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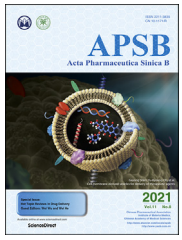

REVIEW

Recent advances in microneedles-mediated transdermal delivery of protein and peptide drugs

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Abstract Proteins and peptides have become a significant therapeutic modality for various diseases because of their high potency and specificity. However, the inherent properties of these drugs, such as large molecular weight, poor stability, and conformational flexibility, make them difficult to be formulated and delivered. Injection is the primary route for clinical administration of protein and peptide drugs, which usually leads to poor patient's compliance. As a portable, minimally invasive device, microneedles (MNs) can overcome the skin barrier and generate reversible microchannels for effective macromolecule permeation. In this review, we highlighted the recent advances in MNs-mediated transdermal delivery of protein and peptide drugs. Emphasis was given to the latest development in representative MNs design and fabrication. We also summarize the current application status of MNs-mediated transdermal protein and peptide delivery, especially in the field of infectious disease, diabetes, cancer, and other disease therapy. Finally, the current status of clinical translation and a perspective on future development are also provided.

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1. Introduction

Proteins and peptides exhibit the most prominent effects in human body, such as molecular transportation, biological scaffold, cellular regulation, and enzymatic catalysis, which have played an important role in almost every medical field[1–4]. Insulin was the first therapeutic protein approved in 1982 and since then remarkable progress has been achieved in the clinical application of numerous protein and peptide therapeutics[5,6]. However, the application of protein and peptide drugs is commonly restrained by certain limitations. The large molecular weight of these drugs substantially decreases their permeability capacity across biological barriers such as skin and mucous membranes. Besides, loss of biological activity in response to external conditions (moisture and temperature) and endogenous proteolytic enzyme put a high difficulty on formulation and delivery technologies[7].

Currently, injection is the primary route for clinical administration of protein and peptide drugs. Intravenous, subcutaneous, and intramuscular injection are the most widely used ways for delivering protein and peptide drugs[2,8–12]. Regardless of the injection method, most protein and peptide drugs are easily degraded by various metabolic enzymes in the body, resulting in short half-life *in vivo*, which means frequent injections are required. Furthermore, injection therapy is inconvenient and unfriendly, especially for patients with chronic diseases such as rheumatoid arthritis and diabetes. Injection safety should also be considered, since contamination of needles during administration can lead to transmission of some infectious diseases such as Hepatitis B and C. Therefore, for the delivery of protein and peptide drugs, there is a great need for an alternative drug delivery system that can be readily administrated with improved therapeutic efficacy, good patient compliance, and safety.

Transdermal drug delivery is a choice that delivers biologically active agents through skin portals for local or systemic effects, which is noninvasive and can be self-administered[13]. There are some requirements for the drugs suitable for transdermal administration, such as a maximum molecular weight of 1000 Da, and a balance between hydrophobicity and polarity due to the stratum corneum barrier[14]. Most protein and peptide drugs are hydrophilic and macromolecular in nature, and therefore they cannot easily penetrate into the skin. Over the past a few decades, various chemical and physical methods such as penetration enhancers[15], microjet[16], laser[17], electroporation[18], sonophoresis[19], and iontophoresis[20] have been developed as feasible strategies to improve transdermal drug permeation. But these techniques are usually expensive and cumbersome to use, and still exhibit limited efficiency for successful transdermal delivery of macromolecular drugs.

Recently, microneedles (MNs) have become a new type of drug delivery technique, and the applications of MNs have been extended to various aspects, including small chemical molecules[21,22], vaccines[23,24], genes[25], proteins[4,26], and nanoparticles[27]. Particularly, MNs provide a great prospect for the transdermal delivery of proteins and peptides[28,29]. MNs are minimally invasive device with needles (<1 mm) arranged orderly on the base. They can directly penetrate the stratum corneum by generating reversible microchannels in the skin. These microchannels can grant access of drugs to the dermal microcirculation located in the interior layers of the skin (Fig. 1). Compared with injection, MNs will not contact with blood vessels and nerves in the deep dermis, which provide better patient compliance and favorable safety profile. Moreover, the mild fabrication condition of MNs will not impact the biological activity of proteins and peptides.

This review provides comprehensive updates on MNs-mediated transdermal delivery of protein and peptide drugs. Emphasis was given to the latest development and advance in representative MNs design and fabrication. Additionally, we summarized the recent studies about the applications of MNs-mediated protein and peptide delivery, particularly focusing in the field of infectious disease, diabetes, cancer, and other disease therapy. Finally, the current status of clinical translation and a perspective on future development were also provided.

2. Representative types of MNs

Gerstel et al. proposed the concept of MNs in 1971, and Henry et al.[30] firstly reported the utilization of MNs for transdermal drug delivery *in vivo* in 1998[30,31]. Since then, various types of MNs have been successfully developed[32]. Based on different drug delivery strategies, MNs can be generally classified into five categories, including solid MNs, coated MNs, hollow MNs, dissolving MNs, and hydrogel-forming MNs (Fig. 2). Each type of MNs has been extensively studied for transdermal drug delivery. However, the protein and peptide drugs are usually sensitive to high temperature, pH value, and organic solvents compared with inert small molecules[33]. To avoid the damage of their biological activity, it is necessary to understand the properties of each type of MNs, and then select reasonable MNs types to formulate them. In

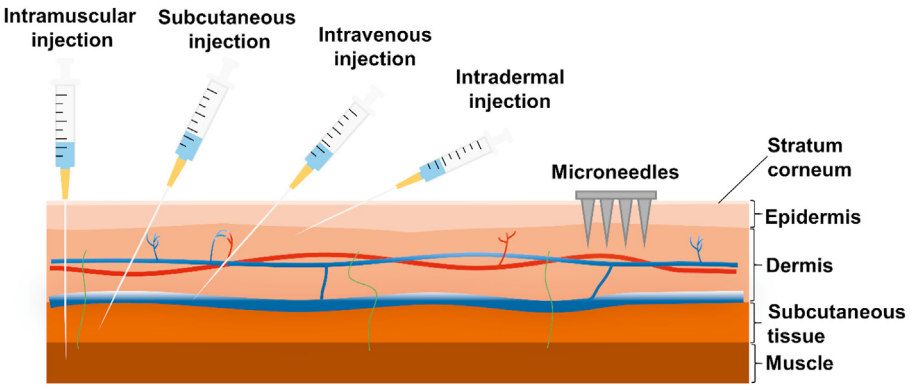

Figure 1 Schematic illustration of protein and peptide drug delivery by conventional injections and microneedles.

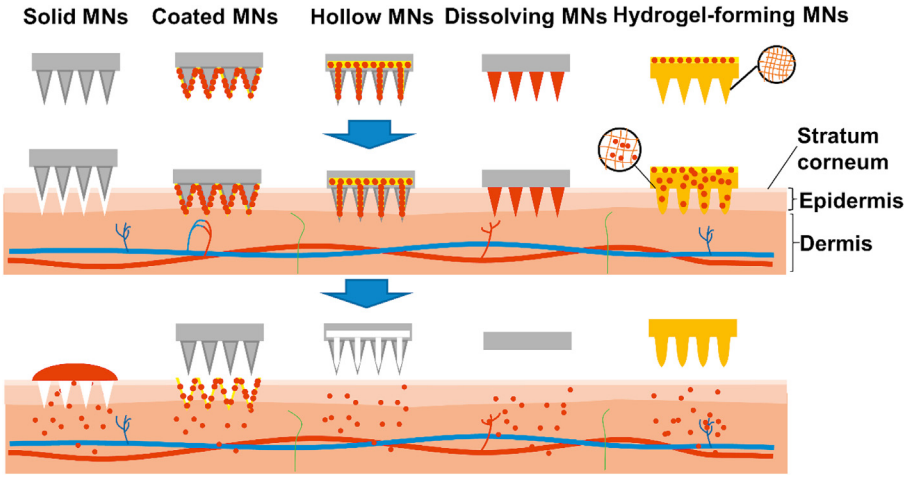

Figure 2 Representative types of MNs for transdermal drug delivery.

this section, the typical applications associated with different MNs-mediated delivery approaches are described in detail.

2.1. *Solid MNs*

Solid MNs usually need a two-step operation for drug delivery. Briefly, solid MNs are first inserted into the skin and subsequently removed to form temporary microchannels. Then, a suitable pharmaceutical dosage form (such as gel, cream, or ointment) is applied to the previously formed microchannels[23,34].

Solid MNs should offer sufficient mechanical strength for successful skin pretreatment by selecting the materials of MNs[23]. Typically, solid MNs are fabricated from silicon[35] and metal[36,37]. It is worth noting that silicon and metal have good properties for solid MNs fabrication, but they may be unsuitable for transdermal drug delivery. Their non-biodegradable nature may cause safety issues after being inserted into the skin. In contrast, polymeric materials usually have good biocompatibility. Various polymeric materials, such as polylactic acid (PLA), polymethylmethacrylate, polycarbonate, and carboxymethylcellulose (CMC) have been developed to prepare solid MNs as an alternative to non-biodegradable metal or silicon[38–40].

Solid MNs deliver drugs by passive diffusion through the generated microchannels in the skin. Therefore, the length and density design of solid MNs used for skin pretreatment will affect drug penetration[41,42]. Moreover, the properties of the drugs also affect delivery efficiency. Contrary to the traditional transdermal delivery, the microchannels formed by the pretreatment of solid MNs will increase the penetration of hydrophilic compounds[43]. McAllister et al.[44] demonstrated that the permeation of bovine serum albumin (BSA) and insulin was increased after skin pretreatment using solid silicon MNs. The molecular weight of drugs can also affect passive transport by using solid MNs[45,46]. Verbaan et al.[46] observed that the transport rate of the larger molecular weight (72 kDa) compound was much lower than the compounds with molecular weight of 10 kDa and 538 Da.

Solid MNs have some inherent drawbacks. A two-step administration process including pretreatment with MNs array and then application of pharmaceutical preparations is considered inconvenient, and it may cause imprecise dosage[47]. Due to the negative impact on patient compliance, drug delivery strategies based on other MNs have now become more prevalent.

2.2. *Coated MNs*

To avoid a two-step application process, solid MNs are coated with drugs on the surface of the needles to obtain coated MNs. Coated MNs provide a more convenient and controllable way for transdermal drug delivery. When coated MNs are inserted, the drug coating layer will dissolve and further deposit the active pharmaceutical ingredients into the skin, then the MNs can be removed[48].

Coated MNs are typically prepared from metal or silicon. To avoid the use of less biocompatible materials, polymeric coated MNs have also been widely investigated. The solid microstructure transdermal system (sMTS) is prepared by a strong polymer, which can retain its structural integrity upon insertion into the skin[49–51]. Kapoor et al.[51] developed the coated sMTS for Peptide A delivery. Two hundred and fifty micrograms of Peptide A were coated on a patch containing 316 needles. The successful transdermal delivery was achieved with the bioavailability being similar to the subcutaneous injection. Besides, the stability of peptide A was significantly improved when coated on the sMTS[51].

Several techniques, such as spray coating, dip coating, and piezoelectric inkjet printing, were applied for the coating of MNs[52]. The spray coating and dip coating are the most common methods using an aqueous drug solution with high viscosity to retain more drugs on MNs surface. The main challenge is how to ensure sufficient therapeutic agents are uniformly coated. Therefore, it is important to optimize the coating process and formulation composition. Surfactants, viscosity enhancers, and peptide stabilizers are usually required in the formulations, to ensure coating stability and uniformity of the drugs[53]. Since most biomolecules are hydrophilic, the coating solution is usually aqueous. Zhao et al.[54] developed a coating formulation, which contained ternary co-solvents and polyvinyl alcohol 2000, for both hydrophilic and hydrophobic peptide loading with maintained bioactivity. Other methods such as layer-by-layer technique are also effective in MNs coating. In this approach, drug molecules can be coated onto MNs by alternately dipping into two solutions containing oppositely charged solutes to form a polyelectrolyte multilayers[55].

Although the mechanical strength of coated MNs is usually retained, their tip sharpness is reduced with the drug loading, which may influence the skin penetration ability[56]. Therefore, the

drug loading amount of coated MNs is compromised, which indicates that proteins and peptides with high potency are suitable for this strategy, such as desmopressin[56], human growth hormone[57], and interferon alpha[58].

2.3. Hollow MNs

Hollow MNs are sub-millimeter devices acted like micron-scale syringes, which can penetrate the stratum corneum to allow the flow of liquid formulation into the epidermis or dermis[59]. In the simplest form, drug delivery using hollow MNs is achieved through passive diffusion. Since the passive diffusion rate in dense tissues is relatively low, faster transport rate through pressure-driven flow or diffusion has been successfully achieved[21,47]. Consequently, compared with solid MNs, hollow MNs can allow the administration of larger doses, and simultaneously provide an exact transport rate[21,60–62].

The digitally controlled hollow MNs injection system (DC-hMN-iSystem) can provide accurate amount of therapeutic vaccine. Immunization study in mice showed that HPV peptide vaccine delivered through the DC-hMN-iSystem induced powerful cytotoxic and T helper response[63]. Hollow MNs-mediated intradermal delivery of nanoparticles is also an effective strategy to improve the effectiveness of vaccine. Antigen-loaded poly(D,L-lactide-glycolide) nanoparticles delivered *via* hollow MNs elicited a remarkably higher antibody response and more lymphocytes than intramuscular injection and soluble antigen delivered *via* hollow MNs[64].

Hollow MNs usually need a more complicated fabrication technology. In addition to preparing a needle with suitable inner holes, hollow MNs should also be combined with some form of drug reservoir. Hollow MNs are usually prepared from metal or silicon with different inner hole diameters, which are inherently weaker than solid MNs and have a greater risk of breakage[65].

2.4. Dissolving MNs

Dissolving MNs are usually prepared from dissolvable materials with therapeutic agents incorporated into the needles, which can effectively deliver drugs into the skin by the dissolution of needle matrix[66–68]. Many materials have been used to prepare dissolving MNs, from low molecular weight carbohydrates to high molecular biodegradable polymers, including dextran, CMC sodium, hyaluronic acid (HA), chondroitin sulfate, polyvinylpyrrolidone (PVP), and polyvinylalcohol (PVA). The use of dissolving MNs is also a one-step administration that is pretty compliant for patients. Dissolving MNs have the unique advantages that they leave no harmful material and do not generate biohazardous sharp waste after application[69–71]. In addition, the mild preparation condition of dissolving MNs makes industrialization easier to achieve, which is quite beneficial to protein and peptide drugs. The solid state of the encapsulated biomolecules can also protect them from cold chain storage and transport[72].

Various methods such as micromolding[73], drawing lithography[74], droplet-borne air blowing[75], electro-drawing[76], and photolithography[77] have been developed for fabricating dissolving MNs. Micromolding method is most widely adopted. Briefly, micromolds are filled by polymer melt or solvent casting, sometimes with the additional use of vacuum and/or centrifugal force. Then the molds are allowed to solidify or *in situ* polymerize of liquid in the microcavities[23]. It should be noted that the above-mentioned methods are usually only suitable for the small-scale preparation of MNs in academic field. For scale-up fabrication, several novel techniques have been designed to manufacture dissolving MNs in a highly effective, controllable, and scalable way[78]. The double-penetration female mold-based positive-pressure microperfusion technique was also developed by our group[79] for scale-up fabrication of dissolving MNs[79].

Heat-sensitive proteins and peptides should be encapsulated in micromolds and solidified at mild conditions that will not destroy their activity. Park et al.[80] fabricated poly-lactide-*co*-glycolide (PLGA) MNs using the micromolding method to encapsulate microparticles containing BSA and calcein. They proved the feasibility of the controlled release of calcein and BSA using polymeric MNs[80]. However, due to the use of elevated temperature in processing, protein activity had a slight loss. To address this issue, Lee et al.[69] employed milder preparation condition to fabricate dissolving MNs from ultra-low viscosity CMC with the full enzymatic activity. Similarly, erythropoietin loaded dissolving MNs were prepared using a thread-forming polymer as a base at room temperature[81].

Although dissolving MNs have significant advantages in transdermal drug delivery, it is hard to control the amount and localization of drugs within needles due to the drug diffusion from needles to base during the micromolding process, which may lead to imprecise dose and limited drug delivery efficiency[82]. To deal with this issue, Prausnitz's group[83,84] concentrated drugs in tips by incorporating an air bubble at the base of the MNs, which effectively prevented drug diffusion. The multilayered dissolving MNs are also useful to achieve controlled drug delivery[85–87]. Li et al.[88] developed a multilayered MNs patch containing an effervescent backing to facilitate rapid separation. Our group[85] also developed a rapidly separating dissolving MNs to realize precise drug delivery as well as rapid separation property. In this approach, the drugs were concentrated in the needle tip, while the blank separating part allowed rapid separation within 30 s in mimic skin[85].

The materials used as matrix for dissolving MNs should be concerned, which may affect the preparation process and the efficacy of the drug. Moreover, it should be noted that long-term use of dissolving MNs may lead to safety problems of polymer accumulation in the skin[89].

2.5. Hydrogel-forming MNs

Hydrogel-forming MNs are usually fabricated from crosslinked polymeric materials, which can pierce the stratum corneum and absorb interstitial fluid to cause the polymeric matrix swell. The drug diffusion through the swollen matrix allows for the delivery to the dermal tissue. Hydrogel-forming MNs can be removed from the skin, leaving almost no polymeric residue behind[22]. Besides, the hydrogel-forming MNs also involve a one-step application, and its drug diffusion will not be blocked by compressed skin tissue like hollow MNs[22].

Hydrogel-forming MNs usually does not contain the drug, and instead, drugs are loaded into a matching reservoir, such as a polymeric film[90]. Therefore, it is not limited by the amount of drug that can be loaded into the needle or needle surface, which significantly increases the drug amount that can permeate into the skin. Recently, other forms of hydrogel-forming MNs have also appeared, in which the drug has not been loaded separately from the needles[73,91]. Novel *in situ* hydrogel-forming MNs were also developed using biocompatible thermosensitive copolymer. Sivaraman et al.[92] utilized the transition property of poloxamer from solution at room temperature to gel at skin temperature

(32 °C) to prepare *in situ* hydrogel-forming MNs. No matter where the drug is located, the swelling degree of the hydrogel matrix plays a key role in drug delivery, and altering the crosslink density of the matrix can control release rate[93]. Hydrogel-forming MNs can also be used for diagnostic purpose through the analysis of interstitial fluid absorbed by the MNs upon insertion into the skin[94].

Hydrogel-forming MNs are fabricated by swellable materials formed by chemically or physically cross-linking polymers[95], such as crosslinked poly (methylvinylether/maleic acid) (PMVE/MA)-poly(ethylene glycol) (PEG) 10,000[96], and PVA-dextran[73]. Hydrogel-forming MNs can be regarded as a subtype of polymeric MNs where the polymers display physicochemical properties of the hydrogel[97]. Typically, micromolding method is widely employed to prepare hydrogel-forming MNs. According to the research conducted by Donnelly et al.[96], an aqueous blend containing PMVE/MA and PEG10,000 was used to produce hydrogel-forming MNs by using silicone micromold. The adhesive drug reservoir patch was prepared in advance and then attached to the needles with moderate pressure, thereby forming an integrated hydrogel MNs system. This system successfully delivered various drugs with different molecular weights, including large molecular weight proteins and peptides (insulin and BSA)[96]. Yang et al.[73] designed a phase-transition MNs system which enabled highly efficient transdermal delivery of insulin by utilizing polyvinyl alcohol as the microneedle material *via* microcrystalline cross-linking strategy. Lutton et al.[98] also designed a scalable manufacturing process for hydrogel-forming MNs, which was conducted at ambient condition utilizing a combination of injection moulding and roller casting.

Since the hydrogel-forming MNs are commonly fabricated from polymeric materials, it should be noted that their mechanical strength and physical stability are possible concerns during the application and storage process.

3. Application of MNs-mediated protein and peptide delivery

Proteins and peptides have become significant therapeutic modalities for various diseases, which continue to enter the market at a steady pace[99–101]. This can be attributed to their target specificity, high potency, and favorable safety compared with traditional small-molecule drugs. As a minimally invasive device, MNs can improve the patient's compliance and offer a multifunctional platform to overcome the skin barrier for hydrophilic and macromolecular drugs[32]. Moreover, the mild fabrication condition and solid state nature are a major advantage of MNs compared to traditional injection of the aqueous solution, which can improve drug stability and reduce the use of cold chain[80].

With the progress of material science and microfabrication technology, many MNs-mediated protein and peptide delivery strategies have been developed. Typically, MNs have been utilized to deliver various forms of cargoes, from native drugs to the nanoparticle or microparticle-based formulations[27]. In this section, we summarized the recent advances in MNs-mediated protein and peptide delivery, especially focused on their application for infectious disease therapy, diabetes therapy, and cancer therapy.

3.1. *Infectious disease therapy*

Infectious diseases such as influenza, measles, and hepatitis B are one of the main causes of human deaths, which is a major public health concern worldwide. Vaccination has been recognized as the most successful, and cost-effective public health intervention strategy to combat infectious diseases[47,102]. Compared with other antigen molecules, only proteins can induce both cellular and humoral immunity[103]. In addition, the versatility and customizability of proteins make protein-based vaccines one of the most effective strategies for artificially immunity induction[103].

Most vaccines are administered by subcutaneous or intramuscular injection, which is relatively painful, resulting in poor patient compliance[104]. There are a large number of antigen presenting cell populations in the skin, such as macrophages, dermal dendritic cells (DCs), and Langerhans cells, making the skin a unique target for immunomodulation[59,105–107]. MNs are easy to use with minimal pain, which provide a promising platform for transcutaneous immunization with improved efficacy[108–110] (Fig. 3). Over the past few decades, MNs have been developed successfully as an experimental delivery system for various protein and peptide vaccines (Table 1).

The MNs-mediated transcutaneous vaccination can effectively present antigens to skin-resident immunocyte which often enables lower dose and stronger topical immunization[143,144]. Matriano et al.[37] compared different routes of OVA (model antigen) administration, and when the protein antigen was delivered, the immune response was most efficient by using coated MNs and intradermal administration as compared to subcutaneous or intramuscular administration. Similarly, the titers of IgG in mice that received 0.5 μg of antigen with MNs were comparable or higher than those received 5 μg of antigen by intramuscular administration[137]. Dissolving MNs for influenza vaccine delivery could also improve the efficiency of virus clearance and enhance cellular recall response, compared with conventional intramuscular injection[72,129].

The key parameter of protein and peptide vaccine formulation is to maintain the stability of the vaccine component, which is crucial during the fabrication, transportation, and storage process. Appropriate formulation techniques using MNs can retain the long-term antigen immunogenicity and allow flexible storage conditions[145,146]. DeMuth et al.[127] found that the sucrose-coated MNs effectively delivered adenovirus into the skin and allowed storage at room temperature for several months without losing the biological activity of adenovirus vectors. Mistilis et al.[130] screened different dissolving MNs formulation combinations to stabilize a trivalent subunit influenza vaccine. After being stored at 25 °C for 24 months, dissolving MNs formulated by combinations of arginine/heptagluconate, sucrose/arginine, and trehalose/sucrose still retained the vaccine immunogenicity. The mice immunization experiment also proved that the antibody titer was equivalent to the fresh liquid vaccine provided by intradermal injection[130].

Many available vaccines are formulated with adjuvant[112,119,121–123]. Balmert et al.[121] used dissolving MNs to

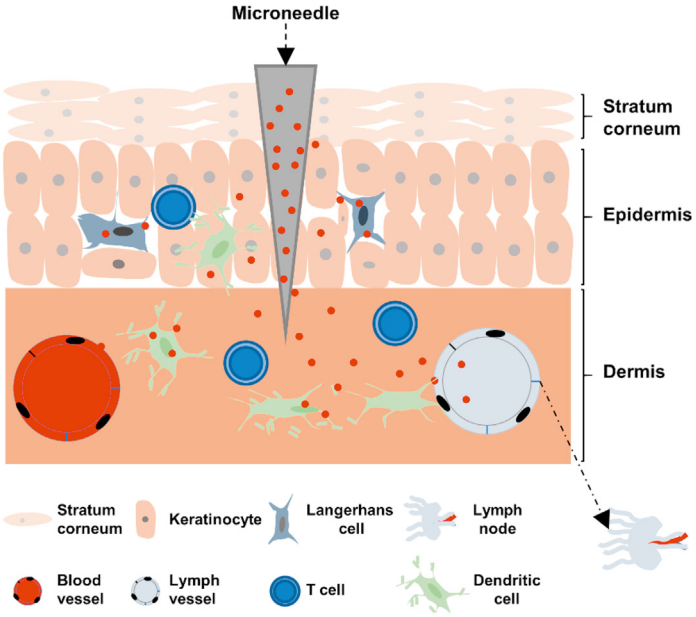

Figure 3 Mechanisms of MNs-mediated transdermal immunomodulation.

deliver OVA and Poly(I:C) adjuvant. Although the addition of Poly(I:C) showed little effect on the IgG1 response, it promoted a moderate increase in IgG2c response. Specifically, many MNs polymeric matrix materials can also be adapted as adjuvants to enhance the immune response ascribed to their intrinsic immunogenicity. For example, poly[di(carboxylatophenoxy)phosphazene] can serve as both vaccine adjuvant and fabrication material. When used in coated MNs for antigen delivery, it exhibited superior activity in pigs and significant antigen sparing potential compared to intramuscular administration[138]. It can be predicted that this will further promote the application of polymeric MNs in immunity.

OVA, a model protein with unique lymph node-targeting ability, is commonly used to assess the performance of MNs for immunization[37,111–119,121,124]. Zaric et al.[118] encapsulated OVA into PLGA nanoparticles which were then delivered to the skin by the dissolving MNs. Skin-derived DCs could deliver nanoparticles to skin draining lymph nodes through afferent lymphatic vessels, thereby inducing a potent antigen-specific immune response. Besides, PLGA nanoencapsulation maintained the stability of antigen in the dissolving MNs which further facilitated antigen retention into the skin[118]. He et al.[114] prepared a layer-by-layer coated MNs based on a synthetic pH-induced charge-invertible polymer to shorten the implantation time, which only required 60 s to implant layer by layer films *in vivo* during the insertion process (Fig. 4). The coated MNs triggered a strong immune response, and the serum OVA-specific IgG1 levels of the coated MNs group were 160 times and 9 times higher than that of the subcutaneous and intramuscular injection groups, respectively[114].

With the rapid development of nanotechnology, recently the MNs have been employed to efficiently deliver macromolecules along with nanoparticle-based therapies. The advantages of both nanoparticles and MNs can be leveraged to improve the transdermal delivery efficiency of proteins and peptides. Du et al.[112] compared intradermal delivery efficiency of four nanoparticulate vaccines using hollow MNs. Although both nanoparticles and solution aroused strong total IgG and IgG1 responses, the nanoparticles significantly increased the IgG2a response[112].

MNs-mediated transdermal immunomodulation has been mostly studied for influenza[72,126–133]. Zhu et al.[126] coated virus protein on stainless MNs and then used them to immunize mice. Four weeks after immunization, all mice immunized with virus-coated MNs survived as well as intramuscular injection, while the mice in the control group died on Day 5−8 after challenge[126]. Littauer et al.[133] demonstrated that incorporation of the thermolabile granulocyte-macrophage colony stimulating factor into the H1N1 vaccine-loaded dissolving MNs could result in improved vaccine-induced immunity, which provides a pipeline to other active recombinant molecules as adjuvants for maximized vaccination efficacy to combat with influenza. MNs-mediated transdermal immunomodulation has also been widely investigated to combat other infectious diseases, such as HIV[134,135], diarrhea[137], hepatitis B[138], plague[139], tuberculosis[140], measles[141], and leishmaniasis[142], with representative examples listed in Table 1. Especially, the recent prevalent COVID-19 coronaviruses have caused a serious threat to public health. Coronaviruses-S1 subunit vaccines are a promising immunization modality against coronaviruses infection. Kim et al.[136,147] incorporated the protein into carboxymethyl cellulose to fabricate the dissolving MNs at room temperature. All dissolving MNs vaccines elicited higher levels of neutralizing antibody, even beyond those induced by subcutaneous injection of monophosphoryl lipid A adjuvanted vaccine.

Although its efficacy and safety need further research, transdermal delivery of proteins and peptides based on MNs represents a promising strategy for combating various infectious diseases. In particular, for vaccines that require multiple administrations, transdermal MNs vaccination provides a much more convenient option.

3.2. *Diabetes therapy*

Diabetes is a chronic disease of glucose metabolism disorder characterized by abnormal accumulation of glucose in the blood[148]. Diabetes is commonly induced by the reduced insulin secretion (type 1) or the defective responsiveness of the body to insulin (type 2)[149,150]. Exogenous insulin administration is

Table 1 MNs-mediated transdermal delivery of proteins and peptides for prophylaxis of infectious diseases.

Disease	Protein/peptide drug	MNs type	MNs material	Ref.
Model	OVA	Hollow MNs	Silica	111,112
	OVA	Coated MNs	Titanium	37,113
	OVA	Coated MNs	PLLA	114
	OVA	Coated MNs	PLGA	115
	OVA	Coated and hydrogel-forming MNs	Zein	116
	OVA	Dissolving MNs	PMVE/MA	117,118
	OVA and platycodin	Dissolving MNs	HA	119
	OVA and Poly(I:C)	Dissolving MNs	CMC, trehalose	120,121
	OVA and Poly(I:C)	Dissolving MNs	PLGA and poly(acrylic acid)	122
	OVA and Poly(I:C)	Dissolving MNs	Silk and poly(acrylic acid)	123
	OVA	Dissolving and hydrogel-forming MNs	PMVE/MA, PEG, sodium carbonate/Gantrez S-97	124
	BSA and recombinant protective antigen	MicroCor™ (dissolving MNs)	PVA, trehalose, maltitol, HP-β-CD	125
Influenza	Inactivated influenza virus proteins	Coated MNs	Stainless steel	126
	Adenoviral serotype	Coated MNs	PLA	127
	Envelope protein Domain III subunit antigen	Coated MNs	Poly(L-lactic acid)	128
	Virus vaccine antigens	Dissolving MNs	PVP	72
	Virus vaccine antigens	Dissolving MNs	Trehalose and sodium CMC	129
	Influenza antigens	Dissolving MNs	Trehalose/sucrose, sucrose/arginine, and arginine/heptagluconate	130
	Hemagglutinin	Dissolving MNs	CMC sodium, ammonium acetate buffer, PVA, sucrose	131
	4M2e-tFliC fusion protein	Dissolving MNs	CMC sodium, arginine/heptagluconate, sucrose	132
	Influenza subunit vaccine and GM-CSF	Dissolving MNs	PVA, BSA, CMC, trehalose	133
HIV	Recombinant HIV-1 CN54gp140	Dissolving MNs	Gantrez® AN-139	134
	Trimer immunogen and adjuvant	Dissolving MNs	Poly(acrylic acid) and silk	135
Pneumonia	Recombinant protein subunit	Dissolving MNs	CMC	136
Diarrhea	Rotavirus vaccine	Coated MNs	Stainless steel	137
Hepatitis B	Surface antigen	Coated MNs	Titanium	138
Plague	F1 antigen	Microchannel Skin System	Plastic	139
Tuberculosis	Protein derivative	Dissolving MNs	HA	140
Measles	1000 TCID50	Dissolving MNs	Sucrose, threonine, and CMC	141
Leishmaniasis	Recombinant protein LiHyp1	Dissolving MNs	Sugar	142

indispensable for the treatment of diabetes[151,152]. Insulin, a 51-amino-acid peptide, is one of the hormones for modulating blood glucose level. However, the great pain caused by frequent and repeated subcutaneous injections adversely affects compliance with treatment[153]. In contrast, transdermal delivery of insulin is an attractive delivery method[105,154]. Introducing MNs into insulin delivery will benefit a large number of diabetic patients because it is minimal pain and easy to administer[26,155,156].

The solid MNs fabricated by different materials, such as silicon[157], metal[158], and polymer[44], have successfully reduced the blood glucose level by improving the insulin permeability through skin pretreatment. Zhou et al.[158] used stainless steel MNs with different needle lengths to evaluate the delivery efficacy of insulin to diabetic rats. The results showed that the skin's permeability to insulin increased, and blood glucose levels decreased rapidly within 1 h[158]. Besides, the integration of solid MNs with other techniques such as iontophoresis can further enhance the transdermal delivery efficiency of insulin[159,160].

Hollow MNs-mediated intradermal insulin delivery results in faster insulin onset, which can be driven by passive diffusion[161], pressure[44], or electricity[162]. McAllister et al.[44] found that hollow MNs allowed microliter of solutions to enter the skin, and a larger pressure triggered a faster decrease in blood glucose levels. Roxhed et al.[162] designed a patch system based on MNs with an electronically controlled liquid dispenser. The plasma insulin concentration of the electrically driven active administration was about 5 times higher than that of the passive diffusion group at 3 h post dosing[162].

Insulin delivery using drug-free MNs (solid MNs, hollow MNs) generally requires two or more steps, which is inconvenient for patients. The drug-loaded MNs (coated MNs, dissolving MNs, hydrogel-forming MNs) can overcome these issues[26]. Ross et al.[163] developed insulin polymeric layers coated metal MNs. The thin and homogeneous layers could retain insulin intact, and rapid insulin release was realized within 20 min, indicating that solid-state insulin delivery by coated MNs is feasible. However, further studies about insulin coated MNs are limited, which probably due to the insufficient dose of coated insulin.

Dissolving MNs encapsulated insulin in the MNs matrix are more promising due to their favorable biocompatibility, relatively simple manufacturing method, and low cost[22]. Since insulin is heat-sensitive, it is important to incorporate insulin in dissolving MNs at mild temperature. Various water-soluble polymers such as HA[164], chondroitin sulfate[12], poly-gamma-glutamic acid[165], and a mixture of starch and gelatin[166] had

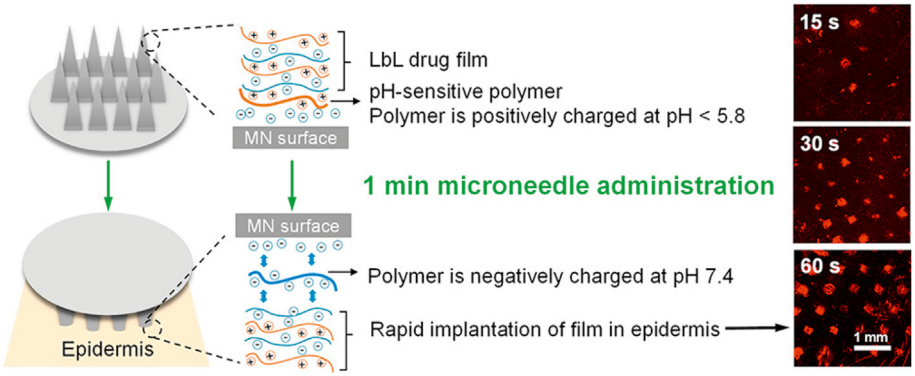

Figure 4 The implantation of layer-by-layer drug films using coated MNs for enhanced transdermal vaccination. Reprinted with permission from Ref. 114. Copyright © 2018, American Chemical Society.

been employed to prepare insulin loaded dissolving MNs at room temperature by using micromold casting method. Liu et al.[164] evaluated the ability of dissolving MNs prepared by HA to deliver insulin to diabetic rats *in vivo*. The results showed that insulin administered through dissolving MNs could effectively enter the systemic circulation, and the hypoglycemic effect was almost similar to subcutaneous injection[164].

Conventional diabetes treatment based on subcutaneous injection is usually associated with poor blood glucose control. The closed-loop drug delivery strategy can delicately control the insulin release profile in response to fluctuations in blood glucose levels, which shows great promise in the diabetes treatment. Hence, glucose-responsive MNs have been developed based on the glucose-sensing elements, such as glucose oxidase (GOx)[167–173] and phenylboronic acid[174,175]. Yu et al.[174] designed an MNs patch loaded with insulin by using a non-degradable glucose-responsive polymer. Under the hyperglycaemic condition, the polymeric matrix swelled and weakened the electrostatic interaction between the negatively charged polymers and insulin,

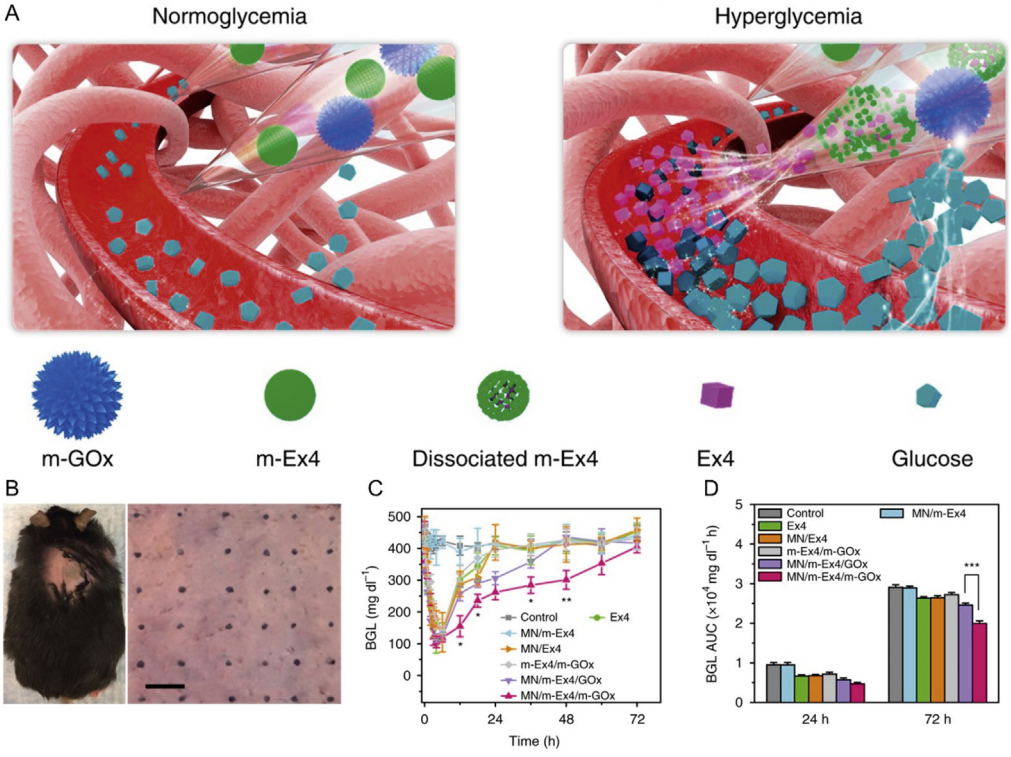

Figure 5 The MNs patch incorporated with dual mineralized microparticles for diabetes therapy. (A) Schematic of glucose-responsive Ex4 delivery mediated by MNs patch. (B) Photograph of the mouse after inserted by an MNs patch. Scale bar, 500 μm. (C) Long-term blood glucose level of mice after different treatments (mean ± SD, $n = 3$). (D) The area under the curve of blood glucose level (mean ± SD, $n = 3$). Reprinted with permission from Ref. 173. Copyright © 2017, Springer Nature.

thereby promoting the release of insulin. When exposed to euglycemic condition, the inhibited volume change and the restoration of electrostatic interaction slowed down the insulin release rate[174].

Another potential approach to combat diabetes is using glucagon-like peptide-1 receptor agonists[173,176,177]. Chen et al.[173] constructed a smart exendin-4 (Ex4), a synthetic 39-amino acid peptide, delivery platform based on MNs incorporated with dual mineralized microparticles separately containing GOx and exendin-4 (Fig. 5). The closed-loop MNs system showed excellent glucose regulation ability by the rapid specific response to hyperglycemia state, thereby significantly improving the therapeutic performance of exendin-4[173].

3.3. *Cancer therapy*

Cancer is the main concern of public health due to the widespread prevalence, high morbidity and mortality[178]. Apart from surgery, radiotherapy, and chemotherapy, immunotherapy has become an effective strategy for cancer treatment. Instead of directly killing the tumor cells, immunotherapeutic drugs are utilized to activate the body's immune system to attack the cancer cells, many of which are evaded when cancer occurs[179]. Therefore, immunotherapy is considered a promising strategy to treat or even cure certain types of cancer. The number of approved immunotherapeutic drugs has been increasing, and there are many treatments in preclinical and clinical stages. Generally, immunotherapeutic agents are mainly divided into five categories: cancer vaccines, checkpoint inhibitors, engineered T cells, lymphocyte-promoting cytokines, agonistic antibodies against co-stimulatory receptors[180], many of which are composed of proteins and peptides. In the preclinical studies, many MNs-mediated transdermal deliveries of proteins and peptides have shown promising efficacy in cancer immunotherapy (Table 2).

Therapeutic cancer vaccines represent a viable option for active immunotherapy of cancers by using a patient's own immune system, which include cell vaccines (tumor or immune cell), genetic (DNA, RNA, and viral) vaccines, and protein/peptide-based vaccines[195]. Vaccination with antigens by MNs can generate a robust antigen-specific cellular immune response. By activating antigen-specific CD8 cytotoxic T-lymphocytes, it can effectively eliminate tumors, just like the complete vaccination protection of the body in infectious diseases[118]. The immune adjuvant can be used simultaneously with the antigen or in advance, which can non-specifically enhance the body's immune response to the antigen. Kim et al.[181] utilized dissolving MNs to deliver model antigen (OVA) and immunostimulatory adjuvant (resiquimod) into lymph nodes to mature and activate antigen-presenting cells (Fig. 6). The dissolving MNs based on amphiphilic triblock copolymer could generate nanomicelles *in situ* after being dissolved in the skin, which facilitated the delivery of poorly water-soluble resiquimod. The results of antitumor immune response showed that the application of the dissolving MNs containing OVA and resiquimod to tumor-bearing mice induced a significant level of antigen-specific cellular and humoral immunity[181].

Proteins and peptides with catalytic abilities can be used as adjuvant agents for other therapeutic modalities or as anticancer drugs themselves[196]. Meanwhile, certain proteins and peptides can also work as drug delivery carriers, due to their biocompatibility and bioresorbable ability. Some cell-penetrating peptides can be combined with vaccines for immunotherapy. Ruan et al.[187] developed an siBraf delivery system based on cell-penetrating peptide octaarginine nanocomplexes combined with coated MNs for targeted anti-melanoma treatment. The results showed that octaarginine presented lower cytotoxicity than polyethyleneimine, while exhibited comparable gene transfection and silencing efficacy. The octaarginine/siBraf coated MNs could successfully pierce into the melanoma site and effectively inhibit tumor growth[187]. Duong et al.[188] developed a dissolving MNs-based polypeptide cocktail to augment cancer immunotherapy. Compared with subcutaneous vaccination, the dissolving MNs induced higher OVA-specific antibody titer and significantly inhibited OVA-expressing metastatic tumor.

The immunomodulatory antibodies can induce a powerful antitumor immune response. However, they usually generate substantial autoimmunity, leading to adverse effects[197]. Targeted and controlled release of antibodies in the desired cell types can achieve minimal off-target effects and reduce toxicity. MNs can directly accumulate sufficient immunotherapies within the topical disease site to effectively target the desired tumor and immune

Table 2 MNs-mediated transdermal delivery of proteins and peptides for cancer immunotherapy.

Therapy	Protein/peptide drug	MNs type	MNs material	Ref.
Cancer vaccine	OVA	Dissolving MNs	PMVE/MA	118
	OVA and resiquimod (R848)	Dissolving MNs	Pluronic F127/PEG	181
	Human melanoma antigens (Trp2) and adjuvant (CpG)	Coated MNs	PLLA	182
	Microparticulate ovarian cancer vaccine	AdminPen™ (Hollow MNs)	Stainless steel	183
	Whole cell lysate of B16F10 cancer cells, GM-CSF	Dissolving MNs	HA	184
	Murine breast cancer whole cell lysate	Solid MNs	Metal	185
	S-91 melanoma cancer cells vaccine antigen	Solid MNs	Dermaroller	186
	HPV E743−63 synthetic long peptide	Hollow MNs	Silica capillaries	63
Gene therapy	Octaarginine/BRAF siRNA	Coated MNs	Stainless steel	187
	Plasmid OVA and poly(I:C)	Dissolving MNs	A cationic polypeptide and PEG	188
Checkpoint inhibitors	aPD-1	Dissolving MNs	HA	189
	aPD-1	Hollow MNs	PVP/PVA	190
	aCTLA-4	Dissolving MNs	PVP	191
	aPD-1 and 1-methyl-D,L-tryptophan	Dissolving MNs	HA	192
	aCTLA-4 and zinc phthalocyanine	Dissolving MNs	HA	193
	1-Methyl-D,L-tryptophan and ICG	Dissolving MNs	HA, PVP, PVA	194

aPD-1: anti-programmed cell death protein 1 antibodies; aPD-L1: anti-programmed death-ligand 1 antibodies; aCTLA-4: anti-cytotoxic T-lymphocyte-associated protein 4 antibodies; ICG: indocyanine green.

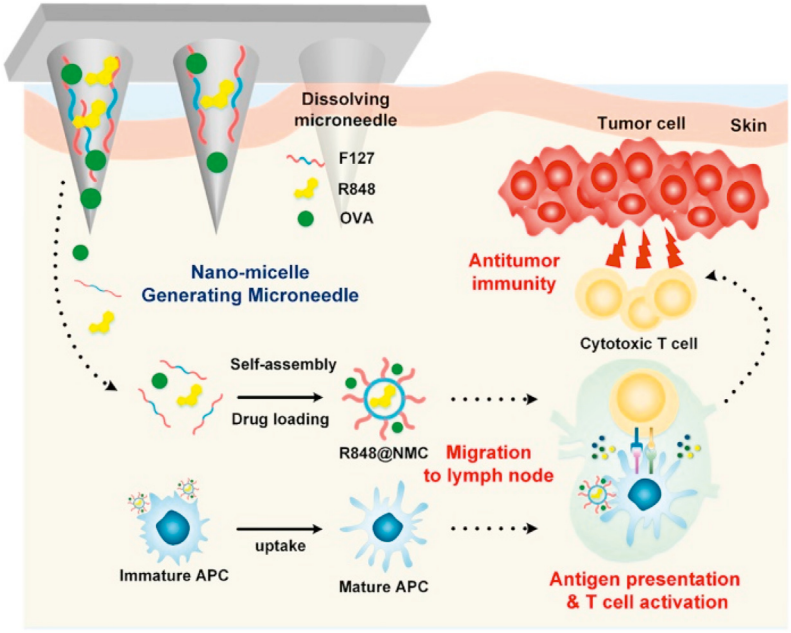

Figure 6 Enhanced cancer vaccination by *in situ* nanomicelle-generating dissolving MNs containing OVA and resiquimod (R848). Reprinted with permission from Ref. 181. Copyright © 2018, American Chemical Society.

cells. Therefore, integrating MNs with immunomodulatory antibody is promising for fighting against malignant tumors. In particular, nanoparticles-encapsulated MNs have been designed to enable controlled release of immune checkpoint inhibitors, including aPD-1/aPD-L1[189,190], aCTLA-4[191,194], and 1-methyl-D,L-tryptophan[192,194]. Wang et al.[189] developed a self-degradable MNs for the sustained delivery of aPD-1. Hyaluronic acid integrated with pH-sensitive dextran nanoparticles containing aPD-1 and GOx were formulated into MNs. The tumor acidic microenvironment promoted the sustained release of aPD-1. *In vivo* antitumor study in mice melanoma model showed that application of the self-degradable MNs induced strong immune response compared to the MNs without degradation trigger or intratumor injection of free aPD-1[189]. The MNs co-loaded with different checkpoint inhibitors resulted in the synergistic treatment of tumors[189,192]. Ye et al.[192] constructed the MNs platform to co-deliver aPD-1 and 1-methyl-D,L-tryptophan. The results demonstrated that the synergistic treatment enhanced effective T cell immunity in a B16F10 melanoma model[192].

Drug delivery based on MNs usually relies on passive diffusion, which may limit the distribution and penetration depth of the therapeutic agents. Lopez-Ramirez et al.[191] loaded magnesium particles into the MNs as a built-in engine to achieve faster and deeper intradermal drug delivery (Fig. 7). The magnesium particles could react with the interstitial fluid to quickly generate H_2 bubbles, thereby providing extremely local high fluid flow to break through the dermal barrier and enhance local payload delivery[191]. *In vivo* antitumor experiments showed that the passive MNs delivering the therapeutic aCTLA-4 initially delayed tumor growth of B16F10 melanoma. However, by day 46, all mice in this group showed exceeding tumor burden of 1500 mm^3. In sharp contrast, 60% of the mice treated with the active MNs exhibited a completely tumor-free state[191].

Immune checkpoint blockade therapy based on MNs can be combined with other cancer therapies. Besides, the activation of the skin immune system can enhance anti-cancer immunity both locally and systemically[190,194]. Chen et al.[190] developed hollow MNs that combined checkpoint inhibitor and cold atmospheric plasma. Cold atmospheric plasma induced tumor cell death, and the released tumor-associated antigens then initiated immune response. Meanwhile, aPD-L1 released from the hollow MNs patch further augmented the antitumor immunity. Immunotherapy combined with phototherapy is also used to further enhance the anti-cancer effect[190]. Chen et al.[193] designed a MNs-assisted platform for synergistic photodynamic and immunotherapy, which simultaneously encapsulated hydrophobic zinc phthalocyanine and hydrophilic aCTLA-4. In this approach, photodynamic therapy worked firstly to kill tumor and triggered the immune response, subsequently facilitated robust immunotherapy with aCTLA-4[193]. Our group[194] also designed a core–shell structure MNs to boost the immune response by combining photothermal therapy and immunotherapy. The obtained system could effectively eradicate primary melanoma tumor and inhibit metastasized tumor[194].

In addition to immunotherapy, proteins can also exert an anti-cancer effect through other therapies. For example, bevacizumab can be used to treat a variety of cancers by inhibiting tumor angiogenesis. Courtenay et al.[198] provided high dose transdermal delivery of bevacizumab using MNs, which highlighted the potential of MNs to provide sustained drug delivery to the systemic and lymph circulation. Collectively, the delivery of proteins and peptides assisted by MNs for cancer treatment is a useful strategy.

3.4. *Other disease therapy*

MNs-mediated transdermal protein and peptide delivery can also be used in other disease therapy, such as hypoglycemia[199], osteoporosis[200], cosmeceuticals[45], and wound healing[201].

The administration of insulin may cause hypoglycemia, a life-threatening condition characterized by abnormally low blood glucose level[202]. To address this issue, GhavamiNejad et al.[199] designed a smart MNs patch to specifically release glucagon at the

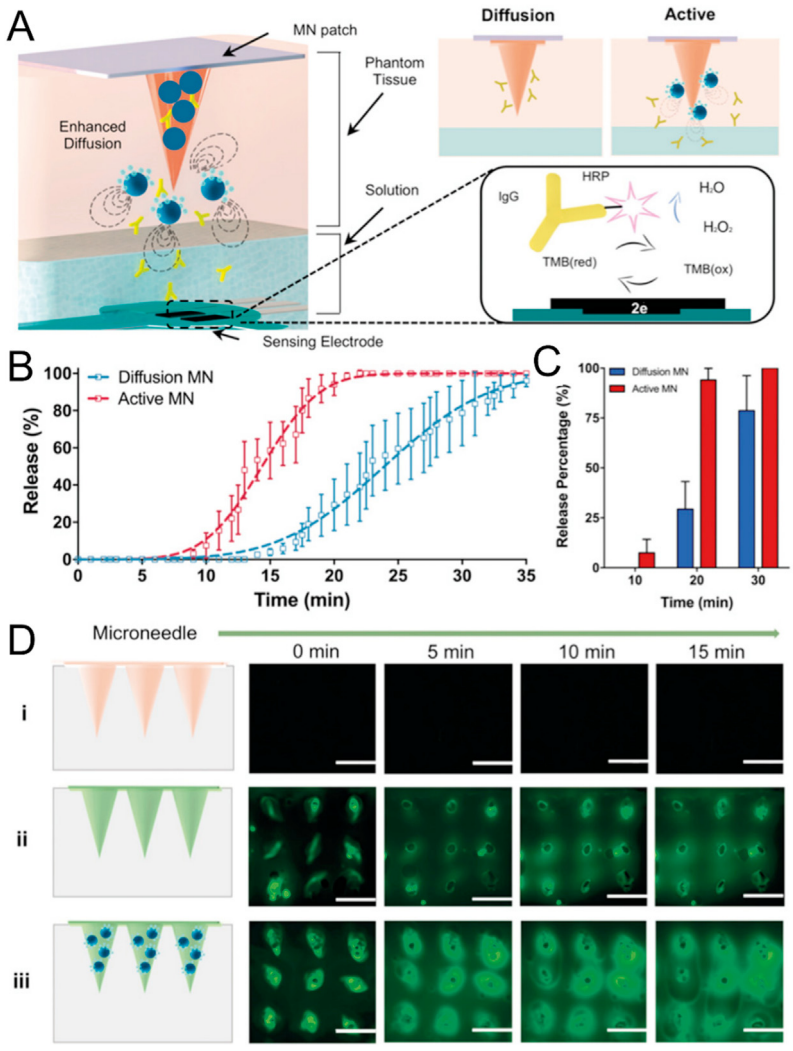

Figure 7 Built-in active MNs patch with enhanced drug delivery. (A) Schematic illustration of the design and mechanism of the active MNs patch. (B) Drug release kinetics of different MNs at pH 6.0. (C) Corresponding release percentage of aCTLA-4. (D) The fluorescence images of MNs patch obtained from top view. (i) Blank MNs, (ii) FITC-loaded MNs, and (iii) FITC-loaded active MNs. Scale bar, 1 mm. Reprinted with permission from Ref. 191. Copyright © 2019, John Wiley and Sons.

hypoglycemia condition. The MNs patch was prepared by a photo-crosslinked methacrylated hyaluronic acid embedded multifunctional microgels, which enabled hypoglycemia triggered release property (Fig. 8). In the type 1 diabetes rat model, the MNs patch successfully prevented hypoglycemia caused by insulin overdose[199].

Naito et al.[200] designed a dissolving MNs patch loaded with human parathyroid hormone to treat osteoporosis. The MNs obviously improved the stability of parathyroid hormone compared to solution. The *in vivo* study showed that the bioavailability of parathyroid hormone-loaded MNs was 100 ± 4% relative to subcutaneous injection. In a rat model of osteoporosis, parathyroid hormone-loaded MNs successfully inhibited the decrease in bone density.

Proteins and peptides play an important role in cosmetic applications. Mohammed et al.[45] investigated the effect of stainless steel MNs on the skin penetration of different chain length peptides, including melanostatin, rigin, and palmitoyl-pentapeptide. They observed that peptides with smaller molecular weight were associated with local delivery enhancement[45].

Chi et al.[201] developed vascular endothelial growth factor encapsulated chitosan MNs to promote wound healing. The drug release could be controlled *via* the temperature rise induced by the inflammation response at the wound site. The *in vitro* antibacterial test and *in vivo* wound healing study suggested that the MNs patch could promote collagen deposition, inflammatory inhibition, and tissue regeneration during wound closure[201].

4. MNs-mediated protein and peptide delivery in the clinic

As mentioned above, the fundamental research has proved the advantages and feasibility of MNs-mediated protein and peptide delivery. At present, many therapies based on MNs-mediated transdermal delivery of protein and peptide drugs have entered clinical use. As shown in Table 3, most currently active clinical trials focus on the vaccination of infectious diseases and insulin delivery for diabetes treatment. These clinical trials mainly utilized the hollow MNs infusion system, and a few investigated

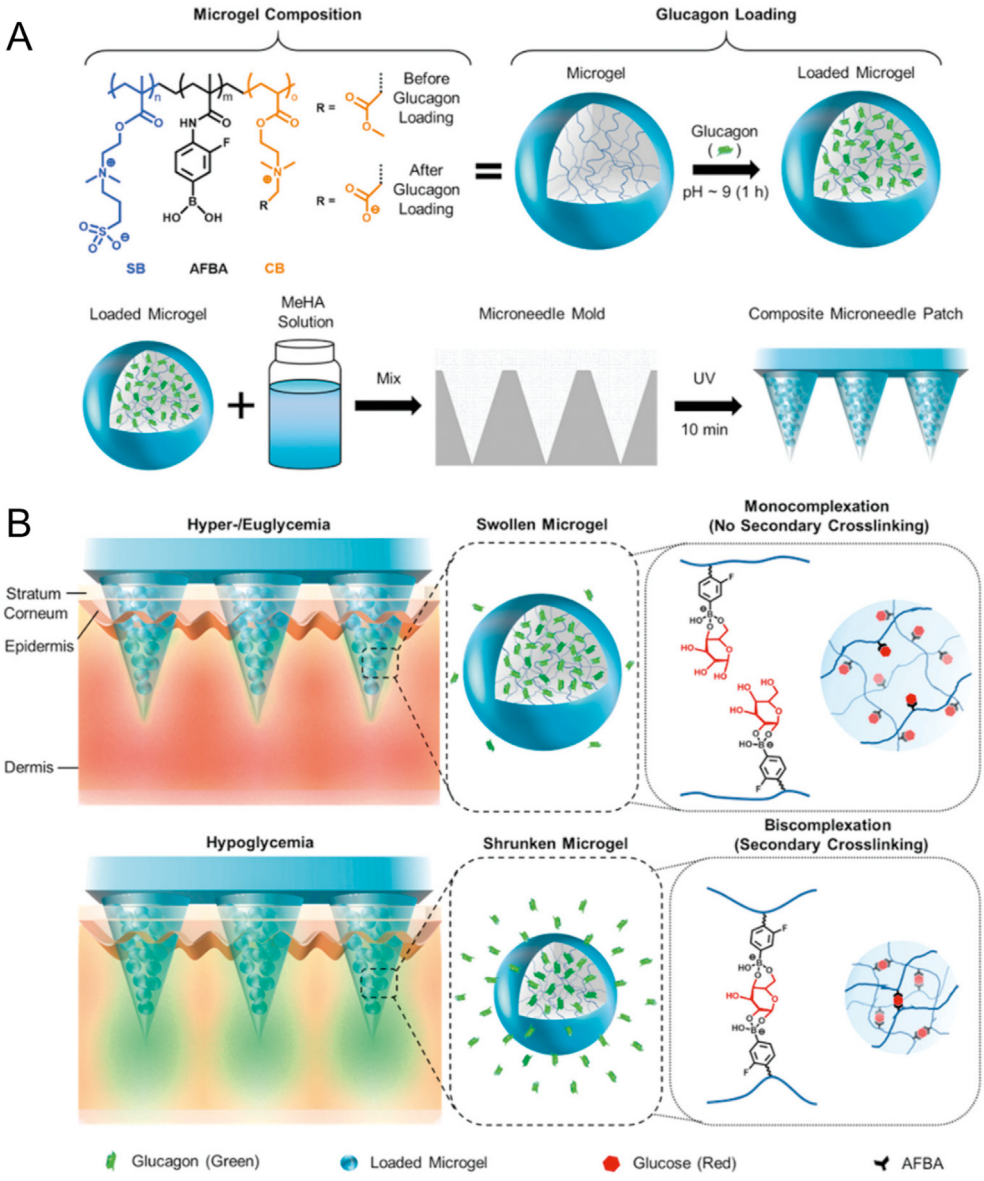

Figure 8 Schematic illustration of the controlled glucagon release from the MNs patch. (A) The fabrication process of MNs patch. (B) The mechanism of glucagon release from the MNs patch. Reprinted with permission from Ref. 199. Copyright © 2019, John Wiley and Sons.

dissolving or coated MNs. This is mainly because the research on coated MNs, dissolving MNs or hydrogel-forming MNs started later. And they usually require more sophisticated MNs design and manufacturing techniques. The interdisciplinary divide between microfabrication and pharmaceutical research also delayed the development of drug delivery[23]. At this stage, the field is at an important transitional point. More MNs products will be translated into clinical and medical practice in the near future.

5. Conclusions and prospects

Proteins and peptides have high specificity and potency compared to small molecules, which have been demonstrated to be effective for the treatment of various diseases. Nonetheless, because of the inherent properties of proteins and peptides, such as large molecular weight, poor stability, and conformational flexibility, they are usually administered by injection, which is inconvenient and unfriendly. MNs can improve the patient's compliance and overcome the skin barrier for protein and peptide drugs. MNs have been developed in several designs with different delivery strategies, which can be generally classified into solid MNs, coated MNs, hollow MNs, dissolving MNs, and hydrogel-forming MNs. Skin plays a unique role in biology and immunomodulation. The active immune environment in the skin can synergize with the MNs-mediated vaccine delivery to fight infectious diseases and treat cancers. It is also an important application for MNs in diabetes treatment, and MNs also make safer closed-loop glucose-responsive therapies possible. MNs-mediated transdermal delivery of checkpoint inhibitors has reduced their off-target effect and achieved local targeted delivery to treat superficial cancers. In short, MNs are a very promising strategy for protein and peptide delivery to treat various diseases.

The successful formulation of proteins and peptides depends on a thorough understanding of their physicochemical and biological characteristics. Notably, the formulation and handling of

Table 3 Currently active clinical trials with MNs for therapeutic protein and peptide delivery.

Condition or disease	Therapeutic agent	MNs type	CT phase	NCT identifier
Influenza	Inactivated influenza vaccine (IIV)	Dissolving MNs	1	NCT02438423
Influenza	Trivalent influenza vaccine	Hollow MNs	1/2	NCT01707602
Influenza	Intanza ®	A micro-needle injection system	4	NCT01368796
Influenza	S-OIV H1N1 vaccine	MicronJet 600 (hollow MNs)	Not applicable	NCT01049490
Influenza	Influenza vaccine (TIV 2010/2011)	Microneedle device (hollow MNs)	Not applicable	NCT01304563
Influenza	Flu vaccine	Microneedle injectors (hollow MNs)	Not applicable	NCT00558649
Healthy	H1N1 pandemic influenza	Microneedle device	Not applicable	NCT01039623
Measles and Rubella	Measles rubella vaccine	Dissolving MNs	1/2	NCT04394689
Renal Failure	HBV vaccine	A novel intradermal microneedle	2/3	NCT02621112
Varicella Zoster infection	Zostavax	A novel intradermal microneedle	2/3	NCT02329457
Atopic dermatitis	Fluzone® intradermal	An ultra-fine micro-needle	1	NCT01518478
Atopic dermatitis	Fluzone® intradermal	An ultra-fine micro-needle	Not applicable	NCT01737710
Intradermal injections	Insulin	MicronJet (hollow MNs)	1	NCT00602914
Diabetes	Insulin	Hollow MNs	1/2	NCT01061216
Diabetes	Insulin	Hollow MNs	2/3	NCT00837512
Diabetes	C19-A3 GNP peptide	Nanopass microneedles	1	NCT02837094
Diabetes	Insulin and glucagon	MicronJet (hollow MNs)	2	NCT01684956
Hypoglycemia	Glucagon	Microneedle patch system	1	NCT02459938
Postmenopausal osteoporosis	Abaloparatide	Solid microstructured transdermal system	3	NCT04064411
Postmenopausal osteoporosis	Abaloparatide	Coated transdermal microarray	2	NCT01674621
Postmenopausal osteoporosis	Zosano Pharma parathyroid hormone	Coated MNs	1	NCT02478879
Primary axillary hyperhidrosis	Botulinum toxin type A	Fractional micro-needle radiofrequency	Not applicable	NCT03054480
Poliomyelitis	Fractional IPV	MicronJet600 (hollow MNs)	3	NCT01813604
Auto-immune/auto-inflammatory diseases	Adalimumab	MicronJet600 (hollow MNs)	1/2	NCT03607903

proteins and peptides need special attention in optimizing their stability and efficacy. The researches for addressing fundamental issues including drug loading, pharmacokinetic and pharmacodynamic profile, safety, and storage of MNs will promote transdermal protein and peptide drug delivery. With the advancement already achieved in the area of microfabrication technologies available in designing MNs, more intelligent MNs systems will gradually emerge. Proteins and peptides are potent active pharmaceutical ingredients, which may break the limit of low drug loading of MNs. The comprehensive characterization methodologies, including both *in vitro* and *in vivo*, have been used to evaluate the ability of MNs to deliver drugs safely and effectively into the skin. The approaches currently used in the field will pave way to the development of standardized protocols for MNs evaluation in the future[97]. It is optimistically expected that extensive academic research in combination with the pharmaceutical industry will further accelerate the clinical translation of MNs-mediated transdermal delivery of protein and peptide drugs.

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Author contributions

Guilan Quan, Xin Pan and Chuanbin Wu conceived the review. Ting Liu wrote the manuscript with assistance of Minglong Chen, Jingtao Fu, Ying Sun and Chao Lu. Minglong Chen, Guilan Quan and Xin Pan revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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