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Nanotechnologies for the delivery of biologicals: Historical perspective and current landscape

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ABSTRACT

Biological macromolecule-based therapeutics irrupted in the pharmaceutical scene generating a great hope due to their outstanding specificity and potency. However, given their susceptibility to degradation and limited capacity to overcome biological barriers new delivery technologies had to be developed for them to reach their targets. This review aims at analyzing the historical seminal advances that shaped the development of the protein/peptide delivery field, along with the emerging technologies on the lead of the current landscape. Particularly, focus is made on technologies with a potential for transmucosal systemic delivery of protein/peptide drugs, followed by approaches for the delivery of antigens as new vaccination strategies, and formulations of biological drugs in oncology, with special emphasis on mAbs. Finally, a discussion of the key challenges the field is facing, along with an overview of prospective advances are provided.

Contents

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1. Introduction

The pharmaceutical technology field has always pursued the optimization of therapies based on the improvement of drugs bioavailability and, ultimately, their efficacy/toxicity balance. In this context, biological drugs, including proteins, peptides and monoclonal antibodies (mAbs), have occupied a prominent place due to their high specificity and potency [1]. Nonetheless, these attributes are due to their complex macromolecule structure, which is also responsible for their high susceptibility to degradation in biological environments and their limited capacity for crossing biological barriers [1,2]. Hence, the scientific community has devoted intense efforts to design drug delivery technologies intended to overcome these limitations that have hampered the widespread use of biological drugs in the practice. In particular, our group has dedicated 3 decades of research to the development of nanotechnologies that enable the transport of macromolecular drugs across biological barriers, including mucosal and epithelial barriers as well as cell barriers. From the experience that our group and many other researchers have gathered, nanotechnology has been revealed as an effective formulation approach for modulating the biodistribution, and preventing the premature degradation of biological drugs following parenteral administration. Not only that, we have reported that nanotechnology has a potential for the delivery of proteins and antigens through non-parenteral mucosal routes.

This review aims to contribute to the celebration of Professor Claus-Michael Lehr 60th anniversary, because we do believe he has enormously influenced the field of polymer nanocarriers for the delivery of a large variety of drugs, and discovered new avenues for overcoming biological barriers. We are honored to have this special opportunity to share with the ADDR readers our perspective about the historical evolution and seminal advances that have shaped the development of Nanotechnologies for the delivery of biologicals. Particularly, our attention will be first directed to the technologies with a potential for the oral and nasal systemic delivery of proteins and peptides. A second major section will analyze different approaches for the delivery of antigens as new vaccination strategies. A final section will cover the analysis of the pharmaceutical nanoformulations of biological drugs in oncology, with particular emphasis on mAbs. Needless to say, that our advances in this field have been possible thanks to the efforts of a broad scientific community (many of them listed in the references section), in which Professor C.M Lehr has taken a leading role.

2. Transmucosal delivery of protein and peptides

The growing number of protein- and peptide-based therapeutics in the pharmaceutical pipelines has prompted the search for new drug delivery routes alternative to parenteral administration, especially in the case of chronic treatments. Non-invasive transmucosal delivery offers a patient-friendly alternative with the possibility of self-administration and, hence, prone to a good treatment adherence. However, the human body is designed by nature to prevent the access of foreign entities, including drugs, and hence, has multiple biological and physiological barriers as a means of protection [3]. Among the transmucosal systemic administration modalities explored so far [4], the oral and nasal routes stand out due to their accessibility. Still, common barriers to drug delivery are faced in both routes, mainly the presence of degradative enzymes, microbiota and mucus, as well as the need to avoid the immune system, and penetrate the epithelium without compromising its integrity [5,6]. These barriers are particularly critical for macromolecules, due to their large size and complex structure. Hence, the scientific community has devoted intense efforts to allow for their transmucosal delivery, with special emphasis in nanotechnology-based tools. Specific barriers posed by either the oral or nasal route, and the most relevant technological advances to overcome them, are reviewed in the following sections, which summarize the key milestones achieved in the development of delivery technologies and emphasize those currently receiving the greatest attention.

2.1. Oral systemic delivery

This modality of administration is particularly attractive because the gastrointestinal (GI) tract offers a highly extensive area for absorption [4] and has a fast recovery against aggressions, such as chemical toxicity or mechanical injury [7]. In addition, the enteral absorption may mimic the endogenous release pathway of certain proteins, especially insulin [8,9]. Nevertheless, orally delivered formulations must confront the heterogeneous barriers derived from the digestive process, which change along the GI tract. Briefly, the stomach presents a low pH along with the presence of gastric enzymes and the thickest mucus layer of the tract [10]; the small intestine has a high activity of degradative enzymes, a complex media composition, a mucus layer and the involvement of hepatic first-pass metabolism [11]; in addition, the colon presents a

limited fluid content for dissolution along with a thick mucus layer and a high presence of bacteria and stool [12]. Finally, the influence of gut microbiota on the *in vivo* effect of formulations and vice versa, along with the impact of pathological conditions on the patient microbiome, is currently attracting increasing attention [13,14].

In addition to the biological barriers and the nature of the sites of uptake in the GI tract, there are some technological limitations for the formulation of a biological drug in an oral solid dosage. Indeed, the need of generating a powder dosage form requires a high drug loading and controlled release properties of the original nanoformulation [6,10].

2.1.1. Historical perspective of nanosystems for oral protein delivery

Soon, after the discovery of insulin in 1922 by Banting and Best, there were attempts to administer it through a needle-free route [15], which proved unsuccessful. Nonetheless, over the years there have been advances showing that peptides with certain characteristics, mainly low molecular weight (MW), lipophilicity and/or cyclic chemical structure, could be absorbed in sufficient extent so as to exert a pharmacological effect, and ultimately reached the market (Table 1) [6,16]. However, proteins and peptides of higher molecular weight and complex structures have been shown to be challenging, and demand innovative drug delivery strategies (Fig. 1). The first GI barrier to be tackled was enzymatic degradation. An initial proof-of-concept was carried out in 1927 by the administration of insulin along with blood serum as an **enzymatic inhibitor** to depancreatized dogs [17]. Years later, aprotinin was introduced in formulations in preclinical trials [18], and currently are in clinical development [6,16]. On the other hand, **emulsions** were proposed in 1968 as absorption facilitators [19], although their impact in the field of oral protein administration has been negligible. Subsequently, in 1984, surfactants and lipids were employed in the form of mixed micelles [20], and this formulation approach was followed by the introduction of paracellular and transcellular **absorption enhancers** [21–23]. An especially relevant advance was later disclosed by Morishita et al. on cell-penetrating peptides (CPPs) [24,25]. Overall, both enzymatic inhibitors and penetration enhancers were, over the years, considered key components of oral delivery systems, and currently they are essential constituents of several formulations in clinical development [6,16].

In the 1990 s, Lehr and Junginger led the introduction of functional biomaterials, such as chitosan [26], carbomer and Eudragit [27] polymers, endowed with **bioadhesive properties** [28,29] as well as with the capacity to open the tight junctions [30,31] and to inhibit enzymatic activity [30,32,33]. These biomaterials were later extensively used as core and coating materials of a variety of micro and nanocarriers.

During this same period of time, Peppas and co-workers started their contribution to the field by pioneering the design of **pH-responsive hydrogels** [34]. This approach offered the possibility to synthesize *de novo* a carrier system specifically adapted to deliver the protein of interest at the intestinal level, by selecting the monomer composition and molecular weight. Using this approach, the team developed a range of pH-responsive micro- [35] and nanocarriers [36] for several protein-based therapeutics [35–39] over the years.

Nanocarriers initially emerged in the field in 1976 [40], when liposomes were proposed to enable oral systemic absorption of insulin in rats. Subsequently, in the 1980 s, Couvreur's team, pioneered the application of polyalkylcyanoacrylate (PACA) nanocapsules (NCs) for the oral administration of insulin [41,42]. These initial works opened a new field of nanotechnology applied to oral protein delivery, which is still evolving. Nanoparticles (NPs) made of poly(lactide acid)- (PLA) and poly(lactide-co-glycolic acid)-(PLGA) based nanoparticles (NPs), introduced by our group [43] and others [44] in 2000, showed the possibility of facilitating protein absorption. A further modification to the PLGA NPs core was also proposed by our lab [45], comprising a PLGA:poloxamer/poloxamine blend that could reduce the interaction with enzymes and improve colloidal stability. During that same period of time, our team proposed the use of Chitosan (CS)-based NPs and NCs as a way to improve the intestinal absorption of salmon calcitonin and insulin [46–49], which brought together the benefits of a lipid-based system, a polymeric shell and the possibility of incorporating additional functional excipients. Later, different modifications of chitosan, i.e. thiolated and timethyl chitosan were also proposed based on their enhanced stability and mucoadhesiveness [50,51]. Finally, solid lipid nanoparticles (SLN), with a hydrophobic structure based on natural and GRAS excipients [52], were introduced in the oral drug delivery field by our group [53]. Overall, the main challenges commonly faced by nanocarriers at the very early stages of development have been ensuring an appropriate protein/peptide loading, while preserving its stability and a subsequent controlled release. Meeting these requirements has been challenging due to the high molecular weight, hydrophilicity and susceptibility to degradation of these drug

Subsequent developments in the field revealed that the size and surface properties of the nanostructures could be conveniently tuned to modulate their interaction with the intestinal epithelium. Overall, small size values and neutral surface charge were considered more convenient for absorption through the enterocytes [54]. Further optimization of the nanoparticle surface led to decorate the surface of nanocarriers with ligands that would bound to specific receptors in the epithelium, when aiming at receptor-mediated transport [55,56]. Especially relevant targeting moieties studied include lectins, which targeted the glycosylated domains of cell surface components and were implemented in liposomes [57] and SLN [58]; vitamin B12, targeting the corresponding uptake pathway [59]; folate, targeting the folic acid GI receptors [60]; mannosamine, proposed by our group for the targeting of M cells [61]; and Fc, targeting the neonatal Fc receptor [62].

Coating of the nanoparticle surface with polymers has also been explored as a means to tune the interaction with the epithelium and the mucus layer. For example, coating with chitosan was extensively applied to liposomes [63], PLGA NPs [64], cyclodextrins [65] and SLN [66], to exploit its mucoadhesive and permeation properties. A TMC derivative was also investigated as a coating agent [51]. Last but not least, coating with polyethylene glycol (PEG), or "PEGylation", also established its place in the field. Our group introduced it first in 2000 on PLGA NPs for improved colloidal stability and enzymatic protection [43]. Years later, it was chosen as the gold standard to render nanoparticles mucodiffusive. The extensive works from the Hanes Lab contributed to identify the key parameters to modulate the mucoadhesive/mucodiffusive role of PEGylation, strongly related to PEG MW, and the extent and configuration of nanoparticle surface coverage [67,68].

2.1.2. Seminal work/current advances

Over the last few years, we have contributed to the subsequent development of the nanoparticles design and composition, and helped make them dynamic delivery systems with combined functionalities. Below, we present recent seminal advances and disruptive technologies of high relevance in the field of oral delivery.

2.1.2.1. Nanocarriers.

2.1.2.1.1. Polymeric nanocarriers. **PLGA NPs** have been functionalized with several moieties in order to target molecules of interest on the surface of the intestinal cells. Among others, the EGP peptide was linked to the surface of PLGA NPs for the targeting of heparan sulfate proteoglycans (HSPGs), aiming at a transcytosis

Table 1
Technologies marketed or in clinical trials for peptide/protein oral transmucosal delivery.

Company - Technology/Product	Indication	Protein/ Peptide	Strategy	Phase	ClinicalTrial.gov Identifier
Novartis AG (Switzerland) Neoral®/ Sandimmune®	Immunosuppression	CsA	Self-emulsifying Drug Delivery Systems (SNEDDS)	Marketed	–
Ferring Pharmaceuticals (Switzerland)/ Generic products (e.g. Actavis Labs FL Inc., NJ, USA) DDAVP® Tablets DDAVP® Melt Minirin®	Central *Diabetes Insipidus*, Primary Nocturnal Enuresis	Desmopressin acetate hydrate (DDAVP)	Chemical modification	Marketed	–
Mitsubishi Tanabe Pharma Corporation (Japan) Ceredist® Ceredist OD®	Spinocerebellar degeneration	Taltirelin hydrate	Chemical modification to avoid enzymatic hydrolysis	Marketed	–
Theranaturals Inc. (ID, USA) Reduced L-Glutathione	AIDS-related cachexia/cystic fibrosis	Glutathione	None	Marketed	–
Emisphere Technologies, Inc. (NJ, USA) with Novo-Nordisk (Denmark) Rybelsus®/Eligen®NN9924/ OG217SC	Diabetes	Semaglutide (long-acting GLP-1)	PE: Sodium N-[8-(2-hydroxybenzoyl) Amino] Caprylate (SNAC)	Marketed	
NOD Pharmaceuticals, Inc. (China) NOD/NodlinTM	Diabetes	Insulin	Nanoparticles with a calcium phosphate core and pegylated salts of fatty acids, coated with carbomer and cellulose acetate phthalate	I	ChiCTR-TRC-12001872*
Oshadi Drug Administration Ltd. (Israel) Oshadi Icp	Diabetes	Insulin	Silica-based nanoparticles	II	NCT01973920
Diasome Pharma (OH, USA) HDV-I	Diabetes	Insulin	Liver-targeted liposomes	III	NCT00814294
Merrion Pharmaceuticals Ltd. (Ireland) with Novo Nordisk A/S (Denmark) Insulin 320 (NN1957)	Diabetes	Insulin	PE: sodium caprate	I	NCT02479022
Insulin 338/ GIPET® I/ OI338GT (NN 1953)	Diabetes	Insulin		II	NCT02470039
NNC0113-0987 (NN9926)/ OI338GT	Diabetes	GLP-1 analog		I	NCT02094521
GIPET®/ ACY-7/ MER-104	Prostate cancer, male oral contraception	Acyline		I/II	NCT00603187
Oramed Pharmaceuticals, Inc. (Israel) POD™/ ORMD 0801	Diabetes	Insulin	PEs: EDTA, bile salts Enzyme inhibitors: soy bean trypsin inhibitor, aprotinin	III	NCT04606576
Proxima Concepts Ltd (UK)/ Bone Medical Ltd (Australia)/ Diabetology (UK) Axess™ / Capsulin™/	Diabetes	Insulin	PE: aromatic alcohols	II	2005–004753-95**
Capsitonin™ (BN002)/	Osteoporosis	sCT		III	N/A
CaPTHymone™ (BN003)/ Perthoxal™	Osteoporosis	PTH		II	N/A
Enteris Biopharma, Inc. (NJ, USA) Peptelligence™	Endometriosis	Ovarest® (oral leuprolide tablet)	PE. Acyl carnitine/ pH modulator, CA/ Peptide with D-stereochemistry resistant to proteases (Cara)	II	NCT02807363
	Chronic Kidney Disease (CKD) associated pruritus, chronic pain	KORSUVA™ (CR845/ difelikefalin)		II	NCT03617536 NCT02524197 NCT02944448 NCT04706975
Emisphere Technologies, Inc. (NJ, USA) with Novo-Nordisk (Denmark) Eligen®/ Novo insulin candidate	Diabetes	Insulin	PE: N-acylated alpha-amino acid (undisclosed)	I	NCT00982254
Emisphere Technologies, Inc. (NJ, USA) with Nordic Biosciences (Denmark) and Novartis (Switzerland) Eligen®	Osteoporosis	sCT (SMC021)	PE: 8-(N-2-hydroxy-5-chlorobenzoyl)-amino-caprylic acid (5-CNAC)	III	NCT00525798 NCT00486434 NCT00704847
Sigmoid Pharma (Ireland) SmPill®/ CyCol™	Immunosuppression	CsA	Oil in water emulsion	I/II	NCT01033305
Tarsa therapeutics, Inc. (PA, USA)/ Enteris Biopharma, Inc./ R-PHARM JSC PeptelligenceTM/ TBRIATM	Osteoporosis	sCT	Local pH modulator: CA	NDA approved for review (2016)	
Chiasma, Ltd. (Israel) TPE®/ MycapssaTM	Acromegaly	Octreotide	PE: sodium caprylate	III	NCT03252353 NCT02685709 NCT01412424

Table 1 (*continued*)

Company - Technology/Product	Indication	Protein/ Peptide	Strategy	Phase	ClinicalTrial.gov Identifier
Biocon Ltd (India) IN-105/ Insulin Tregopil	Diabetes	Insulin-alkylated PEG prodrug insulin conjugates	Chemical modification	II/III (did not meet endpoints)	NCT03430856
RANI Therapuetics RaniPill	Acromegaly	Octreotide	Microneedle capsule	I	NCT03798912

PE: penetration enhancer; CsA cyclosporine; sCT salmon calcitonin;. *Chinese Clinical Trial Registry (www.chictr.org.con); **EU Clinical Trials Register (www.clinicaltrial-sregister.eu). Adapted with permission [6].

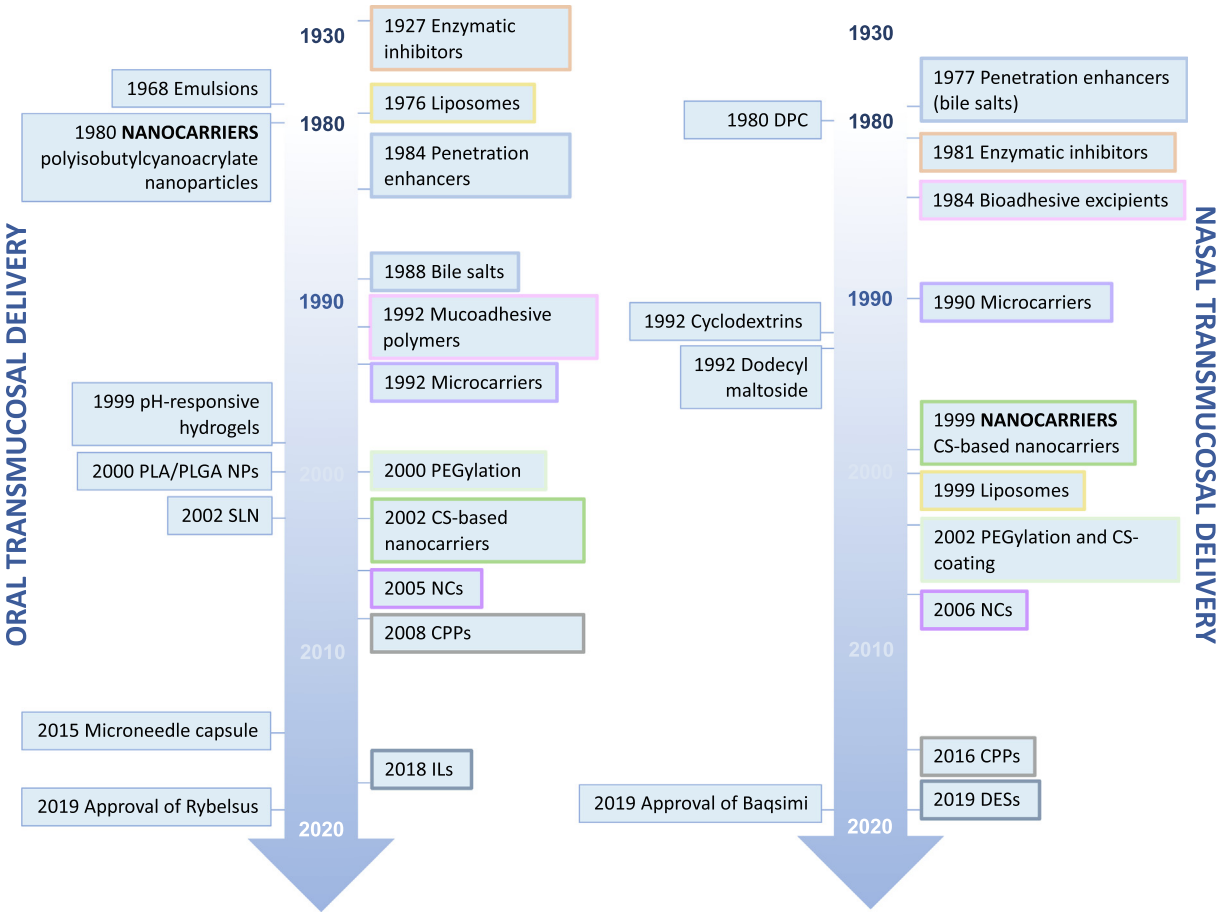

Fig. 1. Timeline of the introduction of seminal advances in drug delivery technologies in the protein/peptide transmucosal oral and nasal delivery fields. Color code highlights those technologies employed in both fields (right side of each panel), introduced at different times. PLA polylactic acid; PLGA poly(lactic-co-glycolide) acid; SLN Solid Lipid Nanoparticles; CS chitosan; NCs Nanocapsules; CPPs Cell Penetrating Peptides; ILs Ionic Liquids; DPC dodecylphosphocoline; DESs Deep Eutectic Solvents.

pathway [69]. Also, functionalization with gambogic acid (GA) was proposed for the targeting of the transferrin-transferrin receptor complex (Tf-TfR) [70]. Alternative approaches have been based on an enzymatically responsive behavior. This was the case of NPs consisting of the octa-arginine (R8) peptide surrounded by a phosphoserine (PhO) layer [71]. In this later design, the negatively charged PhO residues would allow the NPs to navigate through the mucus and, upon hydrolysis by the intestinal alkaline phosphatase (IAP) at the brush border, the positively charged R8 residues would be available for improved cell uptake.

Chitosan-based nanosystems have also maintained their presence in the field, either in the form of modified CS NPs or as a coating material. Novel modifications include the conjugation of L-valine (LV) as a ligand for oligopeptide transporters together with phenylboronic acid (PBA) to trigger glucose-responsive insulin release in the cytoplasm [72]. In a different example, TMC NPs were functionalized with the peptide CRTIGPSVC (CRT) for the targeting of the Tf/TfR complex. The resulting NPs exhibited a 2-fold bioavailability increase compared to the control (TfR-targeting HAIYPRH peptide) [73]. Similarly, the linkage of deoxycholic acid to CS NPs in order to target the apical sodium-dependent bile acid transporter (ASBT) allowed a 2.2-fold increase in bioavailability compared to non-modified nanoparticles [74]. Alternatively, the non-covalent surface coating of TMC NPs with thiolated hyaluronic acid (HA-SH) was proposed to enable mucodiffusion, and subsequent TMC interaction with cells upon HA-SH detachment, achieving a 1.9-fold bioavailability increase compared to their non-coated counterparts [75]. Last but not least, coating with chitosan continued to prove a valuable approach to improve the performance of new nanocarrier designs. For instance, chitosan

coating of zein-carboxymethylated short-chain amylose nanocomposites led to a 15.19% bioavailability increase compared to 11.01% for non-coated nanoparticles [76].

2.1.2.1.2. Lipid nanocarriers. **Liposomes** have also been endowed with new functionalities. For example, chondroitin sulfate-g-glycocholic acid- coated exendin-4 (Ex-4)-loaded liposomes (EL-CSG) were designed to target the bile salt pathway [77]. Another interesting approach was based on the formation of a bovine serum albumin (BSA) protein corona on cationic liposomes (CLs) containing DOTAP [78]. In this case, the hydrophilic protein corona was formed around the liposomes to facilitate their mucodiffusion, while finally exposing the cationic DOTAP residues for cell interaction upon BSA enzymatic degradation, which allowed a 10-fold increase of bioavailability compared to plain CLs.

Lipid-based nanosystems, mainly **lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and nanocapsules (NCs),** continue to hold a consolidated place in the field. They incorporate new functional components and/or coatings for improved performance. For instance, it is worth mentioning that the inclusion of an endosomal scape agent (peptide GLFEAIEGFIENGWEGMIDGWYG (HA2)), aimed at minimizing the lyso-endosomal degradation of the encapsulated peptide in endothelial cells, in SLN allowed a 1.6-fold increase in bioavailability [79]. On the other hand, polymeric lipid-based NCs, including poly(arginine) (pArg) [80], CS [81] and polysialic acid/protamine NCs [82] were recently proposed for enhancing the intestinal absorption of peptides. Finally, Préat et al. investigated the capacity of nanostructured lipid carriers (NLC) [83] and reverse micelle-loaded lipid nanocapsules (RM-LNC) [84] to induce endogenous GLP-1 secretion from enterocytes, enabling a synergistic effect when GLP-1 analogs were delivered from the carriers. In addition, the same group investigated PEGylated RM-LNC, with improved mucodiffusion, and propionate-decorated PEGylated RM-LNC, with the capacity to interact with G protein-coupled receptors (GPCRs) and activate GLP-1 secretion [85]. Although the introduction of the propionate grafting did not exert a significant improvement vs. the plain formulation, PEGylated RM-LNC increased GLP-1 endogenous levels up to 8-fold in normoglycemic mice, and prolonged the antidiabetic effect in obese/diabetic mice following long term (1 month) treatment.

2.1.2.1.3. Silica-based nanoparticles. Silica NPs have also been explored in the area of oral protein delivery. Specifically, Brayden et al. investigated silica-coated nanoparticles (SiNPs) with a core containing zinc and L-arg to encapsulate either insulin or exenatide [86]. While the performance of the carrier was dependent on which peptide they were associated with, the presence of L-arg proved to be critical as a permeation enhancer, while the nanoparticle itself was shown to provide a controlled release of the peptides. Different data were recently reported by Whitehead et al. This group co-administered commercial SiNPs along with insulin and exenatide, and obtained up to 35% oral bioavailability in mice. The authors concluded that the NPs exerted a permeation enhancing effect through tight junction opening, due to binding to integrins, which facilitated the transport of the added peptides [87]. The potential toxicity associated with this extraordinary opening of the tight junctions upon chronic administration remains to be studied.

2.1.2.1.4. Bioinspired nanosystems. Nanocarriers based on novel chemical entities rationally designed for overcoming specific barriers also occupy an important place in the oral delivery scenario. For example, zwitterionic micelles mimicking virus surface for mucus penetration and targeting of the proton-assisted amino acid transporter 1 (PAT1) were reported to promote insulin absorption [88]. Specifically, the micelles were based on the assembly of a 5 kDa polycarboxybetaine (PCB)-lipid derivative with insulin and Zn^{2+}. The PCB chain presented a neutral net charge, providing improved mucodiffusive properties to the nanostructures while, at the same time, interacting with the PAT1 transporter. The Zn^{2+} molecules enabled a sustained release of insulin. Overall, the freeze-dried micelles loaded into an enteric capsule achieved up to a 43% bioavalability in diabetic rats.

2.1.2.2. Self-dispersing ionic liquids-based nanostructures. Ionic liquids (ILs) have been explored for the oral administration of macromolecules by Mitragotri's lab [89]. ILs are composed of polar organic solvents containing an organic cation and an organic/inorganic anion, presenting a melting point below 100 °C. Often, they are also considered Deep Eutectic Solvents (DESs), although some authors differentiate them based on the nature of the interactions between their components (i.e. ionic or hydrogen bonds) [90]. The composition assayed in this case consisted of a mixture of choline and geranate (CAGE) with insulin, which upon dilution with intestinal fluids generate micelles and microemulsions. A combination of several mechanisms for promoting absorption was attributed to the system, including i) stabilization of the protein; ii) thinning of the mucus layer and iii) paracellular permeation. Ultimately, CAGE administration led to insulin absorption *in vivo*, attaining blood glucose decrease with doses as low as 3 IU/kg. More recently, the same authors presented a choline and glycolate IL and DES for the delivery of anti- TNFα IgG [91], which achieved both local and systemic delivery (up to 5-fold higher plasma levels than IgG alone) upon intrajejunal injection in rats. The improved absorption mechanism was attributed to reduced mucus viscosity along with the opening of tight junctions.

2.1.2.3. Microneedle-based devices. The needles-based design reported by Traverso et al. in 2015 [92] was the predecessor of recently reported prototypes, consisting of a central metallic core with hollow 25G needles protruding in radial fashion, covered by a pH-sensitive coating (Fig. 2). Briefly, the enteric coating protects the needles until they reach the intestine, where they will be exposed and then penetrate into the tissue due to peristaltic movements, subsequently releasing the drug at the submucosal level. A proof-of-concept was presented, confirming the generation of hypoglycemia in pigs after individual injections to the intestinal mucosa, and testing the safety of the device upon passage through the GI tract. Following this breakthrough report, Rani Therapeutics presented the robotic pill as an alternative design (https://www.ranitherapeutics.com). The robotic pill consisted of a balloon-like structure supporting hollow microneedles containing the peptide, along with a separate pH sensitive chamber containing sodium bicarbonate and citric acid. Once in the intestine, the acid-base reaction produces CO_2 causing the balloon to inflate, which subsequently drives the microneedles into the intestinal tissue for peptide release [93,94]**.** Preliminary proof-of-concept studies in swine to whom the robotic pill was directly implanted into the jejunal cavity by enterotomy yielded ~ 100% bioavailability [95]. In addition, pilot *in vivo* safety studies focused on the GI transit of the device in dogs and humans were successfully carried out [95], leading to a Phase I clinical trial (NCT03798912, see next section).

More recently, Traverso et al. reported the subsequent development of the initial microneedle capsule concept. One of their designs, the self-orienting millimeter-scale applicator (SOMA), consists of a device reproducing the key morphological aspects of the tortoise's shell, which contains a millipost of insulin connected to a compressed spring fixed with caramelized sucrose (Fig. 2) [96]. Once in the stomach, the SOMA self-orients with its bottom part enclosing the millipost in contact with the mucosa; then, the sugar cap retaining the spring dissolves, thus allowing the injection of the peptide millipost into the mucosa. Following oral administration of this device to swine, it was found that the blood glucose and plasma insulin levels were similar to those obtained upon sub-

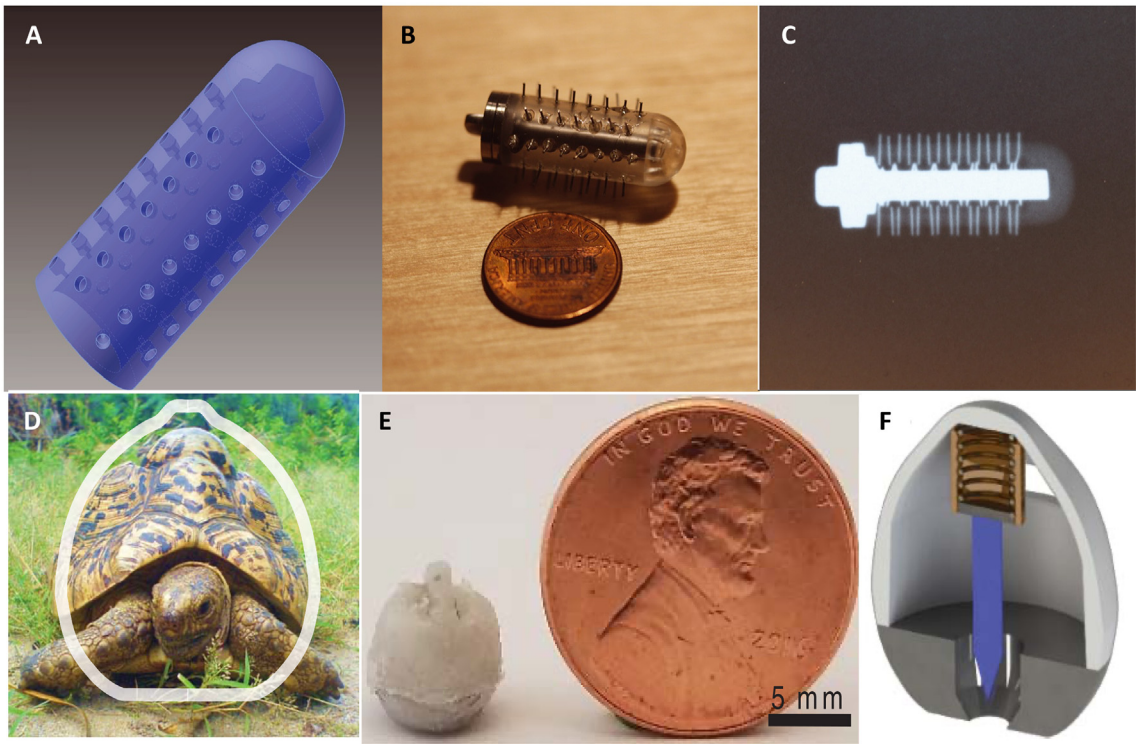

Fig. 2. Images of the initial (**A-C**) and an evolved design (SOMA) (**D-F**) of the microneedle capsule, reproduced with permission from [92,96]. (**A**) Computer-aided design of the initial cylindrical radial prototype. (**B**) Finished cylindrical radial prototype showing a metal endcap and pin. (**C**) Radiography of the prototype in (**B**). (**D**) A comparison between the shape of the leopard tortoise (S. pardalis), which inspired the SOMA device, and that of the device itself. The device orients in the stomach environment and remains stable once reached its preferred orientation. (**E**) A fabricated SOMA. (**F**) Depiction of the SOMA internal mechanism, enclosing a compressed spring fixed in caramelized sucrose (brown) that provides the force for the insertion of the drug-loaded millipost (blue). The spring remains encapsulated within the device after actuation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cutaneous (SC) administrations. Finally, the most recent design from Traverso's lab is the luminal unfolding microneedle injector (LUMI) [97]. The LUMI capsule was coated with a pH sensitive polymer and contained three biodegradable arms, each of them bore a drug-loaded microneedles patch at one end, and was connected to a spring steel core coated with PEG at the other end. Once the capsule reaches the intestine and after the dissolution of the enteric coating, the intestinal fluids dissolve the PEG spring coating, subsequently releasing the spring that propels the arms out of the capsule and, thus, the microneedle injects itself into the mucosa. After this, the capsule breaks apart into fenestrated pieces to allow for their safe passage through the intestine. Such a device led to insulin absolute bioavailability values higher than 10% in swine.

These disrupting prototypes clearly made an impact in the field. Their translation to the clinic may follow after addressing issues related to the manufacturing, toxicological and regulatory evaluation, and controlled performance in a clinical setting. These systems may not represent the ideal solution for peptide drugs needing a precise and responsive daily dosing, such as insulin. However, they might open new avenues for acute treatments.

2.1.2.4. Cells-based therapies. Finally, the use of freeze-dried plant cells as carriers expressing a protein/peptide of interest is gaining a presence in the field. This technology had previously reached FDA approval (Elelyso®) and Phase II-III clinical trials for local delivery of several protein and peptide drugs [6], and was recently applied for their systemic delivery [98,99]. Briefly, genetically-modified plant cells expressing the recombinant protein/peptide of interest are provided with a cellulose wall that protect them from acidic pH. As gut bacteria progressively degrade this cell wall, the protein/peptide is released. Based on this strategy, freeze-dried lettuce chloroplasts expressing an insulin-like growth factor (pro-IGF-1) modified with a CPP (e-peptide) promoted bone regeneration in femoral fractured diabetic mice upon oral administration [99]. Similarly, lettuce chloroplasts expressing Angiotensin Converting Enzyme 2 (ACE2) and angiotensin led to improved plasma levels of the proteins and attenuated pulmonary arterial hypertension (PAH) in a PAH rat model after oral gavage [98].

2.1.3. Note on approved/clinical trials products

An updated summary of the systemic peptide oral delivery technologies in clinical trials can be found in Table 1. The majority of these developments were already described in our previous review [6]. Overall, marketed technologies consist of peptides chemically modified for improved stability and Self-emulsifying Drug Delivery Systems (SEDDSs). Current formulations in Phase III rely on penetration enhancers, in combination or not with enzyme inhibitors, liver targeted liposomes and pro-drug chemical modifications, whereas technologies in Phase II include penetration enhancers, SiNPs and oil-in-water emulsions. Finally, calcium phosphate NPs and other penetration enhancers-based formulations are in Phase I clinical trials.

One of the most prominent advances in the field, recently marketed, is represented by Rybelsus®, an oral semaglutide formulation containing the absorption enhancer sodium N-[8-(2-hydroxybenzoyl) aminocaprylate] (SNAC) [6,100]. SNAC was described to allow for selective semaglutide absorption in the stomach due to buffering capacities that confer protection against acidic and enzymatic degradation, along with fluidification of the epithelial cells membrane, enabling transcellular transport of the peptide. Importantly, this absorption mechanism proved to be compound-specific, since the effect decreased markedly when any of the components involved was interchanged by an analog.

A totally different product entering Phase I clinical trial is the Rani's robotic pill (RaniPill™) loaded with octreotide, previously discussed.

2.2. Nasal systemic delivery

Interestingly, nasal administration offers a direct access to a highly vascularized epithelium with relatively high permeability for drug absorption. As opposed to oral administration, the nasal route presents distinctive advantages for transmucosal delivery, such as the avoidance of gastric degradation and hepatic first-pass metabolism, lower enzymatic activity than the GI tract [4,101], and a rapid onset of action that is comparable to the one obtained after parenteral administration [102]. However, other specific barriers come into play when addressing nasal administration. Among others, the fact that the nasal mucosa presents a certain sensitivity to irritation, the mucociliary clearance mechanism [103] and a low surface area available for delivery [4]. In this regard, recent studies suggested an optimal site for drug deposition for adequate absorption [101,102]. In addition, specific technological challenges for nasal administration include a limited dosing volume, and the need for a specific manipulation of the powders or liquid sprays by the patients [104].

2.2.1. Historical perspective of nanosystems for nasal protein delivery

As in the case of the oral route, small peptides, such as salmon calcitonin, have been administered by the nasal route since 1985. However, the delivery of large macromolecules has proved to be highly challenging [103]. (Fig. 1). In fact, advances in this field have been quite delayed as compared to those related to the oral modality of administration. To the best of our knowledge, the first co-administration of a peptide with an adjuvant dates from 1977 [105] for the nasal route vs. 1927 for the oral route [17], and the use of a nanocarrier was introduced in 1992 [106] in nasal administration vs. 1976 for oral administration [40]. This scenario is logical and based on tradition. It takes time and evidence and practice for patients and doctors to accept a disruptive change in the drug administration protocols. Irrespective of this, the nasal drug delivery field has grown slowly and has become particularly active upon recognition of the potential of nose-to-brain (N-t-B) administration. Readers are encouraged to review the work of Samaridou et al. [107] for further information on the topic.

Penetration enhancers, i.e. sodium glycocholate, were the first functional excipients explored for nasal systemic absorption of insulin, in 1977 [105]. Subsequently, several surfactants, including saponin, sodium glycocholate and polyoxyethylene-9-lauryl ether (BL-9), were assayed in a dog model in 1978 [108], and a 25–30% insulin bioavailability was achieved. Although none of these formulations reached the market, another one containing dodecylphosphocoline (DPC) and glucagon [109,110], was probably the basis for the subsequent development of the glucagon nasal formulation Baqsimi® approved by the FDA in 2019, which in addition to DPC also contain cyclodextrins.

Cyclodextrins were introduced in 1992 [106], when their efficacy for insulin nasal absorption was correlated with the particular cyclodextrin structure and the release of the nasal membrane phospholipid. Three years later, they were combined with the penetration enhancer oleic acid and were shown to achieve increased nasal bioavailability of buserelin in rats [111]. In the 2000 s, our research team introduced a hybrid chitosan-cyclodextrin carrier [112,113], which achieved up to 35% plasma glucose level decrease after nasal administration in rabbits.

Also in 1992, several alkyl saccharides were tested as absorption enhancers in rat rectum [114]. From this chemical family, the compound dodecyl maltoside, a non-ionic penetration enhancer [115], was introduced in the Intravail™ technology, now in clinical trials for the nasal administration of octreotide (NCT03031535). Later, in the 2000 s, the excipient cyclopenta decalactone (CPE-215), a natural compound previously employed in several food products and cosmetics, was revisited as absorption enhancer [116], and was a main component of the Nasulin™ technology. Its mechanism was attributed to a fast, temporary, and reversible phase separation of cells membrane at the target tissue [117]. The technology reached Phase II clinical trials for nasal insulin administration (NCT00850161), although it was soon discontinued. Finally, the excipient macrogol 15-hydroxyestearate (Solutol® HS15) was presented in 2012 as the main component of the CriticalSorb™ technology [118]. This technology was shown to promote macromolecule transport mostly via the transcellular pathway [119], and has reached clinical trials for nasal administration of somatropin (QBR106712, HRA) and teriparatide (NCT01913834).

Enzymatic inhibitors played a substantially less relevant role in the field. Briefly, the inhibitory effect on proteolytic enzymes of bile salts on rat nasal mucosa was highlighted in 1981 [120]. In 1988, enzymatic inhibitors, namely bacitracin and sodium taurodihydrofusidate (STDHF)), were reported to improve nasal absorption in rats [121], setting the stage for other compounds. In 1991, trypsin would be finally regarded as the best performing inhibitor [122].

Bioadhesive polymers have also been explored regarding their use for improving nasal drug delivery. Aside form prolonging the time of residence, several other properties were attributed to these excipients, tight junction opening and enzyme inhibition among others, but the contribution of each functionality was not clear [123]. For example, in 1984, Carbopol® and microcrystalline cellulose were reported to improve the nasal absorption of insulin in dogs. This process achieved a blood glucose decrease up to 68% for up to 6 h [124]. Microcrystalline cellulose is now employed as a functional component in current dry-powder oxytocin formulations already in an advanced stage of development [125]. Chitosan has also been reported as a bioadhesive polymer for insulin nasal delivery [126]

Nanocarriers were first explored for nasal delivery of macromolecules in the 1990 s. Liposomes containing the penetration enhancer sterylglucoside (SG) were reported [127] to attain up to 24.2% of insulin bioavailability in rabbits. In the same decade, our research group developed CS NPs, which were found to be attractive carriers in nasal delivery of insulin [128]. The positive results obtained in rabbits (60% blood glucose decrease) were attributed to improved contact of insulin and chitosan with the nasal mucosa and transient opening of tight junctions. Subsequently, TMC-CS NPs were developed with the purpose of increasing paracellular permeability [129], and chitosan-N-acetyl-L-cysteine (CS-NAC) NPs to improve mucoadhesion through the formation of disulfide bonds with cysteine-rich domains in the mucus glycoproteins [130]. Finally, in 2006, our group presented chitosan nanocapsules (CS NCs) [131] loaded with salmon calcitonin (sCT), in which CS properties were combined with an oily core that would facilitate interaction with the mucosa and stabilize the peptide. A significantly higher hypocalcemic effect was obtained with this formulation compared to a sCT-loaded nanoemulsion and a CS-sCT solution. Another important achievement from our research group was the discovery of the critical role of nanocarriers PEGylation in achieving adequate mucodiffusion and, hence, facilitate the transport of proteins encapsulated in PLA-PEG NPs [132]. This kind of delivery carrier, mainly applied to the administration of vaccines, will be described in section 3.

2.2.2. Seminal work/current advances

The current scenario in nasal protein/peptide transmucosal delivery includes extensive research in penetration enhancers,

including both functional molecules and polymers, and several relevant advanced delivery systems.

2.2.2.1. Self-dispersing ionic liquids-based nanostructures. A novel approach for nasal protein delivery was introduced with the co-administration of insulin and DESs [133], also called ILs, as detailed in section 2.1.3.2. Specifically, this work employed a DESs of choline chloride (ChCl) and malic acid (MA), which, in association with insulin, generated a deep decrease in blood glucose in rats with a 25 IU/Kg dose. This effect was hypothesized to be a transient modification of nasal epithelia fluidity.

2.2.2.2. Penetration enhancers. Morishita's lab compared the effect of several CPPs and the clinically approved enhancer sodium caprate in the nasal systemic absorption of interferon ß in rats [134]. A maximum bioavailability of 8.26% was obtained with D-penetratin. The same research team reported an increase in the nasal systemic and brain absorption of leptin after co-administration with L-penetratin [135]. Finally, several studies investigated the penetration enhancing effect of the human translationally controlled tumor protein transduction domains (L-TCTP-PTD 13), also in a rat model. This last penetration enhancer led to an enhanced nasal bioavailability of insulin (37.1%) [136] and Exendin-4 (23.9%) [137]. This work also investigated the effect of linking the peptide covalently to the enhancer, but the resulting product presented no intranasal (IN) absorption. In a similar approach, Park et al. attained 58% insulin bioavailability when in combination with the protein transduction domain (PTD1) [138]. Later, other researchers obtained 60.71% insulin bioavailability [139]. Additional stabilizing and/or solubilizing excipients were used in both studies (arginine hydrochloride and glycerin, and sucrose, Poloxamer 188 and methionine, respectively).

Similarly, recent results were published using the CriticalSorb™ technology, which is based in the penetration enhancer Solutol® HS15, introduced in 2012 and currently in clinical trials, as discussed above (Section 2.2.2). This formulation consisting of a Solutol® HS15 solution in phosphate buffer reached advanced testing both for human growth hormone (hGH) (Phase I, QBR106712, HRA) and teriparatide (PTH 1–34) (Phase I, NCT01913834). The formulation containing hGH was administered to humans as a spray dried nasal powder formulation (CP024) also containing a gelling agent [140]. Although a low hGH absolute bioavailability (3%) was obtained with this nasal formulation, the IGF-1 levels were similar to the ones obtained by SC injections. Possibly, the IN administration would be beneficial over the sc. administration, as it would resemble the endogenous pattern of GH secretion from the pituitary gland in healthy individuals. The same technology was applied to the teriparatide formulation, and promising results were obtained in rats, when the formulation was administered in a liquid form containing mannitol in acetate buffer. In this case, up to 78% bioavailability was reached [141]. However, this liquid formulation did no exert the expected effect in humans, where a low and highly variable 0.26–1% bioavailability was obtained [142]. This result highlights the difficulties for translating experimental data from animal models to humans.

2.2.2.3. Modified polymers with penetration enhancing properties. The co-administration of the drug of interest with solutions of polymers that have permeation enhancing capabilities, or polymers chemically modified with penetration enhancers has also attracted attention in the last few years. For example, hGH was intranasally administrated along with poly-L-arginine (pArg) of different MW [143]. A concentration of 1% (w/v) of the highest MW (>70 KDa) pArg led to the highest bioavailability value obtained (14.7%). A similar study was carried out for the evaluation of poly-L-ornithine, whose capacity to increase the absorption of fluorescein isothiocyanate-dextran (FD-4) was found to be superior to the one of pArg [144]. The most positive results were observed with 78-KDa MW poly-L-ornithine, where the bioavailability reached 65.9%. Another proposal consisted on the co-administration of Ex-4 with poly(N-vinylacetamide-co-acrylic acid (PNVA-co-AA) polymer modified with D-octaarginine [145], which attained 20% nasal bioavailability in mice.

2.2.3. Note on approved/clinical trials products and prospect view

To the best of our knowledge, up-to-date there are nine approved products in the market for the systemic delivery of protein/peptide drugs via nasal administration (Table 2), eight of which, being small peptides, do not require any delivery strategy [103], and one of them being the recently FDA approved glucagon nasal powder formulation (GNP, also referred to as AMG504-1) (Baqsimi®) (2019) from Eli Lilly. The latter formulation contained synthetic glucagon at a 10% w/w concentration along with beta-cyclodextrin and the penetration enhancer DPC. While the function performed by each component was not disclosed, DPC is known for its paracellular permeation properties [109] and also for its special affinity to glucagon, which leads to the formation of the DPC-glucagon complex [110], as discussed above (Section 2.2.2). On the other hand, beta-cyclodextrins have traditionally out-performed alfa-cyclodextrins in chemical-structure related nasal peptide absorption studies [106,111]. Advanced toxicology studies in several animal models were recently reported with successful outcomes [146].

Regarding clinical trials, three products whose technologies were above described are currently in active evaluation (Table 2): the octreotride Intravail™ formulation (DP1038), based on the penetration enhancer dodecyl maltoside (Phase I, NCT03031535), and the CriticalSorb™ formulations based on the penetration enhancer Solutol® HS15 for hGH (Phase I, QBR106712, HRA) and teriparatide (PTH 1–34) (CP024, Phase I, NCT01913834). It should be noted that the term nasal administration is nowadays used for both, systemic and N-t-B, routes of absorption. As initially mentioned, those products and technologies addressing direct brain delivery do involve systemic delivery and hence are not considered here.

3. Peptide and protein-based vaccines

3.1. Proteins and peptides as antigens

The field of vaccines has significantly evolved in the last decades, bringing innovative antigens as well as new types of adjuvants (or delivery systems) [147]. Progress in immunology and biotechnology allowed researchers to move beyond the traditional live-attenuated and inactivated viral vaccines. In particular, the understanding that the immune response against virus and bacteria was, in fact, directed towards specific epitopes led to the emergence of proteins or peptides as the main antigenic components of vaccines [148]. These protein and peptide antigens are most interesting due to their safety profile, since their use generally leads to more specific immune responses and, unlike traditional vaccines, avoid the risk of viral replication. Additionally, peptides and proteins are more easily produced, reducing production costs. However, these specific antigenic structures are also less immunogenic than the whole microorganisms, increasing the need to use appropriate adjuvant systems to produce vaccines with the ability to induce strong immune responses.

3.2. Challenges and opportunities of different vaccine administration routes

One of the key aspects in the development of any formulation, but particularly in the case of vaccines, is the choice of the admin-

Table 2
Technologies marketed or in clinical trials for peptide/protein nasal transmucosal delivery.

Company - Technology/ Product	Indication	Protein/ Peptide	Strategy	Phase	ClinicalTrial.gov Identifier
Novartis Miacalcin® CSL Behring Stimate® Ferring Pharmaceuticals / Generic products Minirin®; Octostim®; DDAVP® Serenity Pharmaceuticals Noctiva®	Central *Diabetes Insipidus*, Primary Nocturnal Enuresis	Desmopressin	None	Marketed	–
Therapicon Salcatonin®	Osteoporosis	sCT	None	Marketed	–
Pfizer Synarel	Endometriosis, ovarian stimulation	Nafarelin	None	Marketed	–
Hoescht Roussel Canada Inc. with Sanofi Suprefact	Endometriosis, prostate cancer	Buserelin	None	Marketed	–
Elli Lilly Baqsimi® (glucagon nasal powder (GNP), AMG504-1)	Hypoglycemia	Glucagon	Beta-cyclodextrin and dodecylphosphocoline (DPC) in a single-use dosing device	Marketed	–
Dauntless Pharmaceuticals, Inc. and Aegis Therapeutics, LLC DP1034 - Intravail™	Acromegaly, neuroendocrine tumors, chemotherapy-induced diarrhea (CID)	Octreotide	PE: Alkylsaccharide tetradecyl-beta-D-maltoside	I	NCT03031535
Critical Pharm CP024 - CriticalSorb™	Growth disorders, growth hormone deficiency (GHD) related syndromes	Somatropin	PE: macrogol 15-hydroxyestearate (Solutol® HS15)	I	QBR106712*
CP046 PTH - CriticalSorb™	Osteoporosis	Teriparatide		I	NCT01913834
CPEX Pharmaceuticals - Bently Pharmaceuticals NasulIn™	Diabetes	Insulin	PE: cyclopenta decalactone (CPE-215)	II/III	NCT00850096 NCT00850161 (Withdrawn)

PE: penetration enhancer; sCT salmon calcitonin;. *Health Research Authority UK Clinical Trials Register (https://www.hra.nhs.uk).

istration route. Though in Section 2 this review has covered oral and nasal delivery of proteins and peptides, we believe that the singularity of vaccine delivery was worth the more detailed overview we offer in this separate section. The majority of vaccines currently available in the market are administered through parenteral routes, namely through SC or intramuscular (IM) injections. Following SC/IM injection, vaccines can form a local depot at the administration site, and/or drain from there to the lymph nodes (LN) with time. On the other hand, following mucosal administration, vaccines may interact directly with mucosal-associated lymphoid tissue (MALT), generating different immune responses (Fig. 3). Achieving mucosal immune responses may be of interest for certain vaccines, such as those against human immunodeficiency virus (HIV) or urinary tract infections [149]. In these cases, it has been shown that mucosal administration of vaccines is able to elicit important levels of local immunity, which are difficult to achieve with parenteral immunization strategies [150].

Despite the widespread application of the IM route for vaccine administration, with generally reduced local side effects, SC and intradermal (ID) injections are interesting alternatives that may improve lymphatic drainage of these vaccines [152]. Targeting the lymphatic system is a key strategy in vaccine development, since it increases the probability of antigen recognition by antigen-presenting cells (APCs), leading to more potent and long-lasting immune responses [153]. For this purpose, researchers have focused on understanding the most important parameters that promote lymphatic drainage, particularly for nanosized antigen delivery systems. Small particle size (below 100 nm), surface charge, flexibility and hydrophilicity seem to be among the most important characteristics to consider when developing an antigen nanocarrier to target the lymphatic system [151,153]. Moreover, active targeting to APCs, T and B cells through the inclusion of specific ligands for these cells has also been described as a potential strategy to improve lymphatic targeting of nanocarriers [154–156].

Recently, our group has shown how small NCs with neutral surface were able to reach the draining LN following SC injection in mice, more efficiently than their larger or positively charged counterparts [157]. In a subsequent study we showed that positively charged NCs could also drain quickly to the closest lymph node following SC injection in mice, as long as their size is below 100 nm [158]. Nevertheless, it is worth highlighting a recent study performed in macaques which showed that differences between administration routes do not necessarily translate into different immune response levels [159]. In this work, performed with liposomes with an HIV trimer conjugated to their surface, the authors reported differences for SC and IM administration routes in the targeted tissues and immune populations. In particular, liposomes predominantly targeted primary LNs (axillary or inguinal) following SC injection, but drained almost exclusively to secondary LNs (apical or iliac) in the case of IM injections. Despite these differences, the adaptive immune response elicited was comparable in both immunization routes, highlighting the need to appropriately analyzing multiple LNs in this type of studies.

In the case of mucosal vaccination, the oral and nasal routes have taken the lead in the research and development of these products [149,160–162]. This seems to be due to the important role played by MALT in the development of mucosal immunity at the intestinal and nasal levels. At these sites, the antigens are captured by epithelial and/or M cells and taken up by APCs, which then drain to the gut-associated (in the case of the intestinal tract) or to the nasopharynx-associated (in the case of the nasal cavity) lymphoid tissues. Antigen presentation by APCs to the T and B cells resident in these tissues triggers then an immune response not only at a T-cell level (including Th1, Th2. Th17 and regulatory T cells) but also through the production of antigen-specific secretory immunoglobulin A (sIgA) antibodies. These antibodies are particularly relevant as they are secreted back to the mucosal layer and therefore prevent further spreading of the pathogenic organisms

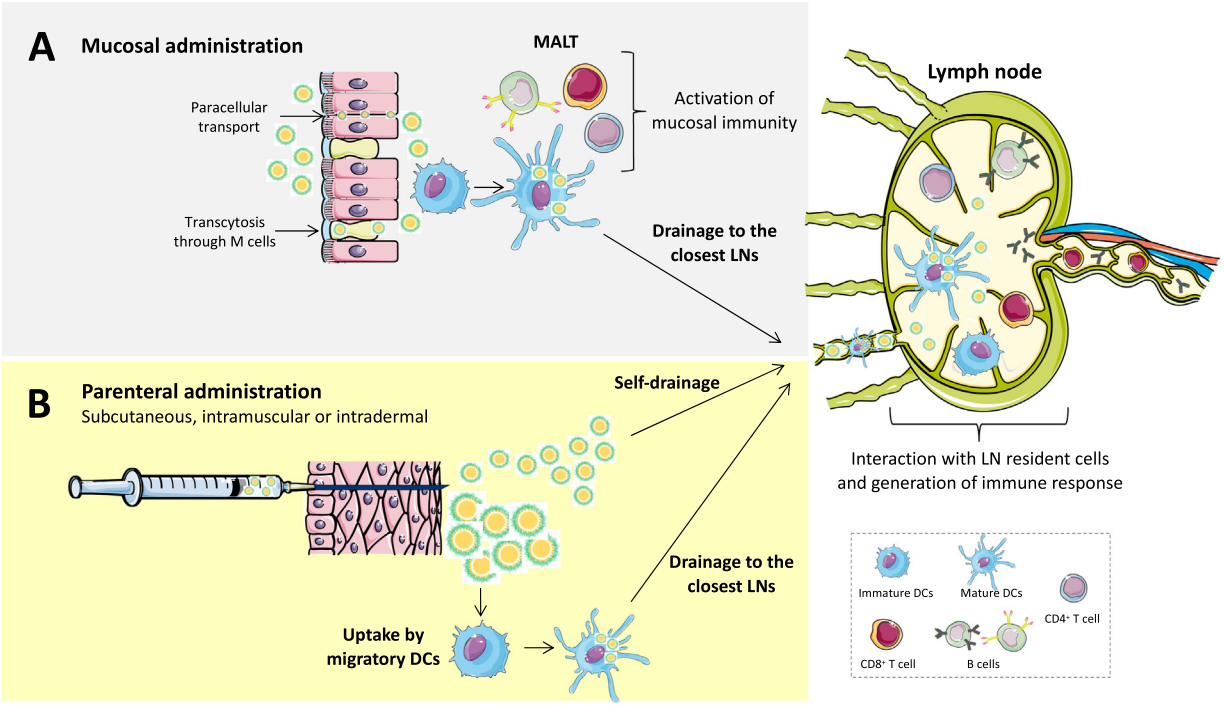

Fig. 3. Illustration of the fate of antigen-loaded nanocarriers depending on the route of administration. Following mucosal administration (A) (nasal, oral and vaginal routes), the nanocarriers may access the mucosal-associated lymphoid tissues (MALT) either by paracellular or transcellular transport across microfold (M) cells. Then, these nanocarriers will encounter and activate resident dendritic cells, inducing mucosal immunity. Simultaneously, some dendritic cells will drain to the nearest lymph node and generate a systemic immune response. In the case of parenteral administration (B), (subcutaneous, intramuscular and intradermal routes), the nanocarriers are deposited in the interstitial space, where they can either passively drain to the lymph nodes or be taken up by migratory dendritic cells, which then migrate themselves to the nearest lymph node. Reproduced with permission from [151]

[149,163,164]. To this date, there are three nasal and ten oral vaccines licensed for human use, targeting specifically influenza and enteric pathogens such as poliovirus, cholera, rotavirus and *Salmonella typhi* [165]. However, all these vaccines are still based on live-attenuated or inactivated pathogens.

In the case of the oral route, protection of the antigen against the harsh conditions of the gastrointestinal tract is key for any vaccine formulation. The highly acidic environment of the stomach, the presence of proteolytic enzymes and significant pH range and mucus layer present throughout the tract are fundamental barriers for these vaccines to overcome [161,162]. For this reason, developing antigen delivery systems that are able to protect the antigen, deliver it intact to APCs within the small intestine residence time, and induce a potent immune response, ideally acting as adjuvants as well, is essential to achieve successful oral vaccines with protein or peptide antigens [162]. Additionally, specific targeting to the M cells present in the intestinal epithelium has also been used as an approach to oral immunization, with successful results [166,167].

On the other hand, when developing nasal vaccines, some physicochemical properties of the antigen delivery systems must be taken into consideration [151,168,169]. Overall, most studies agree that nanometric sizes are more efficient than micrometric ones, and that medium-size NPs might present more advantages than very small ones [170,171]. Nevertheless, according to a recent review, other authors have not found significantly different immune response effects when different nanoparticle sizes were used [172]. The surface composition of the nanosystems is also an important characteristic to bear in mind. In this regard, our group has shown that PEGylation is a promising strategy for nanoparticle transport at the nasal level [173]. Similarly, recent studies using a negatively-charged polysaccharide nanovaccine showed promising protection levels against HIV in macaques [174]. Nevertheless, in other cases, the IN administration of positively-charged nanosystems has also shown positive results *in vivo* [175]. Therefore, it is clear that an adequate balance between muco-adhesive and muco-diffusive properties is needed to elicit potent immune responses after IN administration.

3.3. Historical perspective of nanotechnology and vaccine delivery

The first references to the use of particulate systems in vaccination approaches date back to the 60 s, when Litwin and Singer first demonstrated the adjuvant potential of polystyrene latex particles with human γ-globulin adsorbed to their surface [176]. Some years later, Allison and Gregoriadis reported that diphteria toxoid-loaded liposomes elicited high antibody levels in mice [177], while Birrenbach and Speiser developed polyacrylamide NPs encapsulating the tetanus toxoid, which also provided an important adjuvant effect when intramuscularly administered to guinea pigs [178]. Finally, in 1979, Preis and Langer reported the use of polymeric microparticles for the controlled delivery of protein antigens, with the aim of developing a single-dose vaccine [179]. This approach proved particularly visionary when the World Health Organization launched a campaign promoting the development of single-dose tetanus vaccines in the 90s, leading to numerous efforts being focused on new particulate adjuvant systems.

In the initial approaches focused on single-dose vaccination, several authors, including ourselves, made use of PLA and PLGA-based microparticles [180–182]. In these studies, different variants of PLGA polymers and various protective molecules were tested for their capacity to overcome the lack of antigen stability observed as the polymers naturally degraded. A few years later researchers also began to explore the nasal and oral routes of administration as alternative routes of immunization. In this regard, the oral

administration of polyacrylamide microparticles with ovalbumin (OVA) as a model antigen [183] and the nasal instillation of PLGA microparticles with tetanus toxoid [184] were among the first reports of mucosal particulate vaccine delivery systems. Alongside these studies, the demonstration of the importance of particle size in the development of more potent immune responses against these antigens led to the first reports of NPs being used as vaccine carriers through the oral and nasal routes [170,185]. Our group also demonstrated the importance of modifying the external surface of these particles with polyethylene glycol (PEG) and other materials to improve their stability in mucosal surfaces and, hence, their performance as antigen carriers [43,132,173,186]. However, the challenges posed by protein degradation mediated by PLGA degradation have hindered the clinical development of these prototypes [187].

The difficulties associated with PLGA-based antigen carriers led to the exploration of other materials, among them liposomes and emulsions. In particular, the use of biocompatible oils, such as squalenes, presented an opportunity to overcome the significant adverse effects observed with complete and incomplete Freund's adjuvants, and to improve the tolerability of these vaccine formulations [188–190]. The key importance of introducing oil-in-water emulsions as potential adjuvants is shown by the approval of MF59® in 1997 for human use. It was the first adjuvant ever approved after alum (Fig. 4). This emulsion containing squalene, Span® 85 and Tween® 80 was included in a flu vaccine (Fluad®) and commercialized by Novartis [191]. In recent years, other lipid-based nanocarriers have also been developed as vaccine adjuvants, particularly AS01, AS02, AS03 and AS04. The inclusion of the immunomodulatory molecule monophosphoryl lipid A (MPLA) adsorbed to alum in the AS04 formulation led to the approval of this adjuvant in a human papilloma virus (HPV) vaccine [192]. On the other hand, AS03, a nanoemulsion containing squalene, Tween® 80 and α-tocopherol was approved in 2009 as part of an IN flu vaccine [193]. However, this product was later discontinued due to its unwanted side effects, such as an increase in narcolepsy in children and young adults who received the vaccine [194]. Another squalene-based nanoemulsion, AF03, was developed by Sanofi Pasteur as an adjuvant for an H1N1 pandemic influenza vaccine (Humenza™) [195]. Despite eliciting promising results in clinical trials, the vaccine was never commercialized. Finally, in the case of AS01 and AS02, developed for a malaria vaccine, these adjuvants contained equal amounts of MPLA and the saponin QS-21, although AS01 was developed in the form of liposomes and AS02 in the form of a nanoemulsion containing AS03, MPLA and QS-21 [196]. The results of clinical development ultimately demonstrated the higher adjuvant efficacy of AS01 [197] and its use was recently approved in a malaria vaccine and a recombinant zoster vaccine, both commercialized by GSK (Table 3).

Natural polysaccharides were also explored by researchers in the mid-90s, for vaccine delivery purposes. In 1997, our group reported, for the first time, the use of CS and combinations of this polysaccharide with polyethers for the preparation of NPs containing protein antigens [198]. This initial approach was followed by many others that used a variety of polysaccharides [199]. For example, our group reported the use of hyaluronic acid (HA), alginate and dextran sulfate (DS), in combination with cationic polypeptides such as protamine and polyarginine, for the parenteral and mucosal delivery of the recombinant hepatitis B surface antigen (rHBsAg) [200,201]. Even so, CS has received the greatest deal of attention because of its utility for parenteral [202–206] and mucosal [207–210] immunization of animal models with proteins and peptides. The IN route was a particularly attractive application for the vaccine delivery of this polymer, given its mucoadhesive properties, as reported by Lehr *et al* in 1992 [211]. Various authors have reported on the efficacy of CS-based nanocarriers to deliver antigens across the nasal mucosa and to elicit local and systemic immune responses against the loaded antigens [209,212–214]. It is also worth highlighting a recent trend in the development of nanoparticle-based antigen delivery systems that profits from the capacity of these carriers to co-encapsulate the antigen and additional immunostimulatory molecules. For example, some authors have explored this combination of antigens with molecules such as CpG ODN, lipopolysaccharide (LPS), muramyl dipeptide, cholera toxin B subunit or the TLR-7 agonist imiquimod [215–218].

Beyond mucosal and parenteral immunization strategies, another approach that has gained attention in the last few decades is the use of the skin for transfollicular, ID or transdermal (TD) vaccination. Being the largest organ in the human body, and counting with an extensive population of immune cells within its structure, the skin is a privileged site for immunization. However, there are many challenges related to skin penetration of drugs and antigens, which various researchers have attempted to tackle. In particular, the use of nanotechnology-based strategies, as well as of additional physical or chemical tools to improve skin penetration have been described in this regard. The group of Claus-Michael Lehr, for example, has reviewed the potential of NPs for transcutaneous immunization [219,220], and reported the development of polymeric nanocarriers for vaccine delivery focusing on the transfollicular route. In particular, uncoated and CS-coated PLGA NPs loaded with OVA as a model antigen were able to efficiently deliver the antigen to the hair follicles of excised pig ears [221]. More recently, inverse micellar sugar glass NPs actually outperformed the previously described prototypes in terms of the humoral and cellular immune responses elicited against OVA following transfollicular and ID immunization of mice [222]. Finally, the same group reported a further modification of PLGA NPs with PEG-b-PAGE which led to potent OVA-specific $CD8^+$ T cell responses after SC administration to mice [223].

Another strategy more recently explored for ID and TD immunization is the use of microneedle (MN) arrays [224–227]. The combination of this strategy with nanoparticulate systems has been reviewed elsewhere [228], but it is worth highlighting some studies with promising results with the model protein antigen OVA in animal models. Zaric *et al* reported the encapsulation of OVA in PLGA NPs which were then incorporated in the formulation of dissolving polymeric MN arrays [229]. This approach led to robust antigen-specific cellular responses in mice, as well as complete protection against the development of B16 melanoma tumors and a mouse model of *para*-influenza. Other groups focused on the use of hollow MN arrays, which allow the ID delivery of liquid vaccine formulations through the channel in the structure of the microneedles. In this case, de Groot *et al* reported the use of hollow MN arrays to deliver OVA-loaded PLGA NPs with or without poly(I:C) as an additional adjuvant [230]. This approach elicited protection in vaccinated mice against bacterial challenge with recombinant OVA-secreting *Listeria monocytogenes*, evidencing the potential of the NPs combined with the MN-based delivery for immunization.

3.4. Seminal advances in the field of nanotechnology and vaccine delivery

3.4.1. Vaccines against viral infections

A large number of research efforts in vaccine development are currently focused in viral infections that require effective universal coverage. Unexpected but also continued viral outbreaks such as those caused by Middle East respiratory syndrome coronavirus (MERS-CoV), Ebola or Zika, and more recently the severe acute respiratory syndrome-related coronavirus-2 (SARS-CoV-2) have highlighted the extreme need for a rapid development of effective

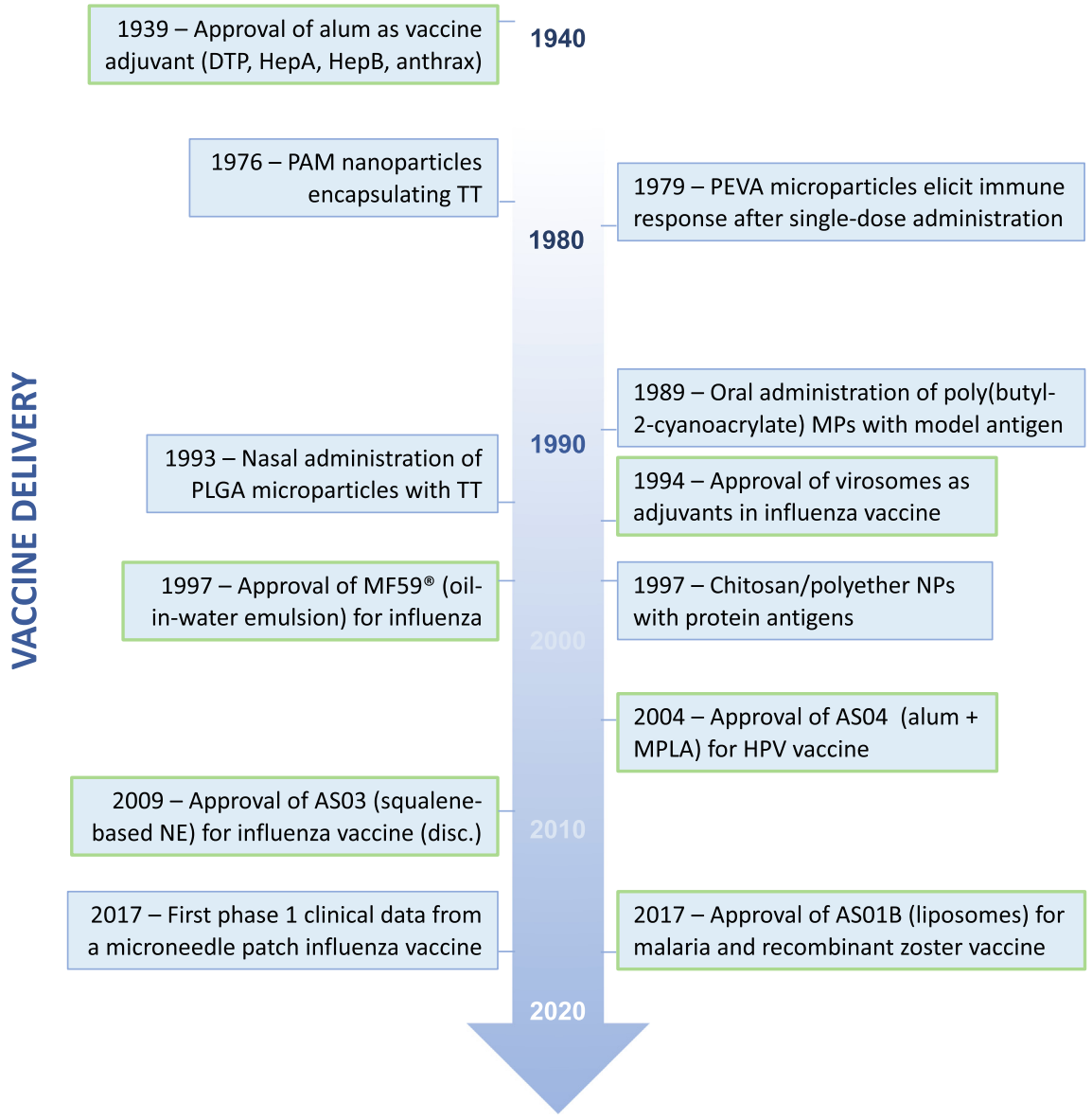

Fig. 4. Timeline of the advances in adjuvant approval and in the application of nanotechnology for vaccine delivery. Alum, aluminum hydroxide; DTP, diphtheria, tetanus, pertussis vaccine; HepA, hepatitis A; HepB, hepatitis B; MP, microparticle; TT, tetanus toxoid; NP, nanoparticle; PEVA, ethylene–vinyl acetate copolymer; PLGA, poly(lactic-co-glycolic acid); MPLA, monophosphoryl lipid A; HPV, human papilloma virus; NE, nanoemulsion; disc., discontinued.

Table 3
Commercialized nanotechnology-based adjuvants for human use.

Vaccine / Company	Antigen	Adjuvant	Adjuvant composition	Disease	Administration route	Licensing year
Fluad® / Novartis	Hemagglutinin + neuraminidase	MF59®	Squalene Span® 85 Tween® 80	Influenza	IM	1997
Mosquirix® / GSK	Portion of *P. falciparum* circumsporozoite protein fused with hepatitis B surface antigen (non-infectious virus-like particles)	AS01	Monophosphoryl lipid A Saponin QS-21	Malaria	IM	2015
Shingrix® / GSK	Glycoprotein E antigen of Varicella Zoster virus	AS01	Monophosphoryl lipid A Saponin QS-21	Herpes zoster	IM	2017

vaccines against these diseases [231]. Additionally, viruses such as influenza, HIV and hepatitis B virus continue to cause high levels of morbidity and mortality across the world, which could certainly be refrained by the use of an effective vaccine against them. The role of nanotechnology in the delivery of antigens against these viral threats has been significant, and is described in the sections below.

3.4.1.1. Influenza. Developing a universal influenza vaccine that could potentially provide cross protection among the different virus strains and avoid the need for seasonal vaccination campaigns remains a key goal in this field [232]. Our group has recently shown the versatility of NCs as vaccine delivery systems, which is due to the fact that their physicochemical properties can be easily modified and optimized by altering their composition and formu-

lation parameters [233]. Through these strategies, we have optimized protamine NCs for the delivery of **H1N1 influenza hemagglutinin**, whose IM administration to mice at low antigen doses elicited higher antibody levels up to 28 weeks, than those obtained with alum and with higher antigen doses [234]. These types of delivery carriers could be easily adapted for the reformulation of the continuously evolving viral vaccines.

The advantages of using NPs for IN influenza vaccination were recently shown by Si *et al*, when comparing the response achieved for a peptide antigen against influenza in a free form or as a nanofiber [235]. According to their results, the **nanofibers** of the MHC-I polymerase peptide epitope induced persistent lung-resident $CD8^+$ T cell responses, while the free antigen did not. Other authors looked at the development of self-assembling protein NPs using ferritin conjugated with a conserved influenza matrix protein [236]. After IN administration, this vaccine was able to generate specific sIgA and T cell responses without any additional adjuvants, and to protect 100% of the immunized mice challenged with H1N1 and H9N2 influenza viruses. In another approach, researchers conjugated the influenza H1N1 nucleoprotein to pH-responsive NPs to which the adjuvant CpG was also associated. Again, the IN administration of this vaccine was more efficient than the parenteral one, eliciting higher levels of specific $CD8^+$ T cells at both airway and lung interstitia [237].

Alternative nanocarriers were also evaluated for the development of influenza vaccine prototypes. For example, Stark *et al* showed the potential of **lipid formulations** from Archaea (natural and semi-synthetic archaeosomes) to induce potent immune responses against hemagglutinin [238]. In this work, the authors demonstrated that antigen-loaded archaeosomes, as well as physical mixtures of the antigen and the carrier, upon IM administration to mice, led to strong immune responses in animals of different ages and in pregnant females, protecting the pups and mothers against viral challenge.

In the field of inorganic nanocarriers, Pham *et al* used **nanodiamonds**, a carbon nanomaterial, for the delivery of recombinant hemagglutinin protein oh H7N9 influenza virus [239]. Results showed that the formulation elicited significantly higher IgG levels than the free protein antigen, following SC immunization of mice. Finally, another approach consisted in the conjugation of recombinant trimeric hemagglutinin onto gold NPs, which were administered intranasally to mice. In combination with flagellin-conjugated gold NPs, this formulation was able to substantially increase the hemagglutinin-specific IgG and IgA titers in mucosal lavages, to induce $CD8^+$ T cell responses, and to increase animal survival in comparison with free antigen and adjuvant [240].

3.4.1.2. Hepatitis B virus. Another pathogen explored in the development of nanocarrier-based vaccines is the hepatitis B (HB) virus. For this purpose, our group developed NCs with different external coatings, observing that CS, protamine and pArg NCs were all taken up by immune cells *in vitro*, and were able to induce ROS production [241]. However, protamine NCs showed better results in terms of complement activation and stimulation of proinflammatory cytokine secretion. Once loaded with rHBsAg, these NCs were administered intramuscularly to mice and the results showed that protamine NCs elicited high antibody response levels. In another study, we demonstrated that these NCs were also able to elicit protective antibody levels against rHBsAg upon IM and/or IN administration [242].

*3.4.1.3. HIV**a.* Undeniably, one of the biggest challenges of current vaccine development is to develop a vaccine against HIV. Recently, our group demonstrated the ability of polysaccharide-based NPs encapsulating an HIV peptide antigen to generate efficient immune responses [243]. To understand the impact of composition, peptide attachment and adjuvant incorporation in the efficacy of these carriers, three prototypes were designed by association of the antigen to the NPs either through ionic interactions, a cleavable or a non-cleavable covalent link. Poly(I:C) was included in some of the NPs to evaluate its potential as an additional adjuvant in these formulations and CS, DS and HA were used as the main components of the NPs. Overall, after IM administration of these formulations to naïve mice, all of them were able to generate high levels of specific IgG antibodies, which were 3-times higher at 16 weeks after receiving the prime dose. Additionally, the study showed that different types of antigen attachment to the NPs led to different kinetics of $CD4^+$ and $CD8^+$ T cell activation. Following these promising results, the prototype based on CS/DS with the peptide antigen attached by ionic interactions was used to attach a cocktail of 12 HIV peptide antigens for further studies [244,245]. This formulation was intranasally administered to female macaques, in combination with IM administration of recombinant vesicular stomatitis virus coding for the same antigens. Antibody responses against the peptide antigens, as well as towards other HIV sequences were generated with this approach [246]. Additionally, the IgG response elicited with this vaccine, both at the mucosal and systemic levels, were higher than the one induced with the traditional HIV antigens Gag and Env [247,248]. Finally, this HIV vaccine that combined a viral vector and an antigen nanocarrier was able to protect 75% of vaccinated macaques after 6 intravaginal challenges with simian immunodeficiency virus (SIV), through peptide-specific $CD8^+$ central and effector memory T cells, as well as $CD4^+$ and $CD8^+$ regulatory T cells [174]. Overall, these studies showed that polysaccharide-based NPs represent new and tunable platforms for the development of vaccines, with the added value of a feasible translation towards industrial manufacturing [249].

Other approaches to nanotechnology-based anti-HIV subunit vaccines include the development of other polymeric NPs and also inorganic carriers. In 2016, Pavot *et al* developed PLA NPs encapsulating NOD ligands and coated with HIV-1 Gag p24 antigen [250]. Results showed increased systemic and mucosal immune responses in mice, following either oral, nasal or SC administration, with efficient induction of dendritic cell activation and T cell differentiation in the draining LN. More recently, Damm *et al* suggested the use of calcium phosphate NPs functionalized with HIV-1 Env trimers as a vaccination strategy against this virus [251]. Aiming at providing "intrastructural help" for B-cell responses, a universal T-helper epitope of tetanus toxoid was also loaded in the core of the NPs, and mice were vaccinated against tetanus before receiving the anti-HIV nanovaccine. Results showed enhanced immune responses in these mice in comparison with those not vaccinated against tetanus, demonstrating the effect of intrastructural help in potentiating antibody responses against Env.

3.4.1.4. SARS-CoV-2. This review would not be complete without highlighting the fundamental role of nanotechnology in the development of vaccines against SARS-CoV-2 [252,253]. The fast development and approval in December 2020 of BT162b2 (Pfizer/BioNTech) in the UK and mRNA-1273 (Moderna) in the United States, as highly effective vaccines against the COVID-19 pandemic [254,255], became a major milestone in the field of vaccine delivery and nanotechnology. Nucleic acid vaccines, and particularly mRNA-based vaccines such as these ones, are appealing due to their simple design and manufacturing, safety and ability to induce potent humoral and cellular immune responses [253,256]. In fact, there are several mRNA-based vaccine candidates against SARS-CoV-2 currently under clinical and pre-clinical development, according to the WHO [257]. However, the delivery of this type of genetic material to APCs is limited without the use of an appropriate carrier. Interestingly, both BT162b2 and mRNA-1273 make use of the same delivery platform – lipid nanoparticles (LNP).

These particles are usually composed of four types of lipids: an ionizable lipid to complex mRNA and promote self-assembly into NPs, a PEGylated lipid to provide stealth properties, cholesterol for stabilization, and natural phospholipids for support. Due to their structure and characteristics, LNP are capable of protecting their mRNA cargo from degradation, target it to the lymphatics and promote protein translation once in the LN (Fig. 5) [256]. Other companies currently have in clinical trials other mRNA vaccines that use similar LNP platforms as delivery systems, including CureVac AG (CVnCoV, Phase 3, NCT04674189), Arcturus Therapeutics and Duke-NUS Medical School (ARCT-021, Phase 2, NCT04668339), the Academy of Military Medical Sciences, Suzhou Abogen Biosciences and Walvax Biotechnology (ARCoV, Phase 2, ChiCTR2100041855), GlaxoSmithKline (CoV2 SAM, Phase 1, NCT04758962), and Imperial College London and Morningside Ventures (COVAC-1, Phase 1, ISRCTN17072692) [257–259]. Zhang *et al* also recently published the development of a thermostable LNP-encapsulated mRNA vaccine candidate targeting the SARS-CoV-2 [260]. In this study, the authors reported robust neutralizing antibody levels and cellular immune responses after IM administration to mice and non-human primates, with full protection against viral challenge achieved with a prime-boost regimen. In an effort to achieve similar responses with lower mRNA doses, other authors focused on the use of self-amplifying mRNA vaccines, derived from an *Alphavirus* genome. These alternatives are particularly interesting because this type of mRNA encodes the alphaviral replicase (besides the gene of interest), allowing for mRNA replication inside the cytoplasm of the target cells. In this regard, McKay *et al* reported high antigen-specific neutralizing IgG levels following repeated IM administration of a self-amplifying mRNA encoding for the virus spike (S) protein, encapsulated in cationic LNP [261].

Apart from these developments in mRNA-based COVID-19 vaccines, the majority of the vaccine candidates currently under clinical development are based on recombinant proteins or peptides, with a particular focus on the SARS-CoV-2 spike (S) protein. The most advanced of these candidates is the vaccine developed by Novavax, which is currently in phase 3 clinical trials for IM administration in two doses (NCT04611802, [258]). In this prototype, the company uses their proprietary Matrix-M™ adjuvant, which is a saponin-based nanoparticulate formulation with demonstrated ability to induce strong and long-lasting antibody and cell-mediated immune responses [262]. Recent reports evidence the high efficacy of the Novavax vaccine even against some of the newest variants of the virus [263]. Other prototypes currently in clinical trials include a spike ferritin NPs loaded in liposomes containing QS-21 (NCT04784767), an MF59®-adjuvanted vaccine (NCT04495933), two AS03-adjuvanted formulations (NCT04405908, NCT04750343), and a formulation adjuvanted with Sepivac SWE™, a squalene-based nanoemulsion similar to MF59® (NCT04702178) [257]. Several other candidates are at the preclinical stage of development, however the information on these approaches and the characteristics of any adjuvants or delivery systems used is still limited [257,264].

3.4.2. Vaccines against bacterial infections

Despite the prevalence of vaccine development approaches directed towards viral infections, the imminent danger of an

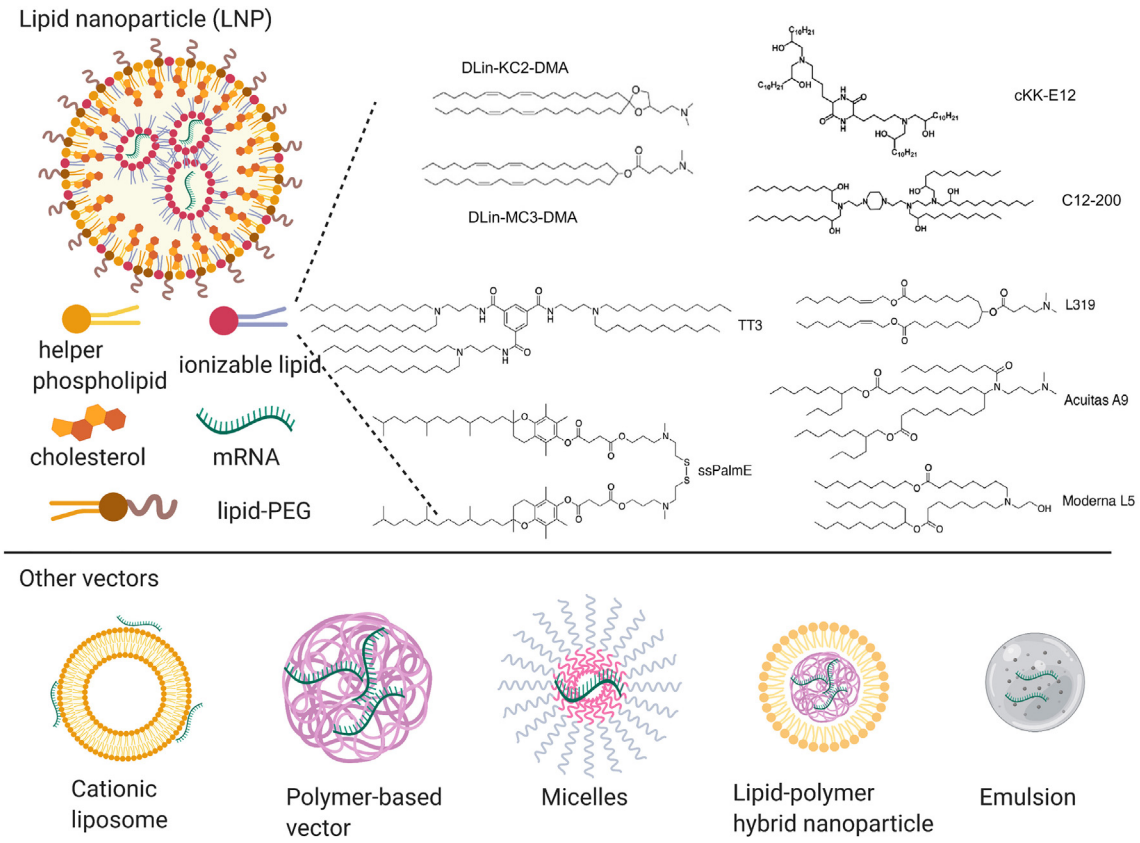

Fig. 5. Non-viral delivery systems for mRNA-based COVID-19 vaccines under development. Lipid nanoparticles (LNP) are composed of four types of lipids: cationic or ionizable lipids (for mRNA complexation), cholesterol (for particle stabilization), helper phospholipids (to facilitate endosomal escape) and PEGylated lipids (to provide stealth properties). Some of the lipids in the formulations currently being developed, or in the market, are shown in the figure. Other types of non-viral systems with potential application in the delivery of mRNA-based vaccines include cationic liposomes, polymer and polymer/lipid hybrid particles, micelles and emulsions. Reproduced with permission from [253]

antimicrobial resistance crisis has also spurred the development of antibacterial vaccines. For example, for the prevention of tuberculosis (TB), IN vaccination is an appealing route to potentially achieve mucosal protection at pulmonary sites. In this regard, the development of self-assembled nanofibers based on CD4 and CD8 peptide epitopes has been reported to enhance the cellular immune response against TB, especially when including a TLR2 agonist in the formulation [265]. In this study, the authors used a heterologous vaccination regime, priming the animals with the commercial BCG vaccine and then intranasally administering a boost with the peptide nanofibers. This strategy was able to significantly decrease the pulmonary viral loads in challenged mice in comparison to the group vaccinated only with BCG. The BCG-prime strategy was also applied by Hart *et al*, who developed yellow carnauba wax NPs coated with a fusion protein of three TB antigens [266]. This formulation, administered intranasally to mice, was able to enhance protection against TB challenge in BCG-primed mice. Unfortunately, a phenotypic and transcriptomic profiling study recently published showed that this protection was partially lost at 7 weeks post-boosting and beyond [267].

Woodworth *et al* also explored the advantages of combining a parenterally delivered prime with a mucosal boost, using a mixture of the liposomal system CAF01 and the fusion protein H56 [268]. Parenteral vaccination with this system was shown to elicit important $CD4^+$ T cell responses, which mediated animal protection. Interestingly, although the mucosal boosting with CAF01:H56 increased the numbers of lung-resident T cells, no improved protection was reported. More recently, our group developed NCs with external coatings of CS or inulin/pArg, as carriers of the *Mycobacterium tuberculosis* fusion protein and loaded with imiquimod as an additional adjuvant [175]. After immunization of mice with these NCs through a SC prime and an IN boost (12 weeks apart), the inulin/pArg prototypes elicited the highest serum IgG and bronchoalveolar IgA levels. These results were in line with biodistribution studies performed earlier in zebrafish [269].

Group A streptococcus (GAS) is a gram-positive bacterium that causes mild to severe diseases in humans. Despite significant efforts, to this date, no effective vaccine has been developed for this pathogen. For example, in one study, cationic liposomes encapsulating a lipopeptide antigen showed better IgA and IgG antibody titers than the commercial adjuvant cholera B toxin, especially when the lipopeptide was a conjugation of B- and T-cell epitopes [270]. The same lipopeptide, this time included in NPs made of dextran, PLGA and TMC induced strong mucosal and systemic immune responses against this bacterium when intranasally administered to mice [271]. Based on these results, further studies included the conjugation of conserved B-cell epitope against GAS and the universal T-helper epitope (PADRE) to polyglutamic acid. This negative-charged conjugate was then formulated as NPs through interaction with the positively-charged TMC. After IN administration of this nanovaccine, a significant reduction in bacterial loads at mucosal sites was achieved [272]. Lastly, these authors also reported this lipopeptide antigen approach with additional epitopes and a mucosal adjuvant (c-di-AMP) for IN immunization of mice increased cellular responses, which allowed antigen dose reduction [273].

The prevention of the *Chlamydia trachomatis* infection could also benefit from mucosal vaccination and protection. In this regard, Rose *et al* developed PLGA NPs modified with a cationic surfactant (DDA) and an immunostimulatory molecule (TDB), further coated with glycol-CS to improve mucoadhesiveness [274]. Intranasal co-administration of this adjuvant with the recombinant fusion protein CTH522 was able to increase the serum and mucosal levels of IgG and IgA, with similar $CD4^+$ T cell activation levels as those obtained when using DDA/TBD liposomes as the adjuvant system. The same authors also developed phytantriol hexosomes, which are lipid particles formed by rod-like arrangements of micelles hexagonally packed, and loaded them with MMG-1, a synthetic analogue of the mycobacterial lipid monomycoloyl glycerol, as an adjuvant [275]. This formulation was mixed with *C. trachomatis* major outer membrane protein (MOMP) as an antigen and given subcutaneously to mice. The results showed an improved adjuvant efficacy of this formulation in comparison with DDA/TBD liposomes.

Uropathogenic *E. coli* (UPEC) is the most common cause of urinary tract infections. Two vaccines have being currently approved in Europe against it, but they have limited efficacy [276]. To tackle this issue, our group developed bilayer NCs composed of DS and CS, loaded with *E. coli* IutA antigen, a specific outer membrane protein for ferric aerobactin from UPEC. When administered subcutaneously to mice, these NCs were able to generate significantly higher IgG levels, in comparison with CS NCs and alum-adsorbed antigen [277]. Enterohaemorragic *E. coli* (EHEC) infections are another cause of concern for the healthcare community, and several efforts have been made towards the development of a vaccine against this pathogen. Khanifar *et al* reported the use of CS NPs encapsulating one or two recombinant protein antigens against EHEC O157:H7 for the oral and SC immunization of mice [278,279]. Results from these studies showed the efficacy of the nanovaccine in eliciting mucosal and systemic antibody responses, and the superiority of a combined oral-SC vaccination regime in comparison with other alternatives. An alternative strategy was presented by Chen *et al*, who developed clay NPs for vaccination against EHEC O26 [280,281]. Initially, the authors reported the ability of the developed NPs to elicit humoral and cellular immune responses against the antigen intimin β upon SC administration to mice, at significantly higher levels than those generated by commercial adjuvants such as QuilA and alum. Furthermore, these authors used the same prototype to load three recombinant EHEC O26 antigens and immunized mice subcutaneously with the formulation, achieving strong, long-lasting and balanced immune responses.

4. Delivery of biological drugs in the context of cancer

Although classical cancer treatments combining chemotherapy, radiotherapy and other small molecules, have led with some positive outcomes [282], there is a clear need to develop advanced oncological therapies with a higher efficacy/toxicity ratio and able to cure severe cancers. In this context, protein therapeutics has emerged as a new promising alternative. Of the 89 biologics approved by the FDA in the last decade, 10 of them were approved this last year, of which 30% were indicated for cancer treatment [283]. Accordingly, it has been estimated that the global protein therapeutics market will reach $155.06 billion by 2025 [284]. Within this frame, monoclonal antibodies (mAbs) represent the leading class of proteins investigated for cancer treatment. As shown in Fig. 2, the number of oncological mAbs approved in the last ten years has grown exponentially. Currently, only in the US, 40 therapeutic antibodies have been authorized for cancer treatment (40.4% of the total Abs approvals), and 9 are under regulatory review [285]. Only a few of them have been withdrawn from the market for commercial reasons or due to the impossibility to ensure a significant clinical benefit as is the case of tositumomab [286] and olaratumab [287]. MAbs operate with high specificity according to different mechanisms of action: i) induction of apoptosis by directly targeting tumor cells, either as receptor agonists or blockers, and ii) induction of vascular and stromal cell disruption or immune-related cell death activation at the level of the tumor microenvironment (TME). This last mechanism, typical of immune checkpoint inhibitors anti-CTLA-4 or anti-PD-1/PD-L1

(discussed in section 4.5) has set up the basis of a new concept of immunotherapy. Although their interaction with the target oncoproteins is very specific, the activation of alternative signaling mechanisms may occur, thus diminishing the efficacy of the treatment [288]. These, among others, are the reasons why mAbs are normally co-administered with other therapeutic options, often involving small molecules.

Despite the great potential of biological drugs in general and mAbs in particular, their full exploitation in cancer is being significantly constrained by a number of biopharmaceutical problems, including their susceptibility to degradation, their incapacity to cross biological barriers and their inadequate biodistribution. In this context, nanotechnology [289], with particle sizes below 200 nm, preferably 100 nm, has emerged as a potential strategy to deal with the above mentioned problems, further discussed in section 4.2. Nevertheless, the design of the appropriate nanocarrier is not a simple and straight-forward approach. For example, a classical problem of nanodelivery carriers is related to their susceptibility to alteration upon contact with the blood stream, a process that impairs their targeting capacity and may exacerbate immune reactions against biological drugs. Although the use of materials such as PEG and other molecules that provide nanocarriers with stealth attributes [290] has been part of the solution, significant variability in the outcomes has been highlighted as a major concern. The use of targeting ligands together with the rational design of the nanocarriers based on the specific physiopathological characteristics of the tumor environment are expected to enhance the chances of the drug-loaded carriers to reach their targets [291]. In this sense it is important to keep in mind that cancer is a dynamic process highly conditioned by the tumor progression and the changes in the surrounding environment. For example, high vascular permeability together with unperfused tumor regions may result in heterogenous blood distribution in tumors. This situation leads to a variable access of the nanoformulations to the different tumor regions [292]. In addition, the stroma composition and the presence of tumor associated macrophages and other immune cells may influence the intratumoral distribution of the formulations. Finally, as the tumor grows, the chances of developing metastatic lesions increase and the access of the nanoformulations to the targeted cells becomes more difficult. The pathogenesis of metastasis involves several events such as angiogenesis progression and the secretion of proangiogenic factors, among others [293]. Consequently, each stage of cancer progression may need different formulation approaches for the effective cure of the disease.

The efforts undertaken by the scientific community in recent years to design nano-oncologicals for the delivery of therapeutic proteins, with special focus on mAbs, are reviewed in the following sections.

4.1. Historical perspective of protein delivery

The first strategy to improve the biopharmaceutical properties of proteins was probably the formulation introduced by Prof. Frank Davis at the end of **1960** [294]**.** It involved the chemical modification of a model protein, the bovine liver catalase, with the hydrophilic polymer PEG. The resulting complex exhibited an increased circulation time and a reduction of its immunogenicity [295]. Despite this early achievement, it was not until **1993** when the first protein-polymer conjugate consisting of neocarzinostatin and a styrene-maleic acid copolymer (SMANCS), known as **Zinostatin stimalamer**®, was marketed for the indication of cancer [296]. Subsequently, three PEGylated proteins have reached the market for cancer indications [297]. In 1994, **Oncaspar**® (PEG-L-asparaginase) became the first PEGylated protein approved for the treatment of acute lymphoblastic leukemia [298,299], soon followed by **Asparlas™,** a PEGylated asparagine enzyme marketed for the same indication but with a lower frequency of administration [300]. In **2011**, a PEGylated interferon-α 2b (**Sylatron™**) was approved as an adjuvant for the treatment of melanoma [301].

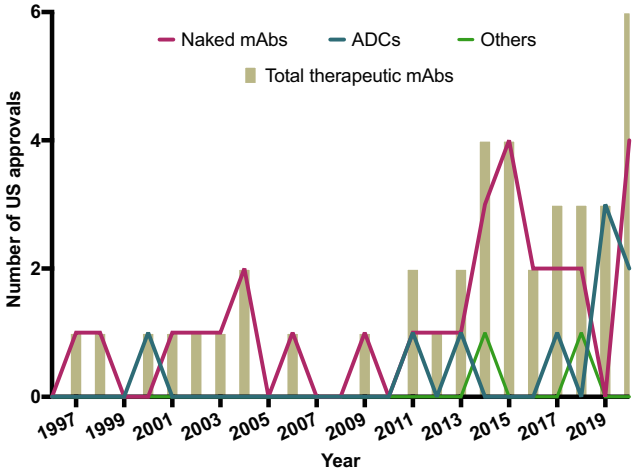

Fig. 6. Number of therapeutic monoclonal antibodies approved in the US for cancer treatment in the period 1997–2020, classified based on their format [i.e., naked mAbs (IgG format), ADCs and others]. *Data available as of February 11, 2021. Biosimilar products were excluded. Products withdrawn or marketing discontinued for the first approved indication were included [285].

Other strategies explored for the formulation of proteins as oncological therapies have involved the use of NPs. In this frame, our lab pioneered in **1997** the encapsulation of model proteins in PLGA nanospheres [302] and CS NPs [303]. The same kind of technologies were later applied to the formulation of the therapeutic enzyme, L-asparaginase [304] and **interferon-α** [305]**.**

In the area of mAbs, it should be highlighted that their production started only in **1975** [306] using the hybridoma technique. The immune related problems encountered with these original mAbs, was soon solved with their humanization. In **1997, rituximab** was the first mAb approved for non-Hodgkin lymphoma treatment, and just one year later, **trastuzumab** was approved for breast cancer therapy [307]. In the same decade mAbs started being produced using phage display libraries [308] and, in **2014, ramucirumab**, the first mAb produced by this technique for cancer indications, reached the market. Since then, there has been an exponential progress in the development of mAbs [307].

The application of nanotechnology to the formulation of these complex started with the antibody-drug conjugates (ADCs) which were first explored in clinical trials in **1983** [309]. After a number of attempts and failures, finally, the first ADC**, Mylotarg™** (Gemtuzumab ozogamicin), was approved in **2000** for the treatment of relapse CD33-acute myeloid leukemia (CD33-AML). However, ten years later, it was withdrawn from the market due to safety issues, and, then, approved again in **2017** for the treatment of relapse or refractory CD33-AML with a different dosing protocol [310]. Currently, there are nine ADCs commercialized for cancer indications (Fig. 6). These technologies are not the focus of this review however because detailed reviews have recently been published on this topic [311,312]. Overall, the studies on protein delivery using nanotechnology in the context of cancer are all quite recent (Fig. 7) and the use of NPs still remain at the preclinical level. Seminal works in this field will be commented in subsequent sections (Fig. 8).

4.2. Recent seminal work in protein delivery in cancer therapy

In this section we cover the most impactful protein delivery nanotechnologies reported in the last years for the treatment of

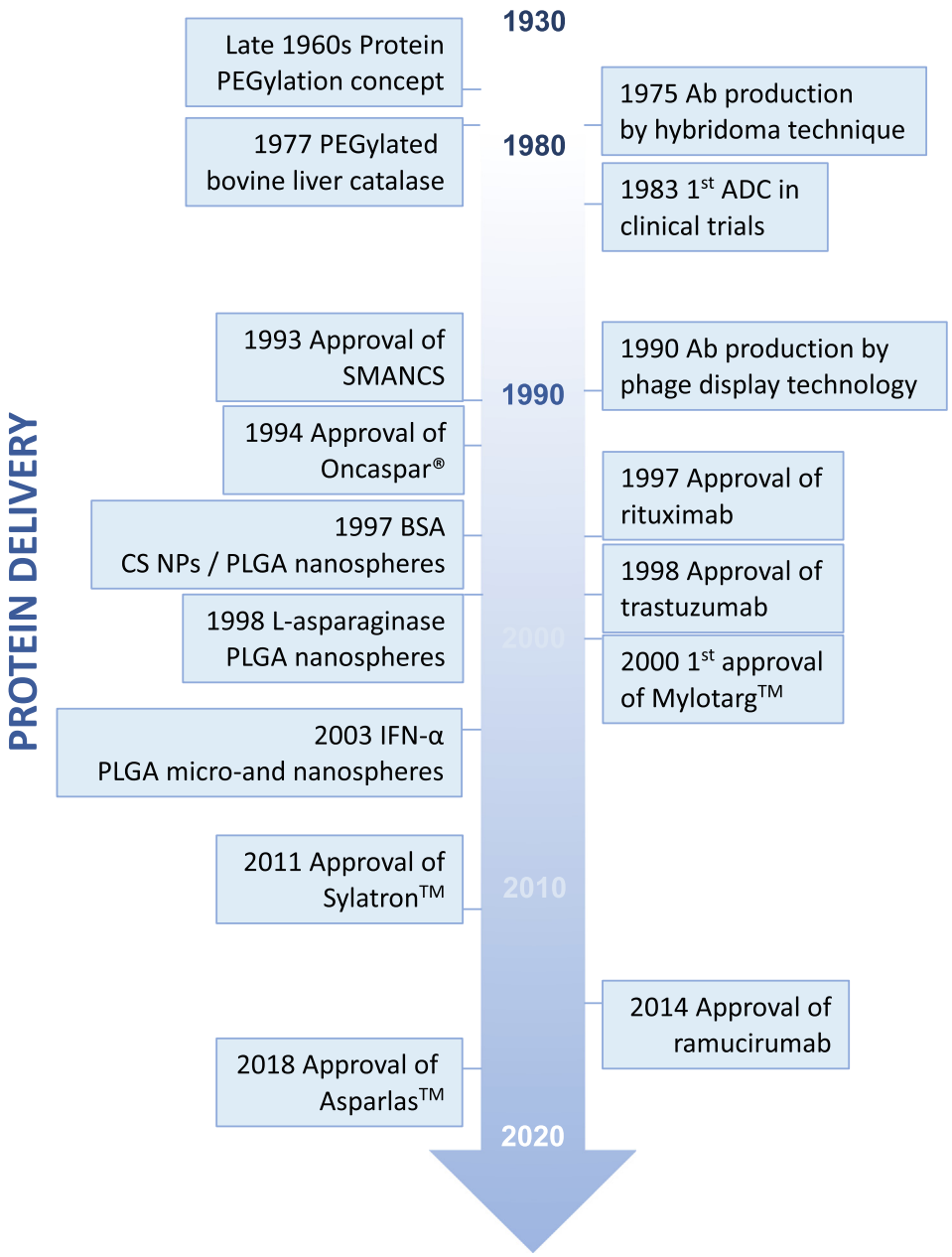

Fig. 7. Timeline of the introduction of seminal advances in drug delivery technologies in the protein parenteral delivery field. PLGA poly(lactic-glycolic acid); PLG poly (lactide-co-glycolide); BSA bovine serum albumin; CS chitosan; IFN-α interferon-α; NPs nanoparticles.

cancer. Due to the different nature and mechanistic aspects of the proteins delivered so far, we have chosen to classify the delivery nanotechnologies in the following groups: enzymes, apoptotic proteins, immunomodulators and, finally, mAbs.

4.2.1. Delivery of enzymes using nanotechnology

4.2.1.1. Tumoral delivery of cancer starvation-enzymes and tumor hypoxia relievers. One of the major physiological characteristics of cancer is the extremely rapid growth of cancer cells and their abnormal consumption of nutrients (e.g., glucose). Besides, the distance from the core of the tumor to the blood vessels makes the diffusion of oxygen difficult, which leads to tumor hypoxia. Different antagonistic strategies have been described to manage the tumor hypoxia: (1) to activate hypoxia by enzymes which will provoke the decrease of tumor oxygen (cancer starvation enzymes), thus preventing the development of the tumor and, on the other hand, (2) to relieve the hypoxia (tumor hypoxia relievers), thus favoring the diffusion of antitumor drugs in order to improve the efficacy of tumor therapies. The use of cancer starvation-enzymes, such as **glucose oxidase**, has attracted interest as it converts glucose and oxygen into gluconic acid and H_2O_2, which results in an increase in cell apoptosis. For example, PEG-liposomes containing glucose oxidase in combination with a liposomal hypoxia-activated pro-drug resulted in a positive outcome in terms of tumor growth in a breast cancer mice model [313]. However, the production of H_2O_2 is a double-edged sword since it may cause DNA damage [314,315]. To confront this problem, Ma and co-workers, developed nanoclustered enzymes consisting of covalently crosslinked **glucose oxidase and catalase,** a catalytic enzyme able to decompose H_2O_2 into H_2O + O_2, via a pH-responsive linker. These nanostructures were coated with a conjugate of BSA and the hypoxia-activated chemotherapeutic, tirapaza-

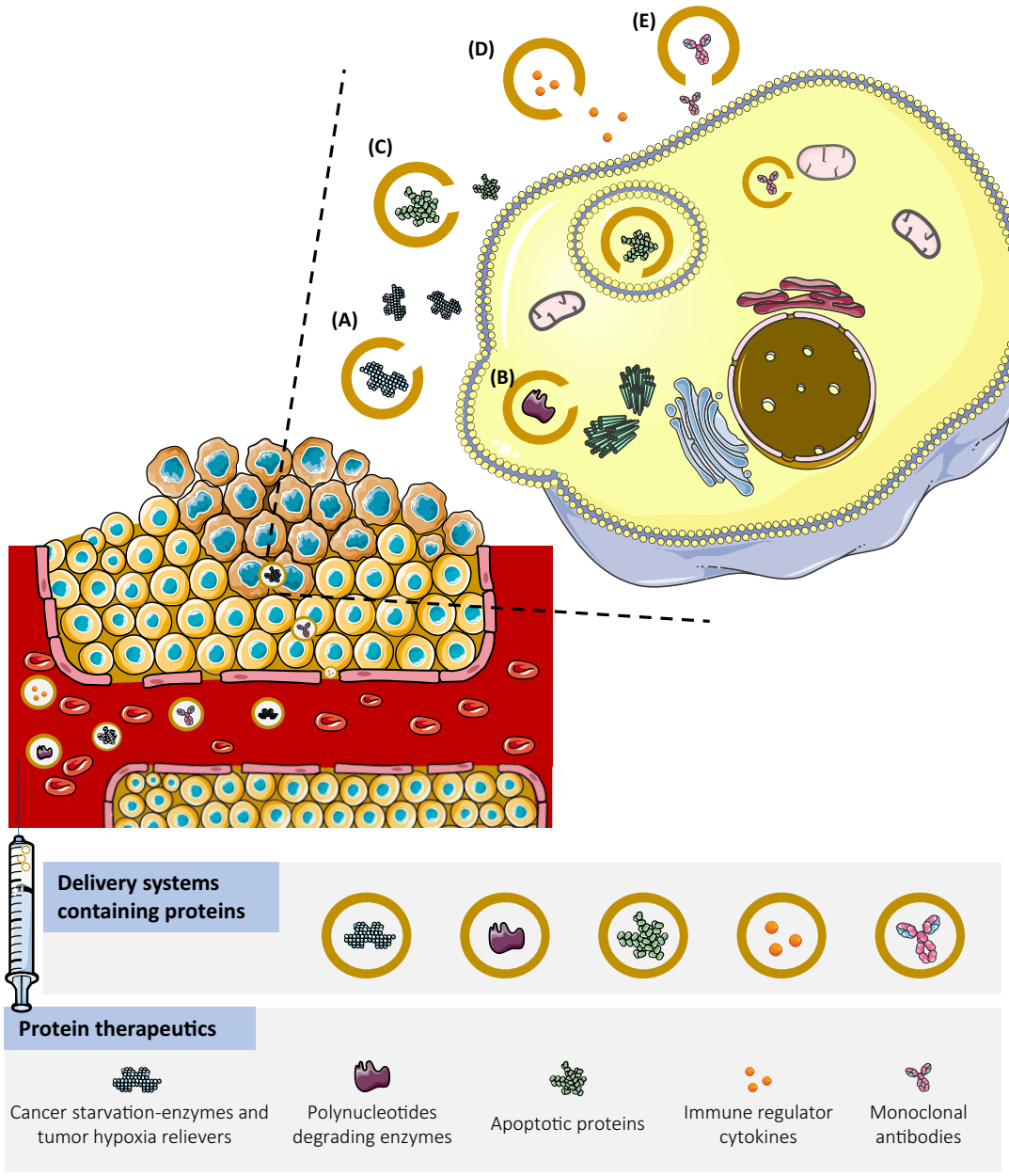

Fig. 8. Summary of current protein delivery strategies for cancer treatment. Delivery of therapeutic proteins into the target tumor is highly challenging. The proper design of nanoplatforms containing proteins, in addition to protecting biologicals, can modify their biodistribution, allowing them to reach the tumor. Once they are in their functional area, nanomedicines will release their cargo extra- and/or intracellularly: **(A)** Nanotherapies containing glucose oxidase or catalase can induce or relieve hypoxia, enhancing the efficacy of tumor therapies. **(B)** Nanomedicines containing DNase or RNase can be applied to catalyse the degradation of DNA or RNA, respectively. **(C)** Delivery of proteins such as Cytochrome *C*, Granzyme B or TRAIL may allow to exploit their apoptotic activities. **(D)** Several nanostrategies can be used to deliver cytokines, exploiting their pro-apoptotic or anti-proliferative activity, or induce the immune response against tumor cells. **(E)** Nanoplatforms encapsulating monoclonal antibodies can induce apoptosis by direct targeting to tumor cells, or induce vascular and stromal cell disruption, or immune-related cell death activation at the TME.

mine. Using this strategy, it was expected that once the enzymes were released in the acid conditions of the TME, the H_2O_2 would be decomposed by catalase to cause lower toxicity. Then, the hypoxia would activate the effect of tirapazamine. Indeed, the result of this treatment was the almost total eradication of the tumor growth in mice bearing EMT-6 mammary cancer cells [316].

A different strategy to mitigate tumor hypoxia has been trying to enhance the efficacy of the treatments. To this aim, catalase was entrapped in NCs or liposomes, in association with therapies, such as photodynamic [317] or radio-immunotherapy [318], respectively. In both cases, the therapeutic effects were remarkably improved. A similar approach to overcome hypoxia involved the encapsulation of catalase in anti-PDL1 surface-modified liposomes. The result of this treatment was an enhanced tumor accumulation, a decrease in tumor growth and an increase in survival time, all of which was attributed to the tumor infiltration of $CD8^+$ T cells and hypoxia relief [319].

So far, the use of nanoplatforms for the efficient delivery of these therapeutic enzymes in the TME has demonstrated the ability to exploit the tumor hypoxia condition, following co-delivery strategies. It remains to be seen whether ongoing research efforts will lead to next-generation strategies based on protein delivery.

4.2.1.2. Tumoral delivery of polynucleotide degrading enzymes. Several approaches have also been explored to achieve the intracellular accumulation of polynucleotide degrading enzymes. In an

example, HA nanogels were developed for the co-encapsulation of **DNase I** and modified-acidic-activatable hyaluronidase (aHAase). The success of this system lies in turning tumor conditions into therapeutic opportunities. Thus, under slightly acidic conditions, aHAase is partially activated, causing the degradation of the major constituent of the HA present in the extracellular matrix, thus allowing a deeper tumor penetration of the system. Once at the intracellular level, the nanogels escape from the endosomal compartment releasing DNase I intracellularly. This formulation led to a significant inhibition of tumor growth in human lung adenocarcinoma epithelial tumor bearing mice [320]. A similar strategy was described for **ribonuclease A (RNase A)**. In this case, nanoassemblies including RNase A-loaded NCs and an antibiotic against cancer stem-like cells, doxycycline, were developed. A marked tumor growth inhibitory activity was observed when these nanoassemblies were intravenously administered in a breast cancer xenograft tumor model [321]. More recently, the encapsulation of RNase into poly (L-glutamic acid)-graft-PEG methyl ether-based nanogels was proven to trigger the protein release under hypoxic conditions thanks to the destruction of the cross-linking point between β-cyclodextrin and azobenzene. The application of this system in a breast cancer model achieved a significant tumor suppression rate (68.7%). Interestingly, the efficacy of this approach was improved when it was combined with a nanosystem containing a vascular disrupting agent, i.e. combretastatin A4 [322].

Recent advances in biotechnology and the identification of case-by-case needs will contribute to the success of polynucleotides degrading enzymes-based therapies.

4.2.2. Delivery of apoptotic proteins

Some strategies have been applied to the intracellular delivery of apoptotic proteins, i.e., **Cytochrome *C* (Cyt C)**. Cyt C is a key component of the electron transport chain in mitochondria that binds to the protease activating factor-1 and triggers cell apoptosis. In a report, Cyt C was encapsulated into HA nanogels [323] and, also co-encapsulated with a plasmid DNA encoding the p53 protein [324]. The results of both approaches were similar in a SC and orthotopic breast tumor bearing mice model, respectively.

In a different work, the pro-apoptotic protein **Granzyme B (GrB)** loaded into HA nanogels could be delivered at the intracellular level, thus causing the reduction of tumor growth in subcutaneous human breast and orthotopic human lung tumor-xenografts mice models [325]. The outcome of the study could be improved by adding to the delivery carrier an epidermal growth factor receptor (EGFR) ligand, the GE11 peptide. The result of this treatment was the almost total suppression of the tumor growth in human ovarian carcinoma and human breast tumor-xenograft in mice [326]. A different approach using again GrB consisted on its conjugation with the cell-penetrating peptide, TAT, and further encapsulation into porous p-2-methacryloyloxy ethyl phosphorylcholine (PMPC)/HA NCs. This strategy led to a significant tumor accumulation which was correlated with a great reduction in tumor growth in a breast cancer model [327]. Similar strategies have been adopted for multiple myeloma (MM) treatment. In this context, polymersomes containing GrB were functionalized with HA for targeting CD44 in MM cells. This delivery approach led to a high accumulation of GrB in subcutaneous LP1 MM tumor as well as in the bone marrow of the orthotopic LP1 MM model. This accumulation was translated into a significant suppression of subcutaneous LP1 tumor and an increase survival in the orthotopic LP1 model [328].

The **Apo2 ligand**, also called **tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL or TRAIL),** has attracted attention due to its ability to induce apoptosis upon binding to the surface death receptors DR4 and DR5 on cancer cells. Unfortunately, so far TRAIL approaches could not be translated to the clinic due the unstable nature of the soluble form of the protein and to its short half-life (60 min in humans) among other reasons [329–331]. To overcome these limitations, TRAIL was associated to the surface of liposomes in combination with the adhesion receptor E-selectin. This receptor facilitates adhesion to selectin ligands leukocytes in blood and on tumor cells. Minimal administration resulted in a significant reduction of the metastatic burden following tumor resection in a 4 T1 breast carcinoma mice model [332]. In this regard, the surface liposomal display of Apo2 ligand/TRAIL protein led to marked tumor growth inhibition in HT-29 colorectal carcinoma, following intraperitoneal administration [333]. Likewise, the combination of RGD peptide and TRAIL with stimuli responsive elastin-like polypeptide-based NPs was reported to increase the half-life and to achieve an almost complete tumor regression in a colon carcinoma tumor xenograft model after single intraperitoneal administration [334].

Overall, the study of the delivery of apoptotic proteins is in its early-stages with only a few examples showing the efficacy of strategies. Subsequent development of this kind of treatments will shed light on its translational potential.

4.2.3. Immune regulator cytokines

Cytokines are immune regulatory proteins that have shown promising results in cancer therapy. However, their poor pharmacokinetic profile and their associated systemic toxicity have limited their therapeutic exploitation. Nanotechnology has been presented as a tool to overcome these drawbacks as illustrated by a number of examples described below.

As previously mentioned, the PEGylation of cytokines has been useful for increasing their half-life, however, other approaches have been later explored to improve their biodistribution and reduce their toxicity. For example, the intravenous administration of **IL-2 Fc and anti-CD137** anchored on the surface of PEGylated liposomes resulted efficacious in terms of reducing tumor growth in the absence of systemic toxicity, while the free drugs caused a lethal immunotoxicity. In this sense, the rapid accumulation of the liposomes in tumors could be responsible for their success [335]. Other combination therapies investigated have involved the co-administration of two cytokines, **IL-2 and IFN-γ,** and doxorubicin (DOX) into nanovesicles (NV). This combination of drugs was administered intravenously to triple negative breast cancer bearing mice leading to a significant inhibition of the primary tumor growth and lung metastasis. These results were explained by the induction of DCs maturation that was accompanied by the infiltration and activation of natural killer cells and CD8 + T lymphocytes, as well as the recruitment of CD45 + immune cells and Ly6G + neutrophils. In addition, the absence of systemic toxicity in terms of body weight was noticed in a murine melanoma model when IL-2-NV-DOX was compared to the free drugs [336].

In brief, the clinical use of IL-2 and IFN-alpha, as the only FDA-approved cytokines, were initially suggested as milestones in cancer treatment [337]. However, early findings of their short half-life and dose-related toxicities prompted the search for novel approaches. As noted above, several technologies have been shown to overcome these drawbacks and could potentially find their way to the clinic. Meanwhile, the development of specific immunotherapies, such as immune inhibitors checkpoints and others described in the following section, have attracted the attention of researchers and clinicians and, as a consequence, this still inefficiently exploited therapeutic approach was abandoned.

4.2.4. Monoclonal antibodies

The discovery of mAbs represented a critical milestone in cancer therapy. However, the therapeutic potential of these complex molecules has not yet been fully exploited due to their biopharmaceutical limitations. In this context, nanotechnology has been shown to be a useful tool for improving the accumulation of mAbs

in tumors, thereby enhancing their efficacy and reducing their systemic exposure. In addition, our laboratory [338,339] and others [340,341] have shown it is possible to design nanocarriers intended to target intracellular oncoproteins that, historically, have been considered "undruggable targets". This section will be devoted to highlighting the most disruptive technologies developed in the last few years for improving the delivery and, hence, the clinical benefit of mAbs. Table 4 shows the delivery carriers developed for either tissue or intracellular targeting, and the results of their proof-of-concept studies in different animal models, following their administration as a mono- or a combination therapy.

4.2.4.1. Delivery of mAbs targeted to the tumor microenvironment. The diversity of the TME composition is responsible for its therapeutic complexity since it supports tumor growth and participates in the development of resistances [363,364]. In this sense, fibrosis and high interstitial pressure have been reported as two of the main causes for the limited tumor accumulation [363]. Therefore, several efforts in the nanotechnology field have been directed towards target different molecules that are relevant in the TME. For example, the modification of the TME organization was possible with the administration of anti-IL6R Ab loaded liposomes decorated with an anti-CD44 Ab. This treatment blocked IL6R-Stat3 signaling and led to the reduction of the expression of several downstream molecules, such as MMP-9 and Sox2, which was accompanied by a reduction of angiogenesis and the macrophages phenotype conversion from M1 to M2. Ultimately, these effects translated into the inhibition of tumor metastasis in different breast cancer models [342].

Similarly, different nano-delivery systems have been proposed for the intratumoral delivery of **trastuzumab**, which targets the

Table 4
Summary of the most relevant nanocarriers for the delivery of mAbs.

mAb	Cellular target (location)	Delivery system	Therapy	Tumor model	Ref.
Delivery of mAbs to the TME					
anti-IL6R	IL-6 receptor (extracellular)	Anti-CD44 Ab-liposomes	Monotherapy	Breast cancer (orthotopic)	[342]
Trastuzumab	HER2 (extracellular)	Micellar nanocomplexes	Monotherapy	Breast cancer	[343]
Trastuzumab	HER2 (extracellular)	PEG-VitE based hydrogels	Monotherapy	Breast cancer	[344]
Bevacizumab	VEGF-A (extracellular)	PEG-HSA NPs	Monotherapy	Colorectal cancer	[345]
Bevacizumab	VEGF-A (extracellular)	Lipid-polymer hybrid NPs	Erlotinib/ bevacizumab	Non-small cell lung cancer	[346]
Bevacizumab	VEGF-A (extracellular)	Liposomes	Doxorubicin-loaded HER2 Ab decorated liposomes/ bevacizumab co-administration	Breast cancer	[347]
Bevacizumab	VEGF-A (extracellular)	HSA-bound paclitaxel (Abraxane®)	Abraxane®/bevacizumab	Melanoma cancer	[348]
Nimotuzumab / trastuzumab	EGFR / HER2 (extracellular)	MPC-peptide crosslinker based NCs	Monotherapy	Glioma (orthotopic)	[349]
Rituximab	CD20 protein (extracellular)	MPC/PLA-PEG-PLA/ GDMA-based NCs	Monotherapy	B-cell lymphoma (orthotopic)	[350,351]
Delivery of immune checkpoint inhibitors to the TME					
Anti-PD-1	PD-1 ligands (extracellular)	PEG-PLGA NPs	Monotherapy	Melanoma cancer	[352]
Anti-PD-1	PD-1 ligands (extracellular)	GRGDS peptide - PEG-PLGA NPs	Iron oxide/perfluoropentane/anti-PD-1	Melanoma cancer	[353]
Anti-PDL-1	PDL-1 ligands (extracellular)	HA-polylysine NPs	Chlorin e6/dextro-1-methyl tryptophan/anti-PDL-1	Melanoma cancer	[354]
Anti-PDL-1	PDL-1 ligands (extracellular)	Liposomes conjugated to Treg cells	Imiquimod/ IL-2/anti-PDL-1	Melanoma cancer	[355]
Anti-CTLA-4	CTLA-4 ligands (extracellular)	Liposomes	Doxil®/anti-CTLA-4 co-administration or monotherapy	Melanoma and colorectal cancer	[356,357]
Intracellular delivery of mAbs					
Antigasdermin B	Gasdermin B protein (intracellular)	HA-NCs	Monotherapy	Breast cancer (orthotopic)	[338]
Anti-KRAS	Mutated KRAS (intracellular)	HA-NCs	Monotherapy	Pancreatic and colorectal cancer (orthotopic)	[339]
Bevacizumab	VEGF-A (extra- and intracellular)	CD44v6 Fab-PEG-PLGA NPs	Monotherapy	N.A.	[358]
Bevacizumab	VEGF-A (extra- and intracellular)	Tween 80/Tween 20/ Brij 97 NPs	Monotherapy	N.A.	[359]
Bevacizumab	VEGF-A (extra- and intracellular)	Liposomes	Benzoporphyrin derivative-loaded PLGA-PEG NPs/ bevacizumab	Pancreatic cancer (orthotopic)	[360]
Bevacizumab	VEGF-A (extra- and intracellular)	Liposomes	Benzoporphyrin derivative/bevacizumab	Pancreatic cancer	[340]
anti-pKi-67	Ki-67 protein (intracellular)	Liposomes	~~Monotherapy~~FITC-anti-pKi-67-Ab conjugate	N.A.	[361]
anti-hTERT	hTERT (intracellular)	EM-coated self-assembling NPs	Monotherapy	N.A.	[362]
anti-S100A4	S100A4 protein (intracellular)	Fusogenic liposomes	Doxorubicin/anti-S100A4	Breast cancer	[341]

N.A.: not applicable, HSA: human serum albumin, Treg cells: regulatory T cells; MPC: 2-methacryloyloxyethyl phosphorylcholine, GDMA: glycerol dimethacrylate, PLA-PEG-PLA: poly(d,l-lactide)-b-poly(ethylene glycol)- b-poly(d,l-lactide)-diacrylate triblock copolymers, HER2: human epidermal growth factor receptor 2, VitE vitamin E; hTERT human telomerase reverse transcriptase; EM erythrocyte membrane; FITC: fluorescein 5(6)-isothiocyanate; VEGF-A: vascular endothelial growth factor- A.

HER2 [343,344] or **bevacizumab (BVZ)**, a VEGF-A blocker [345–348]. For instance, it is worth mentioning that the association of trastuzumab to micellar nanocomplexes resulted in a change in the pharmacokinetic and biodistribution profile of the mAb with a final output in terms of reduction of the tumor volume in a human breast cancer xenograft tumor model, following intravenous administration [343]. In another example, trastuzumab-loaded PEG- vitamin E hydrogels were also investigated for the treatment of breast cancer. Single SC administration near the tumor site led to a remarkable reduction in the tumor growth compared to both, the SC and intravenous administration of the free mAb [344]. Unfortunately, in the specific case of BVZ, the benefit achieved by the encapsulation in terms of improving the biodistribution or efficacy of the encapsulated monoclonal is still under debate. For example, some authors have found that the administration of BVZ-loaded PEG-HSA-NPs led to significantly higher intratumoral levels of BVZ, when compared to the free BVZ. However, this accumulation did not translate to a higher antitumor efficacy when the mAb was administered as a monotherapy [345]. However, more positive outcomes where reported for treatments involving the co-encapsulation of BVZ and other drugs in the same NPs [346–348]. This should not be surprising if we take into account that, at the clinical level, BVZ is always administered as a combination therapy.

Nanotechnology has also been investigated as a way to facilitate the delivery of drugs to tumor areas of difficult access, i.e., the **central nervous system (CNS).** For example, taking advantage of the capacity of choline and acetylcholine analogues to overcome the brain-blood barrier, NCs made of these analogues were developed for the encapsulation of **nimotuzumab or trastuzumab**. The resulting formulations were found to facilitate the accumulation of the mAbs in the cerebrospinal fluid and brain and showed remarkable antitumor efficacy in a glioma mice model [349]. In a different study PLA-PEG-PLA NCs containing **rituximab (RTX),** were found to facilitate, following intravenous administration, the delivery of the mAb to the CNS where significant levels were maintained for up to 4 weeks. The final output of this formulation was a decrease in the brain lymphoma metastases in a non-Hodgkin lymphoma xenograft murine model [350]. The relevance of this approach in immunocompetent animals, rats and non-human primates (NHPs), was also reported. The results showed higher RTX levels in CNS (10-fold) and in LN (2–3 fold) in both animal models when compared with the free molecule. Interestingly, NCs revealed lack of blood, liver or brain toxicity in NHPs [351].

4.2.4.2. Delivery of immune checkpoint inhibitors to the tme. The discovery of ICIs, such as **anti-CTLA-4, anti-PD-1 or anti-PDL-1** represented a major milestone in oncological therapy. However, these mAbs are far from ideal as significant immune-related side effects associated with their indiscriminate biodistribution have been reported [365,366]. Once more, researchers have used nanotechnology to overcome this limitation. For example, Ordikhani et al. proposed the incorporation of anti–PD-1 in NPs composed of mPEG-PLGA for the treatment of melanoma. Results demonstrated the uptake of NPs by CD11c + dendritic cells (DCs) in the spleen, which triggered DCs activation and maturation. As a result, effector T cells were activated, resulting in a strong immune response. The administration of a low dose of anti-PD-1 demonstrated a marked antitumor efficacy in a murine melanoma model, which was mainly attributed to the selective uptake of NPs by DCs in secondary lymphoid tissues [352]. A different technological approach consisted in encapsulating **anti-PD1**, in PLGA NPs, functionalized with PEG and Gly-Arg-Gly-Asp-Ser peptides in combination with a photothermal therapy. This synergistic treatment allowed a controlled release of the anti-PD1 by thermal treatment and increased the infiltration of CD8 + T cells to the TME, reaching an almost complete tumor regression [353]. Another approach combined the use of NPs of HA, polylysine derivatives and anti-PDL1, with photodynamic therapy. This immunostrategy was found to be efficacious against tumor metastasis, relapse, and post-surgical regrowth [354]. Similarly, a combination therapy consisting of **imiquimod, anti-PDL1 and IL-2** encapsulated in liposomes and, then, conjugated on the surface of regulatory T cells, triggered the maturation of DCs, inhibition of the PD-1/PD-L1 immune-checkpoint, and infiltration of CD8 + T cells, resulting in a strong suppression of primary and metastatic tumors [355]. Finally, **anti-CTLA-4 mAb**-loaded PEG-liposomes led to a significant tumor growth reduction and increased CD8 + population and CD8+/Treg ratio in tumor-infiltrated lymphocytes, when compared to the free CTLA-4 administration. Moreover, the administration of anti CTLA-4 liposomes prior to Doxil® led to an improvement of the antitumor efficacy in a melanoma mouse model [356]. These results correlate with previous studies, where the antiCTLA-4 encapsulation into PEGylated liposomes significantly increased the tumor accumulation via the enhanced permeation and retention (EPR) effect [357].

4.2.4.3. Intracellular delivery of mAbs. The majority of the oncoprotein targets remain elusive because they are localized at the intracellular level and do not exhibit the adequate pockets for interaction with small molecules. The term "*undruggable*" has been coined to describe this situation. Although the most frequent oncogene mutation is the Kirsten Rat Sarcoma Viral Oncogene Homologue (KRAS), others such as MYC, MYB, and nuclear factor-κB (NF-κB) have been described as undruggable oncogenes [367]. MAbs could be ideal candidates to interact with these targets, however, so far, their use has been restricted by their inability to enter the cancer cells. Although several approaches such as cell-penetrating peptides and physical methods (e.g., electroporation or microinjection) have been used to this end, alternative strategies must be explored in order to protect the mAb and reach a translation in humans, clearly discarded in the case of physical methods [368].

A few years ago, our group, was a pioneer in demonstrating the feasibility of targeting the intracellular oncoprotein gasdermin B (GSDMB). Indeed, HA NCs were developed for the delivery of **anti-gasdermin B (AbGB)** (Fig. 9)**.** Among the results that support the preclinical relevance of this prototype, it is important to mention the ability of AbGB-loaded NCs in reducing the migration rate of tumor cells and the reduction of the tumor volume in a HER-2 + breast cancer model. These outcomes were a consequence of the accumulation of the AbGB inside the tumor cells and its ability to interact with the target GSDMB [338].

These results encouraged us to move forward and develop a proprietary nanotechnology platform (Multi-functional Polymeric Nanocapsules, MPN Technology®) for the intracellular delivery of **anti-KRAS mAb** [339]. The KRAS target is one of the most relevant and challenging oncological targets. One specific mutation, KRAS-G12C could be finally targeted after decades of efforts [369], however, other mutations of the same target still remain "undrugged". Therefore, our laboratory's efforts have focused on finding ways to reach other KRAS mutations. The results obtained so far have shown promising *in vivo* responses in several murine models harboring G12D and G12V KRAS mutations. To our knowledge, this is the first nanotechnology that has been proven to facilitate the intracellular delivery of the anti-KRAS mAb, thereby eliciting anti-tumoral responses following intravenous injection in different mice cancer models [339].

Other authors have explored the effect of nanoformulations of mAbs whose target is localized either inside or outside the cells. This is the case of the VEGF-A, whose inhibition at the intracellular level has been proposed as a therapeutic strategy. Based on this premise, BVZ was encapsulated into PLGA-PEG NPs decorated with

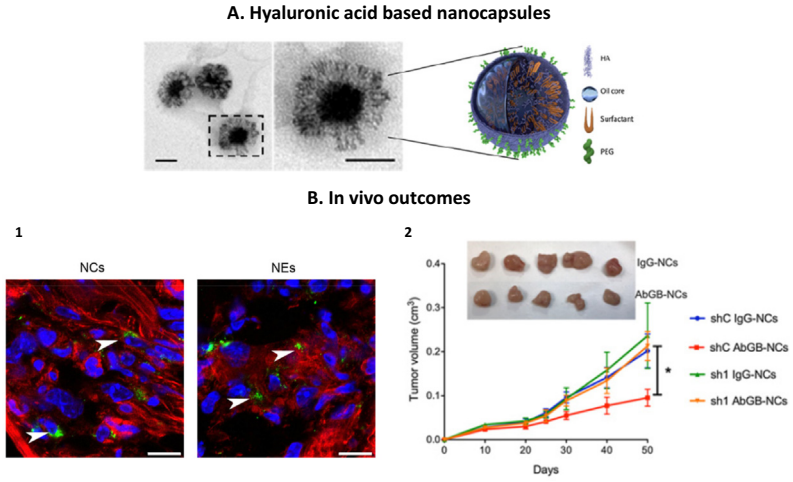

Fig. 9. A. Schematic representation and images of HA NCs obtained by transmission electron microscopy (TEM). Scale bar, 100 nm. **B**. Main outcomes of the *in vivo* assay. **1)** Representative confocal microscope images of breast tumor cryosections showing intracellular *in vivo* accumulation (arrows) of the NCs containing FITC-AbGB (FITC-AbGB-NCs) and nanoemulsion (lack of HA) containing FITC-AbGB (FITC-AbGB-NE) in green; tumors were stained with phalloidin (F-actin; red) and DAPI (blue). Scale bar, 10 μm. **2)** AbGB-NCs reduce tumor growth *in vivo* by increasing cell death rate specifically in GSDMB-positive breast tumors. Mice were inoculated with either control cells (shC) or GSDMB silenced (sh1) cells and treated with AbGB-NCs or NCs loaded with an irrelevant IgG (IgG-NCs *p < 0.05). Inset: tumor size *ex vivo* of shC tumors after the treatment with IgG-NCs or AbGB-NCs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) Reproduced with permission from [338]

an antibody fragment (Fab) specific for CD44v6-expressing human cancer cells and the resulting formulation was found to be effective in terms of reducing VEGF intracellular levels in a human stomach adenocarcinoma epithelial cell line [358]. Additionally, self-associated NPs further demonstrated its effective internalization in a non-small cell lung cancer cell line [359]. In other examples, liposomal strategies for the co-delivery of BVZ and the photodynamic therapy agent benzoporphyrin derivative monoacid (BPD), have been found to significantly reduce tumor growth in a SC pancreatic ductal adenocarcinoma mouse model [340] and orthotopic murine pancreatic model [360]. With both strategies, a BDP synergistic effect and VEFG-A intracellular blocking could be obtained.

Other intracellular delivery approaches explored so far include a liposomal formulation containing a photoactivatable anti–pKi-67 Ab conjugate that aimed to reach the nucleus [361], erythrocyte membrane-coated anti-hTERT mAb NPs [362] and fusogenic liposomes loaded with **anti-S100A4 mAb** and doxorubicin, for cytoplasmatic delivery. This last formulation was also tested in a breast cancer xenograft model, and the results showed not only the reduction of the tumor growth but also the suppression of liver metastasis [341].

5. Conclusions and prospect view

The irruption of biological macromolecules as therapeutics, and the recognition of their limitations to overcome biological barriers have motivated the development of technological designs for their successful delivery. As a result, the pharmaceutical nanotechnology field has devoted significant efforts to leverage formulations intended for the treatment of a variety of diseases following different routes of administration. In this endeavor, particular challenges and opportunities were identified when addressing each administration modality, shaping the current direction of the investigations in each area.

Regarding **the systemic delivery of macromolecules following transmucosal administration,** special attention has been devoted to enable their administration through the oral and nasal route. Still, as it is generally the case for transforming technologies, the field of transmucosal protein/peptide delivery is taking longer than initially anticipated. Of note, initial works exploring the **oral modality of administratio**n showed great promise based on results obtained with relatively simple formulations, whose mechanism of action was uncertain. Subsequent efforts were unable to outweigh previous results, but rather shed light on the importance of the physicochemical properties of the nanocarriers on their capacity to overcome the intestinal barriers. In this context, technical limitations to an accurate comparison of the performance of nanocarriers *in vitro* are manifest. The diverse physicochemical properties of both, the loaded drugs and the nanostructures and the different *in vitro* experimental set-ups make it very difficult to have a comprehensive comparative evaluation. At the *in vivo* level, the experimental divergences among studies influence dramatically the outcome of the studies. As an example, the available animal models for the evaluation of nanocarriers delivering insulin, the model peptide most frequently employed, present several significant limitations. The healthy non-diabetic rat model provides a closer resemblance to a clinical scenario, where blood glucose levels are maintained within certain limits on account of auto regulation mechanisms [42]. However, as a consequence, only modest responses are to be expected in healthy rats [82,370]. On the other hand, the diabetic rat model allows low amounts of absorbed insulin to exert a pronounced effect due to the β-cell deficiency [42]. Notwithstanding, the induction of diabetes, typically through streptozotocin (STZ) injections, results in an artificial and variable animal model [370]. As a result, the evaluation of formulations may vary significantly from study to study. On the other hand, absolute bioavailability is usually regarded as the reference parameter to compare the performance of formulations. However, bioavailability values are highly influenced by the experimental conditions. For instance, the administration route (oral gavage, intestinal injection, or *in situ* intestinal loop), the use of anesthesia, the fasting time, and other factors can significantly alter the outcome of the study [6,371–373]. Thus, the comparative assessment of formulations based on bioavailability values, or in the blood glucose levels in the case of insulin formulations, should be carried out cautiously. Moreover, the divergences between the results obtained in small and large animal models, and in humans, highlights the challenge posed by the anatomical and biological differences among the available experimental models and patients [374].

To overcome these limitations, the scientific community is dedicating efforts to propose guidance criteria for standardization and accurate comparison of results [375]. Hopefully, this initiative will also lead to optimized *in vivo* evaluation models in all the stages of formulation development, accelerating the translation from bench to bedside. In addition, and thanks to the know-how provided by the extensive work on nanocarriers for oral peptide/protein delivery, more complex delivery designs have been proposed, leading to the development of functionalized and multicomponent nanosystems as well as disruptive technologies such as microneedle-based systems, which have yielded promising results. Meanwhile, simple technologies such as ILs and DESs are receiving significant attention, also providing encouraging results. Furthermore, the intense activity in the field has led to much-awaited clinical advances. Briefly, several formulations continue in active evaluation in advanced phases, while new technologies initiated Phase I, including Rani's robotic pill. Moreover, penetration enhancers (1980′s), have made their way to the market with the approval of Rybelsus (2019). Overall, the evolution of the field has taught us that, despite the limitations encountered, the advances made in the last decade have paved the way for the transmucosal protein/peptide oral delivery of a growing number of macromolecules.

In contrast to the oral modality of administration, the **nasal route** has been characterized by a limited activity. In this case, the limitations associated to rodents as animal models are particularly important and the use macaques is recommended to have relevant information about the performance of formulations. The current clinical scenario of nasal protein/peptide delivery relies on the use of functional excipients rather than nanotechnologies. However, there is not a clear argument to discard the potential of nanotechnology. Rather than this, the success obtained through nasal vaccination and the irruption of the N-t-B delivery strategy are now taking a leading role in the nasal field. Time will tell whether this scenario will be translated into a significant clinical input.

Regarding the **development of antigen delivery systems for vaccination**, it is clear that nanotechnology will continue to play a key role, particularly considering the additional adjuvant potential nanosystems can provide to vaccine formulations. This can be achieved through the inclusion of biomaterials with immunomodulatory properties in the composition of the carriers, or through the co-encapsulation of immunomodulatory molecules. Different types of nanocarriers may offer specific advantages for vaccination against distinct diseases while using different modalities of administration. In the development of this field, we should also keep in mind the need for consistent evaluation of these nanocarriers, not only in terms of their physicochemical properties, but also considering the variety of animal models, antigen types, and immune response assays. Specifically, it would be positive to establish standard protocols to evaluate the immune response generated by these vaccine candidates, to favor comparisons between different published studies. This could include, for example, standardization of time points for measuring antibody levels, cytokine production or cellular response, or the inclusion of a well-established SC/IM control formulation.

These hurdles in the interpretation of the literature may justify some difficulties for the translation of vaccine delivery technologies to the clinical development phase. Fortunately, recent developments in SARS-CoV-2 vaccines, fundamentally anchored on nanotechnology to sustain their efficacy, offer a new and more promising scenario. These recent achievements will certainly be remembered as a key milestone in this research field. The delivery of genetic material without the protection provided by nanocarriers, would have limited efficiency, mainly due to the easy degradation of these products by enzymes and their generally low permeability [253,259]. Developing novel nanocarriers for the delivery of these antigens is therefore inevitable, and largely already ongoing. In fact, as millions of people around the world are vaccinated with the approved mRNA vaccines against the COVID-19 pandemic, invaluable safety and efficacy data is being gathered on these formulations, which will certainly pave the way for their application in other areas, such as therapeutic cancer vaccines or immunotherapy.

In addition to this generated knowledge, as the understanding around different nanomaterials and their properties increases, their use as antigen nanocarriers in vaccine development will certainly open a new era in this field. The opportunities for targeted antigen delivery, enhanced adjuvant activity and co-administration of multiple antigens and/or antigens and adjuvants are countless, making nanovaccines the most promising candidates for a new generation of vaccines against infectious and other diseases.

In the context of **oncological therapies**, the development of protein delivery strategies is still in early stages. Despite the number of nanomedicines that have made their way to the market, its clinical and preclinical failures observed lies in numerous factors yet to be resolved such as NPs accumulation in liver and spleen, the limited diffusion across the tumor environment, the difficult access to metastatic niches and, ultimately, the hurdles associated to the intracellular delivery. The true potential of targeted delivery systems is yet to be realized specially for the protein/peptide drugs.

Aiming at confronting these challenges from early stages of formulation design and characterization, including *in vivo* anti-cancer efficacy studies, we must keep in mind the difficulties for mimicking the human tumor complexity, heterogenicity and immune status. Particular considerations such as the EPR effect, tumor size and mouse strains must be prudently considered in order to provide conclusive data. For example, high tumor volumes at the onset of treatment normally reduce the efficacy of the therapy, which might be due to low penetration and increased hypoxia [376]. Moreover, NPs clearance may be governed by the mouse immune status, thus, Th2 strains such as BALB/c elicit faster clearance when compared with Th1 strains (e.g., C57BL/6) [377]. As a whole, the generation of advanced animal models such as patient-derived xenografts, humanized or genetically engineered mouse models with aggressive metastasis are fundamental for the clinical translatability of the nanomedicine products [378]. In this sense, the use of *in silico* predictive models that could merge tumor variability over time, resistance as well as transport barriers faced by NPs, which will allow to improve predictive equations. However, to properly implement this and similar strategies, further knowledge of the physiological scenario, tumor type, NP-cell interaction, and transport mechanisms, must be generated.

It should also be mentioned that the development of nanocarriers for the intracellular delivery of monoclonal antibodies has been an important achievement within this field. We are confident that the design of new mAbs and nanobodies against undruggable targets is a promising next step for nanoncologicals therapeutics. In parallel, the emergence of immunotherapies has changed the concept of tumor therapy, providing the possibility to target immune cells located both in the tumor area and in relevant distant organs such as the spleen. Thus, the application of nanotechnology offers the opportunity to manage the antitumor immune response, either by blocking or by boosting its response.

As a whole, the field of oncological nanotherapies is moving towards introducing special chemical modifications in the nanosystems that can exploit tumor pathophysiological conditions. Ultimately, the design of personalized strategies based on case-by-case needs and a better understanding of oncological demands will be crucial to open up new avenues of treatment.

Finally, in our view, the main common challenge in the development of new nanomedicines, relies on the fact that small changes in the composition may drastically change the pharmacokinetic and pharmacodynamic profile. This, together with the limited standardized regulatory guidelines by the FDA and EMA for nanomedicines, has made difficult the translation of nanomedicines to the clinical development phase. However, now this scenario is moving toward a more positive projection. Of note, significant efforts are being taken in the regulatory sciences framework, as illustrated in the REFINE NANOMED project [379], which aims to provide input for the risk–benefit assessment of nanomedicines. Moreover, the case of COVID mRNA vaccines making use of nanoparticles together with recent increase of nanomedicines in the market, we could anticipate that, overall, the clinical exploitation of biological drugs will significantly benefit from the advances in the nanomedicine field.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of Competing Interest

M. J. Alonso and D. Torres are founders and shareholders of SmartVitamins. M. J. Alonso is founder and shareholder of LiberaBio. The rest of the authors declare no conflict of interest.

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