *LAMB3* cDNA was transduced in keratinocytes to produce an entire, fully functional epidermis in patients with JEB [136-138], and the transgenic epidermis were found to sustain by only a restricted number of long-lived stem cells detected as holoclones, rather than meroclones and paraclones [137].

PC is an ultrarare, autosomal dominant inherited disorder that affects the nails, skin, oral mucosae, larynx, hair and teeth [139]. It was first observed as combined clinical features of thickened toenails and thick/painful plantar calluses. Affecting around one in one million people globally, the disease is caused by mutations in keratin-encoding genes, such as keratin *KRT6a, KRT6b, KRT6c, KRT16* and *KRT17* [140]. Leachman and co-workers have conducted a phase I clinical trial for PC (NCT00716014) utilizing a siRNA-based therapy, TD101, which has been designed to specifically and potently target the keratin 6a (*K6a*) *N171K* mutant. Results from this first-in-human mutation-targeted siRNA research in human skin revealed that TD101 can effectively mediate regression of plantar callus after intralesional injection [141]. This work highlights the potential for other siRNA-based platforms in PC and genetic skin diseases.

The inherited ichthyoses represents a large and clinically and genetically heterogeneous group of keratinization disorders caused by abnormal epidermal barrier function with varying degrees of resultant scaling [142]. There are many different subtypes of ichthyoses which could be therapeutic targets for polymeric gene delivery. A phase 1 clinical trial is recruiting for the treatment of transglutaminase 1 (*TGM1*)-deficient ARCI (NCT04047732). The drug is formulated with a replication-incompetent and non-integrating HSV-1 vector containing *TGM1* encoding genes.

NS is an autosomal recessive skin disease that encompasses ichthyosis linearis circumflexa (ILC) and a predisposition to allergies, asthma and eczema with hypereosinophilia, trichorrhexis invaginata and elevated serum IgE levels. This monogenic disorder is caused by mutations in the gene *SPINK5* and the loss of function of lymphoepithelial Kazal-type-related protease inhibitor (LEKTI-1) [143], and at a functional level the skin barrier in NS is seriously affected. One study proposes using a lentiviral (LV) vector for the genetic engineering of autologous stem cells, and grafting of autologous epidermal sheets generated from these cells for the treatment of NS patients (NCT01545323), however, the recruitment status of this trial remains unknown. Collectively, the main current therapeutic strategies for these hereditary skin diseases in clinical evaluations are shown in Fig. 11, and these delivery methods and routes can be potentially employed by versatile polymeric platforms.

4. Conclusion

Most hereditary skin diseases are severe, life-long and incurable disorders, which require clinically viable gene therapy approaches to alleviate the devastating disease symptoms and complications. Although polymeric vectors show great promise in treating genetic diseases, cancers and vaccination development, their application in hereditary skin diseases is rare. RDEB is a bullous genodermatosis that causes severe damage in response to minor mechanical trauma and imposes a heavy burden on patients and healthcare systems. Numerous efforts have been made to find a cure for RDEB, but each treatment method has its own limitations. In this review, we have summarized the development of an "A2 + B3 + C2" Michael addition platform for the synthesis of HPAE vectors, and

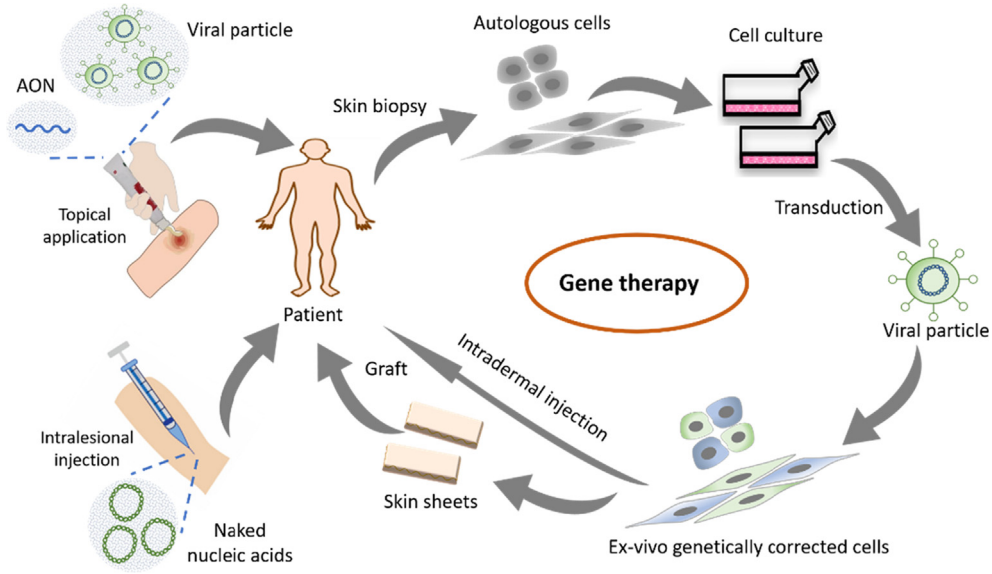

Fig. 11. Current gene therapy strategies for inherited skin disease in clinical application. Generally, *ex-vivo* approaches, topical application and intralesional administration of the naked nucleic acids are the existing systems employed for genetic skin diseases in clinical settings. Autologous cells including keratinocytes, fibroblasts, and epidermal stem cells can be obtained from patient biopsies, and then cultured and expanded, and further processed to prepare epidermal sheets or skin equivalent, which can be grafted back to the wounds of patients, or injected directly to the patient's skin (i.e. fibroblasts); viral vectors carrying the therapeutic gene can be used topically; and naked nucleic acids, for example, AONs and siRNA can be utilized topically and injected to the lesion, respectively.

highlighted the best-performing HPAEs derived from their LPAE counterparts for the treatment of incurable RDEB. Based on the monomer combinations and "bottom-up" design, HPAEs with favorable properties to deliver the therapeutic gene of interest with high-performance gene transfection have been designed. The efficiency and cytotoxicity can be controlled by the structure and M_w of HPAE polymers. The impressive performance of HPAEs in gene transfection work have been validated in skin cells including keratinocytes and fibroblasts, and other cell types such as stem cells, cancer cells and astrocytes. The gene construct of *COL7A1*-encoding pDNA has been optimized by synthesizing a minimized MCC7 for pursuing higher gene delivery efficiency and biosafety. In preclinical studies, complexed with regular *COL7A1*-encoding pDNA and MCC7, HPAEs have successfully restored long-term recombinant C7 expression along the skin BMZ in a *COL7A1*-null mice model and a xenograft RDEB mice model, respectively. Structure-function relationship studies demonstrate these polymeric particles have the capacity to overcome multiple cellular and extracellular barriers in gene transfer. Besides, frozen HPAE/DNA particles can be stored for 1 year without losing transfection efficiency. Our work shows that high efficiency, excellent biocompatibility, and facile manipulation together with long-term stability make the HPAE/MCC7 system a promising bench-to-bed candidate for treating the debilitating condition RDEB.

Cutaneous gene therapy has its own characteristics for gene transfer. The skin is an organ barrier that requires the particles to move through different pathways, such as transcellular route, intercellular route, follicular route and appendageal route. Cellular targets of delivery include keratinocytes, fibroblasts, epidermal stem cells and dendritic cells, depending on the therapeutic indications. Topical, intralesional and intradermal administrations are favorable routes for gene delivery to the skin, and *ex-vivo* methods are very popular, in which autologous skin cells are transfected and the skin sheets are grafted back to the patients. Polymeric particles are also required to surmount systemic barriers and cellular barriers for better gene delivery performances. Currently, traditional viral vectors having been more extensively researched than nonviral technologies in the treatment of genetic skin diseases. However, our design, synthesis, and development of HPAE polymers highlights significant evidence supporting their efficacy and safety, and the future potential for utilizing polymeric vectors for the treatment of other hereditary skin diseases, such as JEB, PC, ichthyosis and NS.

Acknowledgement

This work was funded by National Science Foundation of China (NFSC) (81903240, 51903202, 51573129, 51873179, 82073422 and 81874239), Science Foundation Ireland Industry Fellowship (19/IFA/7435), Government of Ireland Postdoctoral Fellowship (GOIPD/2019/861), Science Foundation Ireland Frontiers (19/FFP/6522).

References

[1] L. Naldini, Gene therapy returns to centre stage, Nature 526 (2015) 351–360.
[2] M.A. Kay, State-of-the-art gene-based therapies: the road ahead, Nat. Rev. Genet. 12 (2011) 316–328.
[3] H. Yin, R.L. Kanasty, A.A. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, Non-viral vectors for gene-based therapy, Nat. Rev. Genet. 15 (2014) 541–555.
[4] M. Ramamoorth, A. Narvekar, Non viral vectors in gene therapy- an overview, J. Clin. Diagn Res. 2015;9:GE01-6.
[5] M. Foldvari, D.W. Chen, N. Nafissi, D. Calderon, L. Narsineni, A. Rafiee, Non-viral gene therapy: Gains and challenges of non-invasive administration methods, J. Control Release 240 (2016) 165–190.
[6] D.W. Pack, A.S. Hoffman, S. Pun, P.S. Stayton, Design and development of polymers for gene delivery, Nat. Rev. Drug. Discov. 4 (2005) 581–593.
[7] A. Aied, U. Greiser, A. Pandit, W. Wang, Polymer gene delivery: overcoming the obstacles, Drug. Discov. Today 18 (2013) 1090–1098.
[8] P.A. Khavari, O. Rollman, A. Vahlquist, Cutaneous gene transfer for skin and systemic diseases, J. Int. Med. 252 (2002) 1–10.
[9] L. Engelke, G. Winter, S. Hook, J. Engert, Recent insights into cutaneous immunization: how to vaccinate via the skin, Vaccine 33 (2015) 4663–4674.
[10] E. Gorell, N. Nguyen, A. Lane, Z. Siprashvili, Gene therapy for skin diseases, Cold Spring Harb Perspect. Med. 4 (2014) a015149.
[11] X. Sun, L. Zeng, Y. Huang, Transcutaneous delivery of DNA/mRNA for cancer therapeutic vaccination, J. Gene Med. 21 (2019) 1–14.
[12] Y. Liu, Y. Li, D. Keskin, L. Shi, Poly(β-amino esters): synthesis, formulations, and their biomedical applications, Adv. Healthc Mater. 8 (2019) e1801359.
[13] R.A. Cordeiro, A. Serra, J.F.J. Coelho, H. Faneca, Poly(β-amino ester)-based gene delivery systems: from discovery to therapeutic applications, J. Control Release 310 (2019) 155–187.
[14] R.W. Pearson, Studies on the pathogenesis of epidermolysis bullosa, J. Invest. Dermatol. 39 (1962) 551–575.
[15] C. Has, A. Nyström, A.H. Saeidian, L. Bruckner-Tuderman, J. Uitto, Epidermolysis bullosa: Molecular pathology of connective tissue components in the cutaneous basement membrane zone, Matrix. Biol. 71–72 (2018) 313–329.
[16] J.-D. Fine, L. Bruckner-Tuderman, R.A.J. Eady, E.A. Bauer, J.W. Bauer, C. Has, et al., Inherited epidermolysis bullosa: updated recommendations on diagnosis and classification, J. Am. Acad. Dermatol. 70 (2014) 1103–1126.
[17] J. Uitto, H. Vahidnezhad, L. Youssefian, Genotypic heterogeneity and the mode of inheritance in epidermolysis bullosa, JAMA Dermatology 152 (2016) 517.
[18] E. Rashidghamat, J.A. McGrath, Novel and emerging therapies in the treatment of recessive dystrophic epidermolysis bullosa, Intractable rare Dis. Res. 6 (2017) 6–20.
[19] D.T. Woodley, T. Atha, Y. Huang, M. Chen, G.G. Krueger, C.M. Jorgensen, et al., Normal and gene-corrected dystrophic epidermolysis bullosa fibroblasts alone can produce Type VII collagen at the basement membrane zone, J. Invest. Dermatol. 121 (2003) 1021–1028.
[20] J. Tolar, J.E. Wagner, A biologic velcro patch, N Engl. J. Med. 372 (2015) 382–384.
[21] J.D. Fine, J.E. Mellerio, Extracutaneous manifestations and complications of inherited epidermolysis bullosa. Part II. Other organs, J. Am. Acad. Dermatol. 61 (2009) 387–402.
[22] J.-D. Fine, L.B. Johnson, M. Weiner, K.-P. Li, C. Suchindran, Epidermolysis bullosa and the risk of life-threatening cancers: the national EB registry experience, 1986–2006, J. Am. Acad. Dermatol. 60 (2009) 203–211.
[23] E. Pfendner, J. Uitto, J.-D. Fine, Epidermolysis bullosa carrier frequencies in the US population, J. Invest. Dermatol. 116 (2001) 483–484.
[24] D.F. Murrell, Life with epidermolysis bullosa (EB): Etiology, diagnosis, multidisciplinary care and therapy, J. Am. Acad. Dermatol. 61 (2009) 1092–1093.
[25] J. Uitto, L. Bruckner-Tuderman, J.A. McGrath, R. Riedl, C. Robinson, EB2017—progress in epidermolysis bullosa research toward treatment and cure, J. Invest. Dermatol 138 (2018) 1010–1016.
[26] A. Nystrom, K. Thriene, V. Mittapalli, J.S. Kern, D. Kiritsi, J. Dengjel, et al., Losartan ameliorates dystrophic epidermolysis bullosa and uncovers new disease mechanisms, EMBO Mol. Med. 7 (2015) 1211–1228.
[27] G. Petrof, M. Martinez-Queipo, J.E. Mellerio, P. Kemp, J.A. McGrath, Fibroblast cell therapy enhances initial healing in recessive dystrophic epidermolysis bullosa wounds: results of a randomized, vehicle-controlled trial, Br. J. Dermatol. 169 (2013) 1025–1033.
[28] S.S. Venugopal, W. Yan, J.W. Frew, H.I. Cohn, L.M. Rhodes, K. Tran, et al., A phase II randomized vehicle-controlled trial of intradermal allogeneic fibroblasts for recessive dystrophic epidermolysis bullosa, J. Am. Acad. Dermatol. 69 (2013) 898–908.e7.
[29] J.E. Wagner, A. Ishida-Yamamoto, J.A. McGrath, M. Hordinsky, D.R. Keene, D.T. Woodley, et al., Bone marrow transplantation for recessive dystrophic epidermolysis bullosa, N Engl. J. Med. 363 (2010) 629–639.
[30] P. Conget, F. Rodriguez, S. Kramer, C. Allers, V. Simon, F. Palisson, et al., Replenishment of type VII collagen and re-epithelialization of chronically ulcerated skin after intradermal administration of allogeneic mesenchymal stromal cells in two patients with recessive dystrophic epidermolysis bullosa, Cytotherapy 12 (2010) 429–431.
[31] G. Petrof, S.M. Lwin, M. Martinez-Queipo, A. Abdul-Wahab, S. Tso, J.E. Mellerio, et al., Potential of systemic allogeneic mesenchymal stromal cell therapy for children with recessive dystrophic epidermolysis bullosa, J. Invest. Dermatol. 135 (2015) 2319–2321.
[32] T. Kühl, M. Mezger, I. Hausser, R. Handgretinger, L. Bruckner-Tuderman, A. Nyström, High local concentrations of intradermal MSCs restore skin integrity and facilitate wound healing in dystrophic epidermolysis bullosa, Mol. Ther. 23 (2015) 1368–1379.
[33] D.T. Woodley, M. Chen, Recessive dystrophic epidermolysis bullosa: advances in the laboratory leading to new therapies, J. Invest. Dermatol. 135 (2015) 1705–1707.
[34] J. Remington, X. Wang, Y. Hou, H. Zhou, J. Burnett, T. Muirhead, et al., Injection of recombinant human type VII collagen corrects the disease phenotype in a murine model of dystrophic epidermolysis bullosa, Mol. Ther. 17 (2009) 26–33.
[35] X. Wang, P. Ghasri, M. Amir, B. Hwang, Y. Hou, M. Khilili, et al., Topical application of recombinant type VII collagen incorporates into the dermal-epidermal junction and promotes wound closure, Mol. Ther. 21 (2013) 1335–1344.

[36] D.T. Woodley, X. Wang, M. Amir, B. Hwang, J. Remington, Y. Hou, et al., Intravenously injected recombinant human type VII collagen homes to skin wounds and restores skin integrity of dystrophic epidermolysis bullosa, J. Invest. Dermatol. 133 (2013) 1910–1913.
[37] L. Bruckner-Tuderman, Can type VII collagen injections cure dystrophic epidermolysis bullosa?, Mol Ther. 17 (2009) 6–7.
[38] D.T. Woodley, J. Cogan, Y. Hou, C. Lyu, M.P. Marinkovich, D. Keene, et al., Gentamicin induces functional type VII collagen in recessive dystrophic epidermolysis bullosa patients, J. Clin. Invest. 127 (2017) 3028–3038.
[39] C. Chamorro, A. Mencía, D. Almarza, B. Duarte, H. Büning, J. Sallach, et al., Gene editing for the efficient correction of a recurrent COL7A1 mutation in recessive dystrophic epidermolysis bullosa keratinocytes, Mol. Ther. - Nucleic Acids 5 (2016) e307.
[40] S. Hainzl, P. Peking, T. Kocher, E.M. Murauer, F. Larcher, M. Del Rio, et al., COL7A1 editing via CRISPR/Cas9 in recessive dystrophic epidermolysis bullosa, Mol. Ther. 25 (2017) 2573–2584.
[41] Z. Siprashvili, N.T. Nguyen, E.S. Gorell, K. Loutit, P. Khuu, L.K. Furukawa, et al., Safety and wound outcomes following genetically corrected autologous epidermal grafts in patients with recessive dystrophic epidermolysis bullosa, JAMA 316 (2016) 1808.
[42] C.E. Thomas, A. Ehrhardt, M.A. Kay, Progress and problems with the use of viral vectors for gene therapy, Nat. Rev. Genet. 4 (2003) 346–358.
[43] C. Rinaldi, M.J.A. Wood, Antisense oligonucleotides: the next frontier for treatment of neurological disorders, Nat. Rev. Neurol. 14 (2017) 9–21.
[44] S. Turczynski, M. Titeux, L. Tonasso, A. Décha, A. Ishida-Yamamoto, A. Hovnanian, Targeted exon skipping restores type VII collagen expression and anchoring fibril formation in an in vivo RDEB model, J. Invest. Dermatol. 136 (2016) 2387–2395.
[45] H. Lv, S. Zhang, B. Wang, S. Cui, J. Yan, Toxicity of cationic lipids and cationic polymers in gene delivery, J. Control Release 114 (2006) 100–109.
[46] D.M. Lynn, R. Langer, Degradable poly(β-amino esters): synthesis, characterization, and self-assembly with plasmid DNA, J. Am. Chem. Soc. 122 (2000) 10761–10768.
[47] D.G. Anderson, D.M. Lynn, R. Langer, Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery, Angew Chemie Int. Ed. 42 (2003) 3153–3158.
[48] A. Akinc, D.M. Lynn, D.G. Anderson, R. Langer, Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery, J. Am. Chem. Soc. 125 (2003) 5316–5323.
[49] A. Akinc, D.G. Anderson, D.M. Lynn, R. Langer, Synthesis of poly(β-amino ester)s optimized for highly effective gene delivery, Bioconjug Chem. 14 (2003) 979–988.
[50] D.G. Anderson, W. Peng, A. Akinc, N. Hossain, A. Kohn, R. Padera, et al., A polymer library approach to suicide gene therapy for cancer, Proc. Natl. Acad. Sci. 101 (2004) 16028–16033.
[51] D.G. Anderson, A. Akinc, N. Hossain, R. Langer, Structure/property studies of polymeric gene delivery using a library of poly(β-amino esters), Mol. Ther. 11 (2005) 426–434.
[52] G.T. Zugates, W. Peng, A. Zumbuehl, S. Jhunjhunwala, Y.-H. Huang, R. Langer, et al., Rapid optimization of gene delivery by parallel end-modification of poly(β-amino ester)s, Mol. Ther. 15 (2007) 1306–1312.
[53] J.J. Green, G.T. Zugates, N.C. Tedford, Y.-H. Huang, L.G. Griffith, D.A. Lauffenburger, et al., Combinatorial modification of degradable polymers enables transfection of human cells comparable to adenovirus, Adv. Mater. 19 (2007) 2836–2842.
[54] G.T. Zugates, N.C. Tedford, A. Zumbuehl, S. Jhunjhunwala, C.S. Kang, L.G. Griffith, et al., Gene delivery properties of end-modified poly(β-amino ester)s, Bioconjug Chem. 18 (2007) 1887–1896.
[55] Y.-H. Huang, G.T. Zugates, W. Peng, D. Holtz, C. Dunton, J.J. Green, et al., Nanoparticle-delivered suicide gene therapy effectively reduces ovarian tumor burden in mice, Can. Res. 69 (2009) 6184–6191.
[56] N.S. Bhise, R.S. Gray, J.C. Sunshine, S. Htet, A.J. Ewald, J.J. Green, The relationship between terminal functionalization and molecular weight of a gene delivery polymer and transfection efficacy in mammary epithelial 2-D cultures and 3-D organotypic cultures, Biomaterials 31 (2010) 8088–8096.
[57] S.Y. Tzeng, H. Guerrero-Cázares, E.E. Martinez, J.C. Sunshine, A. Quiñones-Hinojosa, J.J. Green, Non-viral gene delivery nanoparticles based on poly(β-amino esters) for treatment of glioblastoma, Biomaterials 32 (2011) 5402–5410.
[58] A.A. Eltoukhy, D.J. Siegwart, C.A. Alabi, J.S. Rajan, R. Langer, D.G. Anderson, Effect of molecular weight of amine end-modified poly(β-amino ester)s on gene delivery efficiency and toxicity, Biomaterials 33 (2012) 3594–3603.
[59] N.S. Bhise, R.B. Shmueli, J. Gonzalez, J.J. Green, A novel assay for quantifying the number of plasmids encapsulated by polymer nanoparticles, Small 8 (2012) 367–373.
[60] J.C. Sunshine, D.Y. Peng, J.J. Green, Uptake and transfection with polymeric nanoparticles are dependent on polymer end-group structure, but largely independent of nanoparticle physical and chemical properties, Mol. Pharm. 9 (2012) 3375–3383.
[61] H. Guerrero-Cázares, S.Y. Tzeng, N.P. Young, A.O. Abutaleb, A. Quiñones-Hinojosa, J.J. Green, Biodegradable polymeric nanoparticles show high efficacy and specificity at DNA delivery to human glioblastoma in vitro and in vivo, ACS Nano 8 (2014) 5141–5153.
[62] A. Mangraviti, S.Y. Tzeng, K.L. Kozielski, Y. Wang, Y. Jin, D. Gullotti, et al., Polymeric nanoparticles for nonviral gene therapy extend brain tumor survival in vivo, ACS Nano 9 (2015) 1236–1249.
[63] A.A. Eltoukhy, D. Chen, C.A. Alabi, R. Langer, D.G. Anderson, Degradable terpolymers with alkyl side chains demonstrate enhanced gene delivery potency and nanoparticle stability, Adv Mater 25 (2013) 1487–1493.
[64] Z. Kadlecova, Y. Rajendra, M. Matasci, L. Baldi, D.L. Hacker, F.M. Wurm, et al., DNA delivery with hyperbranched polylysine: a comparative study with linear and dendritic polylysine, J. Control Release 169 (2013) 276–288.
[65] T. Zhao, H. Zhang, B. Newland, A. Aied, D. Zhou, W. Wang, Significance of branching for transfection: synthesis of highly branched degradable functional poly(dimethylaminoethyl methacrylate) by vinyl oligomer combination, Angew Chemie – Int. Ed. 53 (2014) 6095–6100.
[66] M. Ahmed, R. Narain, The effect of molecular weight, compositions and lectin type on the properties of hyperbranched glycopolymers as non-viral gene delivery systems, Biomaterials 33 (2012) 3990–4001.
[67] Y. Lim, S.-M. Kim, Y. Lee, W. Lee, T. Yang, M. Lee, et al., Cationic hyperbranched poly(amino ester): a novel class of DNA condensing molecule with cationic surface, biodegradable three-dimensional structure, and tertiary amine groups in the interior, J. Am. Chem. Soc. 123 (2001) 2460–2461.
[68] Y. Liu, D. Wu, Y. Ma, G. Tang, S. Wang, C. He, et al., Novel poly(amino ester)s obtained from michael addition polymerizations of trifunctional amine monomers with diacrylates: safe and efficient DNA carriers, Chem. Commun. (Camb) 20 (2003) 2630–2631.
[69] J.-Y. Huang, Y. Gao, L. Cutlar, J. O'Keeffe-Ahern, T. Zhao, F.-H. Lin, et al., Tailoring highly branched poly(β-amino ester)s: a synthetic platform for epidermal gene therapy, Chem. Commun. 51 (2015) 8473–8476.
[70] Y. Gao, J.Y. Huang, J. O'Keeffe Ahern, L. Cutlar, D. Zhou, F.H. Lin, et al., Highly branched poly(β-amino esters) for non-viral gene delivery: high transfection efficiency and low toxicity achieved by increasing molecular weight, Biomacromolecules 17 (11) (2016) 3640–3647.
[71] D. Zhou, L. Cutlar, Y. Gao, W. Wang, J. O'Keeffe-Ahern, S. McMahon, et al., The transition from linear to highly branched poly(β-amino ester)s: branching matters for gene delivery, Sci. Adv. 2 (2016) e1600102.
[72] D. Zhou, Y. Gao, J. O'Keeffe Ahern, A S, Q. Xu, X. Huang, et al., Development of Branched Poly(5-Amino-1-pentanol- co -1,4-butanediol Diacrylate) with High Gene Transfection Potency Across Diverse Cell Types, ACS Appl Mater Interfaces 2016;8:34218–34226.
[73] M. Zeng, D. Zhou, S. Ng, J.O. Ahern, F. Alshehri, Y. Gao, et al., Highly branched poly(5-amino-1-pentanol-co-1,4-butanediol diacrylate) for high performance gene transfection, Polymers (Basel) 9 (2017) 161.
[74] M. Zeng, D. Zhou, F. Alshehri, I. Lara-Sáez, Y. Lyu, J. Creagh-Flynn, et al., Manipulation of transgene expression in fibroblast cells by a multifunctional linear-branched hybrid poly(β-amino ester) synthesized through an oligomer combination approach, Nano Lett 19 (2019) 381–391.
[75] M. Zeng, F. Alshehri, D. Zhou, I. Lara-Sáez, X. Wang, X. Li, et al., Efficient and robust highly branched poly(β-amino ester)/minicircle COL7A1 polymeric nanoparticles for gene delivery to recessive dystrophic epidermolysis bullosa keratinocytes, ACS Appl Mater Interfaces 11 (2019) 30661–30672.
[76] J.W. Fountain, W.K. Lockwood, F.S. Collins, Transfection of primary human skin fibroblasts by electroporation, Gene 68 (1988) 167–172.
[77] E.T. Jordan, M. Collins, J. Terefe, L. Ugozzoli, T. Rubio, Optimizing electroporation conditions in primary and other difficult-to-transfect cells, J. Biomol. Tech. 19 (2008) 328–334.
[78] M. Sharifi Tabar, M. Hesaraki, F. Esfandiari, F. Sahraneshin Samani, H. Vakilian, H. Baharvand, Evaluating electroporation and lipofectamine approaches for transient and stable transgene expressions in human fibroblasts and embryonic stem cells, Cell J. 17 (2015) 438–450.
[79] A.K. Patel, J.C. Kaczmarek, S. Bose, K.J. Kauffman, F. Mir, M.W. Heartlein, et al., Inhaled nanoformulated mRNA polyplexes for protein production in lung epithelium, Adv Mater 31 (2019) 1–7.
[80] K.-A. Chang, J.-H. Lee, Y.-H. Suh, Therapeutic potential of human adipose-derived stem cells in neurological disorders, J. Pharmacol. Sci. 126 (2014) 293–301.
[81] F. Yang, S.-W. Cho, S.M. Son, S.R. Bogatyrev, D. Singh, J.J. Green, et al., Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric nanoparticles, Proc. Natl. Acad. Sci. 107 (2010) 3317–3322.
[82] Y.-T. Tai, C.N. Svendsen, Stem cells as a potential treatment of neurological disorders, Curr. Opin. Pharmacol. 4 (2004) 98–104.
[83] S. Liu, Y. Gao, D. Zhou, M. Zeng, F. Alshehri, B. Newland, et al., Highly branched poly(β-amino ester) delivery of minicircle DNA for transfection of neurodegenerative disease related cells, Nat. Commun. 10 (2019) 3307.
[84] F. Faurez, D. Dory, V. Le Moigne, R. Gravier, A. Jestin, Biosafety of DNA vaccines: new generation of DNA vectors and current knowledge on the fate of plasmids after injection, Vaccine 28 (2010) 3888–3895.
[85] M.M. Munye, A.D. Tagalakis, J.L. Barnes, R.E. Brown, R.J. McAnulty, S.J. Howe, et al., Minicircle DNA provides enhanced and prolonged transgene expression following airway gene transfer, Sci Rep 6 (2016) 23125.
[86] A.-M. Darquet, B. Cameron, P. Wils, D. Scherman, J. Crouzet, A new DNA vehicle for nonviral gene delivery: supercoiled minicircle, Gene Ther 4 (1997) 1341–1349.
[87] A.-M. Darquet, R. Rangara, P. Kreiss, B. Schwartz, S. Naimi, P. Delaère, et al., Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer, Gene Ther 6 (1999) 209–218.
[88] V. Gaspar, D. de Melo-Diogo, E. Costa, A. Moreira, J. Queiroz, C. Pichon, et al., Minicircle DNA vectors for gene therapy: advances and applications, Expert Opin. Biol. Ther. 15 (2015) 353–379.

[89] Z.-Y. Chen, S.R. Yant, C.-Y. He, L. Meuse, S. Shen, M.A. Kay, Linear DNAs concatemerize in vivo and result in sustained transgene expression in mouse liver, Mol. Ther. 3 (2001) 403–410.
[90] Z.Y. Chen, C.Y. He, L. Meuse, M.A. Kay, Silencing of episomal transgene expression by plasmid bacterial DNA elements in vivo, Gene Ther. 11 (2004) 856–864.
[91] Z.-Y. Chen, C.-Y. He, A. Ehrhardt, M.A. Kay, Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo, Mol. Ther. 8 (2003) 495–500.
[92] C.S. Yoon, H.S. Jung, M.J. Kwon, S.H. Lee, C.W. Kim, M.K. Kim, et al., Sonoporation of the minicircle-VEGF165 for wound healing of diabetic mice, Pharm. Res. 26 (2009) 794–801.
[93] M.J. Kwon, S. An, S. Choi, K. Nam, H.S. Jung, C.S. Yoon, et al., Effective healing of diabetic skin wounds by using nonviral gene therapy based on minicircle vascular endothelial growth factor DNA and a cationic dendrimer, J. Gene Med. 14 (2012) 272–278.
[94] S. Stenler, P. Blomberg, C.E. Smith, Safety and efficacy of DNA vaccines, Hum. Vaccin. Immunother. 10 (2014) 1306–1308.
[95] C.-W. Chang, L.V. Christensen, M. Lee, S.W. Kim, Efficient expression of vascular endothelial growth factor using minicircle DNA for angiogenic gene therapy, J. Control Release 125 (2008) 155–163.
[96] H. Yi, Y. Kim, J. Kim, H. Jung, Y.A. Rim, S.M. Jung, et al., A new strategy to deliver synthetic protein drugs: self-reproducible biologics using minicircles, Sci. Rep. 4 (2015) 5961.
[97] T.-Y. Chang, C.-Y. Chung, W.-M. Chuang, L.-Y. Li, L.-B. Jeng, W.-L. Ma, Durable expression of minicircle DNA-liposome-delivered androgen receptor cDNA in mice with hepatocellular carcinoma, Biomed. Res. Int. 2014 (2014) 1–9.
[98] L. Cutlar, D. Zhou, X. Hu, B. Duarte, U. Greiser, F. Larcher, et al., A non-viral gene therapy for treatment of recessive dystrophic epidermolysis bullosa, Exp. Dermatol. 25 (2016) 818–820.
[99] Y. Tao, L. Zhang, P.C.K. Cheung, Physicochemical properties and antitumor activities of water-soluble native and sulfated hyperbranched mushroom polysaccharides, Carbohydr. Res. 341 (2006) 2261–2269.
[100] L. Cutlar, D. Zhou, Y. Gao, T. Zhao, U. Greiser, W. Wang, et al., Highly branched poly(β-amino esters): synthesis and application in gene delivery, Biomacromolecules 16 (2015) 2609–2617.
[101] D. Zhou, Y. Gao, A. Aied, L. Cutlar, O. Igoucheva, B. Newland, et al., Highly branched poly(β-amino ester)s for skin gene therapy, J. Control Release 244 (2016) 336–346.
[102] A. Verma, O. Uzun, Y. Hu, Y. Hu, H.-S. Han, N. Watson, et al., Surface-structure-regulated cell-membrane penetration by monolayer-protected nanoparticles, Nat. Mater. 7 (2008) 588–595.
[103] J. Shi, J.L. Choi, B. Chou, R.N. Johnson, J.G. Schellinger, S.H. Pun, Effect of polyplex morphology on cellular uptake, intracellular trafficking, and transgene expression, ACS Nano 7 (2013) 10612–10620.
[104] A. Parodi, C. Corbo, A. Cevenini, R. Molinaro, R. Palomba, L. Pandolfi, et al., Enabling cytoplasmic delivery and organelle targeting by surface modification of nanocarriers, Nanomedicine 10 (12) (2015) 1923–1940.
[105] C. Chamorro, D. Almarza, B. Duarte, S.G. Llames, R. Murillas, M. García, et al., Keratinocyte cell lines derived from severe generalized recessive Epidermolysis Bullosa patients carrying a highly recurrent COL7A1 homozygous mutation: models to assess cell and gene therapies in vitro and in vivo, Exp. Dermatol. 22 (2013) 601–603.
[106] D.T. Woodley, D.R. Keene, T. Atha, Y. Huang, R. Ram, N. Kasahara, et al., Intradermal injection of lentiviral vectors corrects regenerated human dystrophic epidermolysis bullosa skin tissue in vivo, Mol. Ther. 10 (2004) 318–326.
[107] S. Heinonen, M. Männikkö, J.F. Klement, D. Whitaker-Menezes, G.F. Murphy, J. Uitto, Targeted inactivation of the type VII collagen gene (Col7a1) in mice results in severe blistering phenotype: a model for recessive dystrophic epidermolysis bullosa, J. Cell Sci. 112 (1999) 3641–3648.
[108] A. Fritsch, S. Loeckermann, J.S. Kern, A. Braun, M.R. Bösl, T.A. Bley, et al., A hypomorphic mouse model of dystrophic epidermolysis bullosa reveals mechanisms of disease and response to fibroblast therapy, J. Clin. Invest. 118 (2008) 1669–1679.
[109] P.A. Khavari, Therapeutic gene delivery to the skin, Mol. Med. Today 3 (1997) 533–538.
[110] T.-M. Tuan-Mahmood, M.T.C. McCrudden, B.M. Torrisi, E. McAlister, M.J. Garland, T.R.R. Singh, et al., Microneedles for intradermal and transdermal drug delivery, Eur. J. Pharm. Sci. 50 (2013) 623–637.
[111] A.M. Bodles-Brakhop, R. Heller, R. Draghia-Akli, Electroporation for the delivery of DNA-based vaccines and immunotherapeutics: current clinical developments, Mol. Ther. 17 (2009) 585–592.
[112] B.C.-Q. Seah, B.M. Teo, Recent advances in ultrasound-based transdermal drug delivery, Int. J. Nanomed. 13 (2018) 7749–7763.
[113] M. Roberts, Y. Mohammed, M. Pastore, S. Namjoshi, S. Yousef, A. Alinaghi, et al., Topical and cutaneous delivery using nanosystems, J. Control Release 247 (2017) 86–105.
[114] D. Wenzel, J. Bayerl, A. Nystrom, L. Bruckner-Tuderman, A. Meixner, J.M. Penninger, Genetically corrected iPSCs as cell therapy for recessive dystrophic epidermolysis bullosa, Sci Transl Med 6 (2014) 264ra165–264ra165.
[115] A. Patzelt, W.C. Mak, S. Jung, F. Knorr, M.C. Meinke, H. Richter, et al., Do nanoparticles have a future in dermal drug delivery?, J Control Release 246 (2017) 174–182.
[116] J.D. Bos, M.M.H.M. Meinardi, The 500 Dalton rule for the skin penetration of chemical compounds and drugs, Exp. Dermatol. 9 (2000) 165–169.
[117] R.F. Donnelly, T.R.R. Singh, Novel delivery systems for transdermal and intradermal drug delivery. Chichester, UK, John Wiley & Sons, Ltd, 2015. DOI: 10.1002/9781118734506.
[118] K.S. Paudel, M. Milewski, C.L. Swadley, N.K. Brogden, P. Ghosh, A.L. Stinchcomb, Challenges and opportunities in dermal/transdermal delivery, Ther. Deliv. 1 (2010) 109–131.
[119] Mohd. Yasir, Som I, Bhatia K: Status of surfactants as penetration enhancers in transdermal drug delivery. J Pharm Bioallied Sci 2012;4:2.
[120] G. Tan, P. Xu, L.B. Lawson, J. He, L.C. Freytag, J.D. Clements, et al., Hydration effects on skin microstructure as probed by high-resolution cryo-scanning electron microscopy and mechanistic implications to enhanced transcutaneous delivery of biomacromolecules, J. Pharm. Sci. 99 (2010) 730–740.
[121] T. Maeba, T. Yonezawa, M. Ono, Y. Tomono, R. Heljasvaara, T. Pihlajaniemi, et al., Collagen XVIII deposition in the basement membrane zone beneath the newly forming epidermis during wound healing in mice, Acta Med. Okayama 73 (2019) 135–146.
[122] T. Kazama, E. Yaoita, M. Ito, Y. Sato, Charge-selective permeability of dermo-epidermal junction: tracer studies with cationic and anionic ferritins, J. Invest. Dermatol. 91 (1988) 560–565.
[123] C. Blanpain, E. Fuchs, Epidermal stem cells of the skin, Annu. Rev. Cell Dev. Biol. 22 (2006) 339–373.
[124] M. Aragona, C. Blanpain, Transgenic stem cells replace skin, Nature 551 (2017) 306–307.
[125] C. Georgiadis, F. Syed, A. Petrova, A. Abdul-Wahab, S.M. Lwin, F. Farzaneh, et al., Lentiviral engineered fibroblasts expressing codon-optimized COL7A1 restore anchoring fibrils in RDEB, J. Invest. Dermatol. 136 (2016) 284–292.
[126] B. Mattix, T. Moore, O. Uvarov, S. Pollard, L. O'Donnell, K. Park, et al., Effects of polymeric nanoparticle surface properties on interaction with brain tumor environment, Nano Life 03 (2013) 1343003.
[127] M. Longmire, P.L. Choyke, H. Kobayashi, Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats, Nanomedicine 3 (2008) 703–717.
[128] M. Hamidi, A. Azadi, P. Rafiei, Pharmacokinetic consequences of pegylation, Drug Deliv 13 (2006) 399–409.
[129] J.S. Suk, Q. Xu, N. Kim, J. Hanes, L.M. Ensign, PEGylation as a strategy for improving nanoparticle-based drug and gene delivery, Adv. Drug Deliv. Rev. 99 (2016) 28–51.
[130] K. Kunath, T. Merdan, O. Hegener, H. Häberlein, T. Kissel, Integrin targeting using RGD-PEI conjugates for in vitro gene transfer, J. Gene Med. 5 (2003) 588–599.
[131] D.V. Schaffer, D.A. Lauffenburger, Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery, J. Biol. Chem. 273 (1998) 28004–28009.
[132] T. Ahmadzada, G. Reid, D.R. McKenzie, Fundamentals of siRNA and miRNA therapeutics and a review of targeted nanoparticle delivery systems in breast cancer, Biophys. Rev. 10 (2018) 69–86.
[133] S. Guan, J. Rosenecker, Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems, Gene Ther. 24 (2017) 133–143.
[134] J. Uitto, J.A. McGrath, U. Rodeck, L. Bruckner-Tuderman, E. Clare Robinson, Progress in epidermolysis bullosa research: toward treatment and cure, J. Invest. Dermatol. 130 (2010) 1778–1784.
[135] F. Mavilio, G. Pellegrini, S. Ferrari, F. Di Nunzio, E. Di Iorio, A. Recchia, et al., Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells, Nat. Med. 12 (2006) 1397–1402.
[136] J.W. Bauer, J. Koller, E.M. Murauer, L. De Rosa, E. Enzo, S. Carulli, et al., Closure of a large chronic wound through transplantation of gene-corrected epidermal stem cells, J. Invest. Dermatol. 137 (2017) 778–781.
[137] T. Hirsch, T. Rothoeft, N. Teig, J.W. Bauer, G. Pellegrini, L. De Rosa, et al., Regeneration of the entire human epidermis using transgenic stem cells, Nature 551 (2017) 327–332.
[138] L. De Rosa, S. Carulli, F. Cocchiarella, D. Quaglino, E. Enzo, E. Franchini, et al., Long-term stability and safety of transgenic cultured epidermal stem cells in gene therapy of junctional epidermolysis bullosa, Stem Cell Rep. 2 (2014) 1–8.
[139] S.A. Leachman, R.L. Kaspar, P. Fleckman, S.R. Florell, F.J.D. Smith, W.H.I. McLean, et al., Clinical and pathological features of pachyonychia congenita, J. Investig. Dermatol. Symp. Proc. 10 (2005) 3–17.
[140] D.A. Mordaunt, Sharing (data) is caring for patients with pachyonychia congenita, Br. J. Dermatol. 182 (2020), 537 537.
[141] S.A. Leachman, R.P. Hickerson, M.E. Schwartz, E.E. Bullough, S.L. Hutcherson, K.M. Boucher, et al., First-in-human mutation-targeted siRNA phase Ib Trial of an inherited skin disorder, Mol. Ther. 18 (2010) 442–446.
[142] C.R. Hill, A. Theos, What's new in genetic skin diseases, Dermatol. Clin. 37 (2019) 229–239.
[143] K. Özyurt, M. Atasoy, R. Ertaş, Y. Ulaş, M.R. Akkuş, A. Kiraz, et al., Netherton syndrome previously misdiagnosed as hyper IgE syndrome caused by a probable mutation in SPINK5 C, Turk. J. Pediatr. 61 (2019) 604–607.