Biopharmaceutical nanoclusters: Towards the self-delivery of protein and peptide therapeutics

Danielsen, Mia; Hempel, Casper; Andresen, Thomas L.; Urquhart, Andrew J.

Published in:
Journal of controlled release

Link to article, DOI:
10.1016/j.jconrel.2022.04.050

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

Journal of Controlled Release 347 (2022) 282–307

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Review article

Biopharmaceutical nanoclusters: Towards the self-delivery of protein and peptide therapeutics

Mia Danielsen, Casper Hempel, Thomas L. Andresen, Andrew J. Urquhart *

Department of Health Technology, Technical University of Denmark, 2800 Kongens Lyngby, Denmark

ARTICLE INFO

Keywords:
Nanoparticles
Nanoclusters
Biopharmaceutical
Biologic
Methodology
Drug delivery

ABSTRACT

Protein and peptide biopharmaceuticals have had a major impact on the treatment of a number of diseases. There is a growing interest in overcoming some of the challenges associated with biopharmaceuticals, such as rapid degradation in physiological fluid, using nanocarrier delivery systems. Biopharmaceutical nanoclusters (BNCs) where the therapeutic protein or peptide is clustered together to form the main constituent of the nanocarrier system have the potential to mimic the benefits of more established nanocarriers (e.g., liposomal and polymeric systems) whilst eliminating the issue of low drug loading and potential side effects from additives. These benefits would include enhanced stability, improved absorption, and increased biopharmaceutical activity. However, the successful development of BNCs is challenged by the physicochemical complexity of the protein and peptide constituents as well as the dynamics of clustering. Here, we present and discuss common methodologies for the synthesis of therapeutic protein and peptide nanoclusters, as well as review the current status of this emerging field.

1. Introduction

Biopharmaceuticals (e.g., therapeutic proteins and peptides) are increasingly being used for the treatment of a range of diseases such as diabetes, rheumatoid arthritis, and different types of cancer [1,2]. Biopharmaceuticals are characterized by having high specificity and affinity towards targets and a reduced risk of side effects compared to many other types of therapeutics [3]. However, the instability of proteins and peptides can complicate manufacturing, formulation, and storage as well as impair the in vivo pharmacokinetic and pharmacodynamic performance. The therapeutic potential of biopharmaceuticals is further challenged by the poor absorption and high susceptibility to acid- and enzyme-catalyzed degradation in the gastrointestinal (GI) tract. For this reason, biopharmaceuticals are routinely dosed via invasive administration routes, which are associated with poor patient compliance [3]. Thus, to improve compliance, there is a need for new formulation strategies enabling the delivery of proteins and peptides via alternative administration routes and/or to reduce the dosing frequency.

Encapsulation in a nanocarrier system (e.g., liposome or polymeric), co-delivery of absorption enhancers (e.g., cell-penetrating peptides) and protein modifications are strategies to improve stability and uptake and prolong circulation times [1,3–6]. Nanocarriers can improve macromolecular stability, reduce clearance, and prolong the physiological fluid half-life due to the protection of individual macromolecules in the formulation [3,7]. Furthermore, nanoparticles have shown potential to be taken up by cells via different transcellular endocytotic mechanisms albeit limited by size [8] (Fig. 1). This can be an advantage in oral drug administration where GI uptake is essential for the biopharmaceutical to reach the blood circulation. Potential disadvantages of the cellular uptake property of nanoparticles are only expected to precede in few specific cases such as local invasive administration of drugs with cell surface targets, or in the case of non-specific uptake in reticuloendothelial organs causing clearance of the drug before onset of its therapeutic effect. The use of proteins and peptides as nanocarriers are increasingly making an impact in the clinic but these systems predominantly transport small molecule drugs. As an example, Abraxane® is an albumin nanocarrier loaded with paclitaxel, which represents a protein-nanocarrier formulation that has reached the market [9]. A number of reviews of protein and peptide nanoparticles for delivery of small molecule drugs exist, and we direct the reader's attention to these [7,10,11].

The transition of nanoparticle formulations for delivery of biopharmaceuticals into the clinic is often limited by formulation challenges such as instability and low drug loading. Compared to small

* Corresponding author at: Department of Health Technology, Technical University of Denmark (DTU Health Tech), Denmark.
E-mail address: anur@dtu.dk (A.J. Urquhart).

https://doi.org/10.1016/j.jconrel.2022.04.050
Received 10 March 2022; Received in revised form 27 April 2022; Accepted 28 April 2022
Available online 13 May 2022

molecule drugs, proteins and peptides are relatively sensitive structures that are prone to misfolding and aggregation, thereby reaching a thermodynamically more favorable energy state [12–14]. Aggregation and misfolding of high molecular weight proteins (e.g., antibodies) is usually associated with a loss of biopharmaceutical activity and potential cytotoxicity. Furthermore, the use of nanocarriers and non-therapeutic additives can potentially be associated with a risk of reduced therapeutic efficacy due to reduced drug loading, limited drug release, as well as a risk of long-term side effects. Complete drug safety profile of many additives recognized as being safe by the FDA are still not established, and some compounds may accumulate in the body over time to give toxic and immunogenic reactions [15–17]. Furthermore, inconveniently large doses may be required of nanoparticles with low drug loading. Finally, since the cellular uptake of nanoparticles appears to occur via active transport mechanisms, there will likely be an upper internalization limit, which may not allow delivery of the minimum effective dose within a certain timeframe if the bulk of the nanocarrier is composed of non-therapeutic and the biopharmaceutical is to be delivered intracellulary [16].

Although nanocarrier systems with low drug loading capacities can be sufficient for therapy with high-potency drugs and many biocompatible additives exist, the above-mentioned challenges have motivated the development of novel delivery systems. Here, the therapeutic protein or peptide comprises the bulk of the nanocarrier system, and the non-therapeutic carrier material is eliminated or significantly reduced. Such nanocarrier delivery systems have been developed the past decade

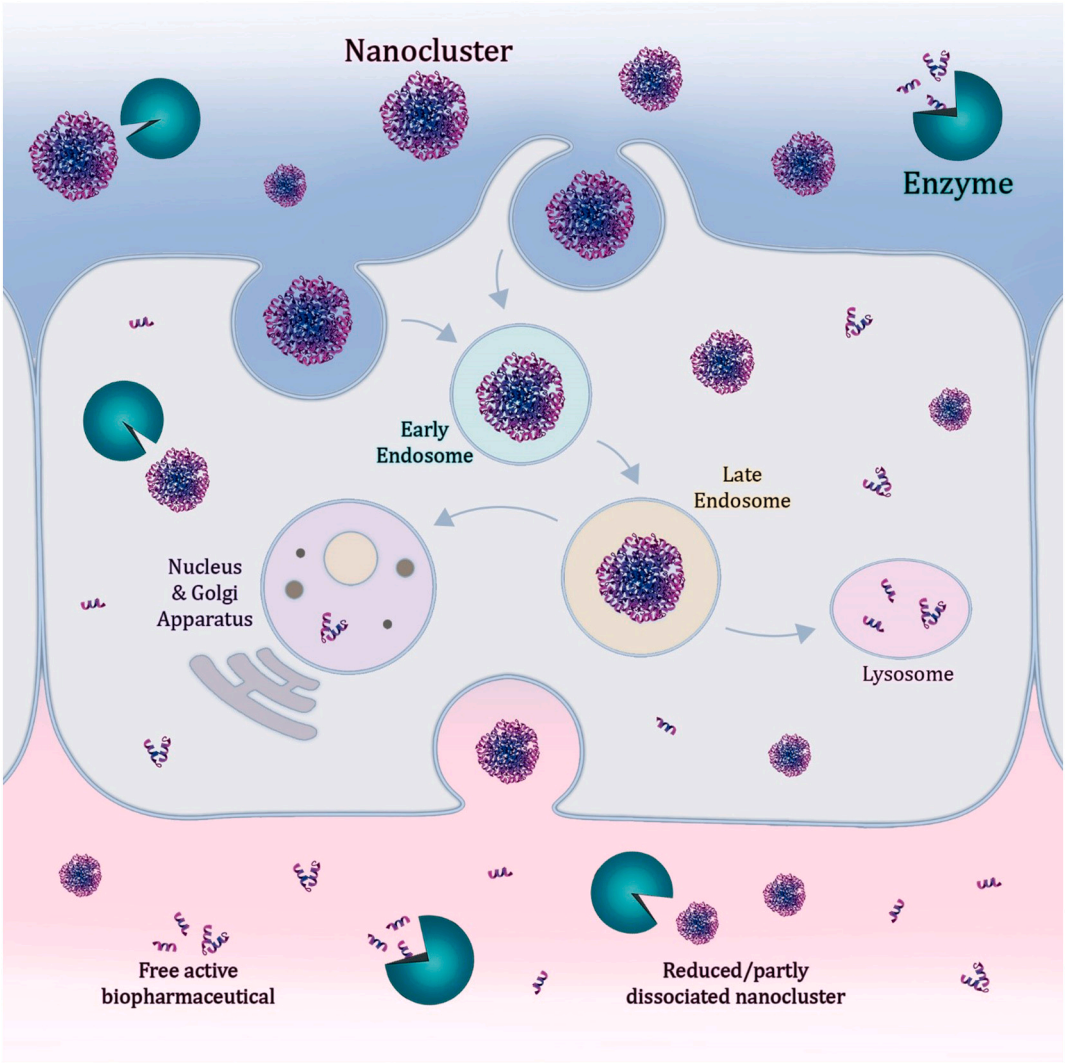

Fig. 1. The ideal biopharmaceutical nanocluster (BNC) formulation; improved enzymatic resistance, internalization, and pharmacokinetics. The structure, size, and high sensitivity to degradation in physiological fluid are major limiting factors in the administration of biopharmaceuticals that may be overcome by clustering of the protein or peptide molecules into reversible BNCs. In an intact BNC structure, enzymes will likely have a limited access to biopharmaceuticals. This will inhibit the otherwise rapid degradation of biopharmaceuticals in physiological fluids and allow time for cellular uptake of the BNCs as well as reaching of their target to exert a biopharmaceutical effect. BNCs as well as nanoparticles in general have potential to be taken up by cells via transporters or in a facilitated manner, e.g., receptor-mediated endocytosis. In contrast, the uptake of the free form of biopharmaceuticals is usually limited to few mechanisms if any at all. The ideal BNC will survive the degradative processes following drug administration while slowly dissociating into the free and active therapeutic. Depending on the exact BNC structure, some biopharmaceuticals may even be in a position in the BNC structure that allows for interaction with their target molecules without significant BNC break-up. While some biopharmaceuticals have extracellular targets (i.e., surface receptors), others will work intracellularly. The fate of a BNC following cellular uptake may involve intracellular processing (i.e., trafficking via endosomes to the nucleus and ultimately the lysosomes for degradation), recycling to the surface, or even survival of the intracellular environment allowing for exocytosis to reach other organs of the body (e.g., the bloodstream depending on the route of administration). Successful development of BNCs will contribute to the development of formulations that require less frequent drug administration and maybe even delivery via non-invasive administration routes. True BNCs consisting solely of the biopharmaceutical will furthermore have a decreased risk of side effects from additives.

and have been referred to as nanoclusters (NCs) [18–25]. The general use of the NC term dates to the beginning of the 20th century, and it has since been widely applied within different scientific fields, examples being gaseous ion clusters, metal clusters, and protein clusters [26]. Early studies used the cluster term to describe reversible aggregates of proteins including antibodies formed in highly concentrated solutions [19,20,27–30]. In 2015, the concept of protein NCs was presented as a relatively new type of protein formulation that needed further evaluation [31]. Despite a wide application of the term, protein-based studies seem to agree on the definition that protein NCs are reversibly self-assembled protein structures [18–25,27–30]. Correspondingly, these are defined as biopharmaceutical nanoclusters (BNCs) in this review. Here, we present the status in the emerging field of BNCs (Fig. 1). Since several methods are used to synthesize the BNCs, the achievements are sectioned according to the methodology of BNC synthesis (Fig. 2). The main characteristics are summarized with respect to the formulation strategy and associated challenges, the class of therapeutics to which the formulation strategy has been applied, as well as in-vitro and in-vivo activity including routes of administration and stability considerations (Table 1). A detailed breakdown of the studies included in the review is presented as well (Tables 2 and 3).

2. Desolvation and crosslinking

Desolvation represents the most popular method for preparing BNCs and is one of several synthesis methods commonly categorized as coacervation. In desolvation, a miscible pair of a solvent and antisolvent is used to precipitate the protein or peptide in a controlled manner. Addition of a poor solvent (antisolvent) to a solution of the biopharmaceutical will displace the water molecules from the surface of the biopharmaceutical into the bulk solvent. As a result, the solubility of the biopharmaceutical decreases, which promotes clustering and results in protein nuclei formation and BNC growth. Desolvation is associated with a favorable entropy with the displacement of relatively high-order water molecules from the protein surface to the bulk solution reducing the protein entropy while increasing the disorder of the surrounding system [61,62]. Sufficiently strong repulsion between the molecules will increase the resulting BNC stability by preventing excessive aggregation. To increase interparticle electrostatic repulsion, the process of desolvation is usually completed at a pH value some distance from the isoelectric point (pI) of the biopharmaceutical [62]. Desolvation can be followed by crosslinking to stabilize the particle structure. The use of glutaraldehyde (GTA) as a crosslinking agent post-desolvation has been "the golden standard" for many years since its premiere in the late 1970s [63]. However, in more recent years the use of other types of crosslinkers has increased due to their potential for targeted delivery and toxic considerations related to GTA [64,65]. In contrast to the irreversible crosslinking mechanism of GTA, the application of alternative reversible crosslinking methods is increasing. These include UV-induced crosslinking and as well as chemical crosslinkers that are designed to be cleaved in response to specific pH values, reducing or oxidative agents, or the presence of enzymes. Crosslinkers can have a stabilizing effect on biopharmaceutical molecules and BNCs, but one should be aware of the risk of introducing structural changes in the protein [39,66–68].

2.1. Immunotherapeutic nanoclusters synthesized by desolvation

BNCs of peptide subunit epitopes of the oncofetal antigen (OFA) previously identified as T-cell epitopes for killing of cancer cells have been successfully developed with sizes ranging from 148 to 261 nm. The peptides were crosslinked using trithiol followed by desolvation using HFIP/DEE as solvent/antisolvent pair for BNC formation. The 3,3′-

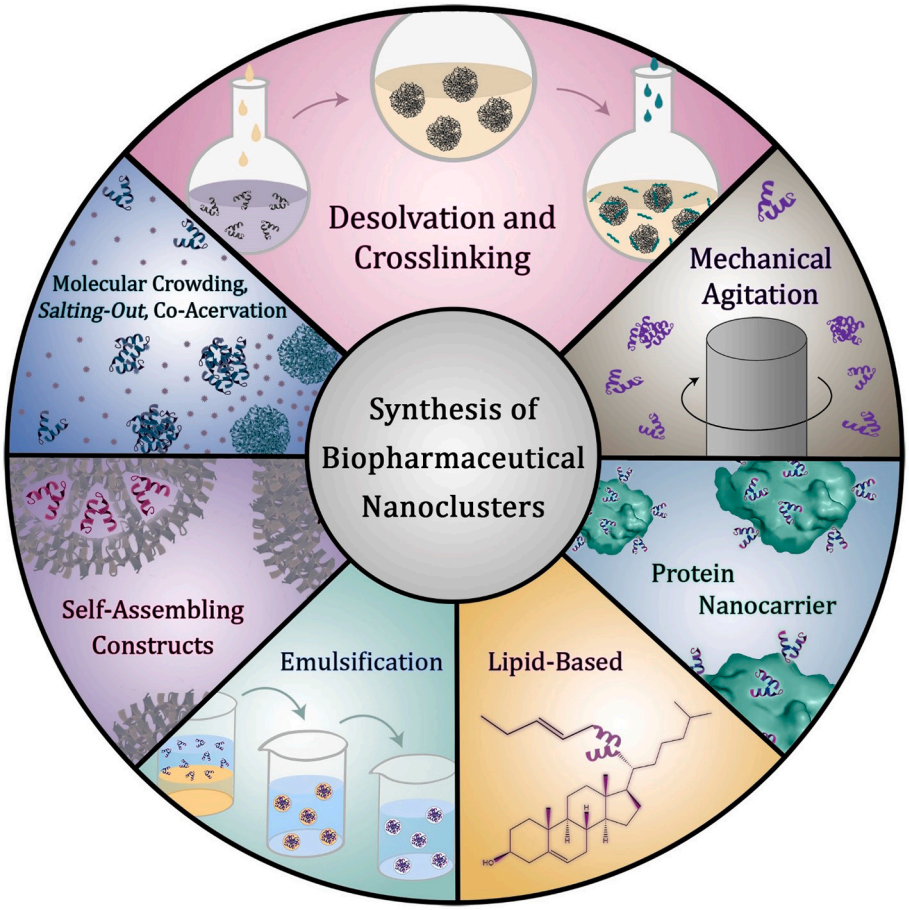

Fig. 2. Methodologies for synthesis of biopharmaceutical nanoclusters (BNCs). The solubility of a biopharmaceutical can be reduced by addition of a poor solvent to form BNCs, which may be stabilized by surface crosslinking (Desolvation and Crosslinking). Cluster-promoting agents such as sugars or salts as well as phase-separation by change in solvent polarity or pH value will promote short-range protein interactions and promote BNC formation (Molecular Crowding, Salting-Out, and Co-Acervation in general). The sensitive structural behavior of proteins and peptides which makes them prone to misfolding and aggregation may be utilized in a controlled manner to form well-defined BNCs (Mechanical Agitation). Self-assembling sequences have been identified and have potential for the design of constructs containing the biopharmaceutical of interest that self-assemble into BNC structures under specific conditions (Self-Assembling Constructs). Dispersion of two or more immiscible phases can be utilized to form nanosized droplets in which therapeutic proteins can be loaded (Emulsification). Liposomes and solid lipid nanoparticle formulations are challenged by very low drug loading and currently represents a field for which true BNCs have not shown potential (Lipid-Based). Protein nanocarriers can be used for delivery of biopharmaceuticals by use of any of the above-mentioned formulation strategies (Protein Nanocarrier). Synthesis of a true high-loaded BNC formulation is challenged by the presence of non-therapeutic such as crowding agents, crosslinkers, self-assembling sequences, lipids, or nanocarriers.

Table 1
Overview of biopharmaceutical nanocluster (BNC) formulation strategies.

Synthesis method	Method description	Main formulation challenges	Class of therapeutics	In-vitro stability and structural considerations	In-vitro biological activity	Applied routes of administration	In-vivo biological performance
Desolvation	Addition of a poor solvent (antisolvent) to a solution of the biopharmaceutical Often followed by crosslinking of the BNC surface Few times protein crosslinking has been applied prior to desolvation and NC formation	Structural damage caused by harsh antisolvents (usually organic solvents) which may be irreversible Crosslinking may reduce the drug loading significantly	Proteins and epitopes for the treatment of cancer and for vaccination against influenza Enzymes	Improved shelf-life stability and resistance towards enzymatic degradation achieved for some BNCs, while others suffer from aggregation or dissociation upon storage	Improved cellular uptake Reduced biological activity of structurally damage proteins	ID IN IM	Higher immune responses following BNC treatment compared to treatment with the free biopharmaceutical BNCs appear to have a self-adjuvanting effect
Molecular Crowding and Salting-Out	Addition of crowding agent at relatively high concentration or until the interactions between the biopharmaceutical molecules causes clustering into BNCs	Low drug loading in the final drug formulation (due to high concentration of crowding agent)	Antibodies Proteins and peptides for glycemic control Peptides for treatment of bone disease	No or minor reversible structural changes in the biopharmaceutical BNC dissociation upon dilution in physiological relevant media of most BNCs	Improved cellular uptake	IV SC IP	The BNC structure allows for delivery of higher concentrations using smaller dosing volumes Prolonged therapeutic effect and retention at site of SC injection compared to free therapeutic for some BNCs while others did not show any therapeutic improvement
pH coacervation	Titration to reach a pH value of low biopharmaceutical solubility	N/A	Antibodies Protein for glycemic control	Conformational damage to larger biopharmaceutical structures (i.e., antibodies)	Improved cellular uptake	IM IG IV	Slower uptake, prolonged duration of action and/or higher pharmacological response following IM and IG administration but not IV administration
Mechanical Agitation	Application of mechanical forces to introduce structural changes	Identification of controlled conditions for BNC formation without misfolding	Enzymes Antibodies	Increased stability under GI-like conditions Poor shelf-life stability	No or slight decrease in biological activity	N/A	N/A
Lipid-Based	N/A	N/A	Antithrombotic peptide	Preserved native structure and very good shelf-life stability	Improved cellular uptake	IV	Faster onset of action and longer plasma half-life with reduced risk of side effects
Emulsification	Dispersion of two or more immiscible phases (i.e., one with the biopharmaceutical)	Conformational damage by harsh chemicals (e.g., DCM commonly used for emulsification)	Enzymes Immunotherapeutic and anti-cancer proteins	Protein modification (e.g., PEGylation) prior to emulsification may protect the therapeutic from solvent-induced structural damage by increasing its solubility in the aqueous phase	Loss of biological activity after modification and emulsification	N/A	N/A
Self-Assembly	Chemical modification to induce or improve the self-assembling properties of the biopharmaceutical (e.g., amino acid modification, conjugation to self-assembling structures, or loading into cage-like protein carriers)	The design of constructs and conditions under which BNCs are formed in a controlled manner	Wound-healing peptides Proteins and peptides for glycemic control Epitopes for vaccination against HPV Antibodies	Poor shelf-life stability of some BNCs (aggregation) Some BNCs had improved resistance towards proteolytic degradation compared to the free biopharmaceutical	Improved cellular uptake	Topical SC	Improved therapeutic effect (stronger or prolonged response) compared to the free therapeutic with reduced risk of side effects Improved resistance towards proteolysis

Non-applicable (N/A) used in case of limited number of studies (e.g., lipid-based BNCs) or in case the experiments providing the information have not been completed (e.g., no in-vivo testing) meaning that no general conclusions can be made. The listed routes of administration do not mean that all studies tested the BNCs via this administration route. Dichloromethan (DCM). Polyethylene glycol (PEG). Conjugation of PEG (PEGylation). Gastro-intestinal (GI). Human Papillomavirus (HPV). Intradermal (ID). Intragastric (IG). Intramuscular (IM). Intranasal (IN). Intraperitoneal (IP). Intravenous (IV). Subcutaneous (SC).

Table 2
Overview of biopharmaceuticals nanoclusters (BNCs) prepared via desolvation and/or crosslinking chemistries.

Protein/ peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
α-lactalbumin (α-LAC)	Desolvation using water as solvent and ethanol as antisolvent followed by GTA crosslinking and resuspension in 10 mM NaCl solution pH 9	150–325 (DLS, TEM)	0.104–0.383	Surface-crosslinking-independent degradation in simulated gastric and pancreatic juices (pepsin in pH-adjusted saline solution)	Reduced antioxidant activity of BNCs compared to free protein (ABTS radical-scavenging assay)	N/A	Synthesis yield ~100% ZP of ~ +10 mV at pH 3.2 ZP of ~ −40 mV at pH 5.5 Despite a lower antioxidant activity, BNCs with the highest antioxidant activity may have potential as protein nanocarriers for oxidation-sensitive compounds	[32]
	Desolvation using 10 mM NaCl pH 9 as solvent and 80% acetone as antisolvent followed by GTA crosslinking and resuspension in 10 mM NaCl solution pH 9	100–184 (DLS, TEM)	0.07	Lower denaturation enthalpy of BNCs compared to free α-LAC (DCS) Limited stability at high temperature and low pH (DCS)	N/A	N/A	Structural changes with loss of α helical content (CD) Low drug loading of ~4% (*w*/w)	[33]
	Desolvation using water as solvent and ethanol as antisolvent followed by GTA crosslinking and resuspension in water	300 (DLS, FESEM, AFM)	N/A	Stable at a range of temperatures (20–70 °C) and pH values (5–10) (CD, fluorescence microscopy) Increased proteolytic resistance stability of BNCs compared to the free protein (proteinase kinase K digestion)	Anti-proliferation/ cytotoxicity in different cell lines (MCF-7, MDAMB231, HeLa, and A549), enhanced cell uptake (MCF-7), and reduced hemolysis of BNCs compared to α-LAC-oleic acid-complex (MTT assay, confocal microscopy)	N/A	ZP of +40 mV Structural changes with small increase in α helical and β-sheet content (CD, FTIR) Lower surface hydrophobicity of BNCs than the free protein (fluorescence spectroscopy)	[34]
Catalase (CAT)	Desolvation using PBS as solvent and 20 or 75% acetonitrile as antisolvent followed by resuspension in PBS	~ 163–176 (DLS, SEM)	0.189–0.196	Increased resistance to α-chymotrypsin in simulated intestinal fluid Small increase in size and PDI after 3 days at 4 °C Sedimentation ≤12 h when stored at RT Reduced loss of catalytic activity of BNCs compared to free CAT after 4 °C storage (ammonium molybdate-based H_2O_2 degradation assay)	50% and 90% decrease in enzymatic activity compared to free CAT using 20% and 75% acetonitrile, respectively (ammonium molybdate-based H_2O_2 degradation assay)	N/A	Structural changes; CD data suggested loss of α helical structure, FT-IR suggested preserved secondary structure Exposure of Trp and Tyr (fluorescence spectroscopy)	[35]
Cholesterol oxidase (CHOX)	Desolvation using water as solvent and ethanol as antisolvent followed by GTA crosslinking and resuspension in water	100–200 (TEM, SEM)	N/A	50% loss of enzymatic activity of BNC electrode when stored in 0.1 M phosphate buffer (corresponds to regular use of 150 times over 90 days)	Increased enzymatic activity compared to the free enzyme, improved biosensor characteristics (linearity between current and cholesterol levels, low limit of detection, minimal interference of other compounds)	Blood samples of healthy and hyper-cholesterolimic humans	Limited relevance – not a BNC formulation intended for treatment of disease	[36]
LYZ	Desolvation using water as solvent and ethanol as antisolvent followed by GTA	300–400 (DLS, AFM, FESEM)	N/A	Compared to the free protein, BNCs had increased thermal stability in temperature range	BNC uptake in MCF-7 cells (fluorescence microscopy) Higher cytotoxicity of BNCs than free	Insignificant level of hemolysis of BNCs in red blood cells isolated from human	ZP of −39.1 mV Structural changes with increased β-sheet content (CD)	[37]

(*continued on next page*)

Table 2 (*continued*)

Protein/ peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
	crosslinking and resuspension in water			25–80 °C and resistance towards enzymatic degradation (CD, absorbance-based proteinase K assay)	LYZ in MCF-7 cells but not in HaCaT and murine fibroblast 3 T3 cells (MTT assay) Higher MCF-7 cell specificity by folic acid conjugation to BNCs (MTT assay) ROS-mediated mechanism of cytotoxicity of BNCs (use of NAC in MTT assay)	blood (UV spectroscopy)	Exposure of Trp in the BNC structure (fluorescence spectroscopy) Crosslinking decreased enzymatic and lytic activity of the BNCs	
β-GAL	Desolvation of a mixture of HSA, 30Kc19 and β-GAL using Tris-HCl buffer pH 9 as solvent and ethanol as antisolvent followed by GTA crosslinking and resuspension in PBS	170–350 (ELS, SEM)	~ 0.15–0.25[a]	Stable in water, PBS, and cell culture media (data not shown)	Higher intracellular enzyme activity of 30Kc19-HSA-BNCs compared to HSA-only BNCs (colorimetric β-GAL assay) 30–50% release within 24 h followed by a sustained release >200 h (fluorescence microscopy) Cell uptake and no cytotoxicity (HeLa and HEK293 cells)	N/A	Synthesis yield of 80–95% ZP of −20 to −30 mV	[38]
	Desolvation of β-GAL and eGFP using water, a NaCl solution, or imidazole in a 0–250 mM phosphate buffer pH 8 as solvent and ethanol as antisolvent followed by DTSSP, BS^3 or GTA crosslinking and resuspension in PBS	270–700, 400 (no eGFP) and 280 (with eGFP) (DLS)	N/A	Aggregation (reversible upon sonication) and loss in enzymatic activity after 6 months of storage in PBS at 4 °C	Loss of enzymatic activity from desolvation and crosslinking with 25% (without eGFP) and 75% (with eGFP) activity retained compared to free β-GAL (ONPG hydrolysis-based β-GAL assay) Improved uptake in NIH3T3, HeLa, and SK-BR-3 cells of BNCs compared to the free enzyme (confocal microscopy, flow cytometry)	N/A	ZP of −12.9 mV in PBS and − 26.4 mV in HEPES of BNCs (with eGFP)	[39]
OFA peptides	Desolvation of DTSSP-crosslinked peptides using HFIP as solvent and DEE as antisolvent and resuspension in water, 150 mM arginine or 1% Tween-80	148–261 (DLS, SEM, AFM)	0.152–0.297 (DLS)	Tendency to aggregate – two of three formulations aggregated after 3–4 days while the last BNC formulation was stable in water for 7 days (DLS)	Higher cellular uptake, epitope presentation and cell activation in M3 DCs (flow cytometry, confocal microscopy)	Significantly higher retention at injection site of the BNCs compared to free peptide after ID injection in alfalfa-free fed BALB/c mice (near-IR fluorescent imaging)	Synthesis yield ~100% ZPs ranging from −6.9 and − 27 mV Arginine had to be added to 5% fluorophore BNCs to ensure stability (DLS)	[21]
M2e	Desolvation of tetrameric M2e using ethanol as antisolvent followed by GTA crosslinking and resuspension in PBS	227	0.42	Stable for a month stored in PBS at 4 °C ~ 100% retention of CpG ODN retention in BNCs after 2-weeks storage at 4 °C	N/A	IN immunization at day 0, 28, and 56 of tM2e, tM2e BNCs and a combination of tM2e and CpG ODN BNCs, respectively, to give higher levels of M2e-specific IgG antibodies, M2e-specific T cell responses, IgG and IgA levels in nasal and lung mucosa, INF-γ levels in the spleen and lungs, and	87% of tM2e and 30% of CpG ODN was incorporated into the BNCs during synthesis The M2e-specific IgG titers in sera of mice treated with tM2e BNCs alone or in combination with CpG ODN were around 100-fold higher than from mice treated with soluble tM2e	[18]

(*continued on next page*)

Table 2 (*continued*)

Protein/ peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
						frequency of IL-4 secreting cells in the spleens compared to the free, soluble M2e (unknown mice species, ELISA, ELISpot)		
	DTSSP crosslinking of M2e or HA-epitope-M2e	47–82	N/A	N/A	TLR5 bioactivity and IL-1β secreting DCs after treatment with the BNCs (HEK293-based TLR-5 bioactivity assay and ELISA-based assay on JAWS II DCs)	IN immunization in BALB/c mice with a booster dose 4 weeks after the initial dose to give high levels of 4M2e-specific IgG titers, more IL-2 secreting splenocytes, induce antibodies against the HA stalk domain which can potentially increase the overall protection efficacy (ELISA, ELISpot) Therapeutically active BNCs and the HA domain seemed to improve this activity	ZP ranging from −7.1 mV to −10.9 mV No comparison to free, soluble protein	[24]
	Desolvation of tetrameric M2e using ethanol as antisolvent followed by M2e surface-coating using DTSSP as crosslinker with or without HA domains	178–229 195 (4MtG BNCs) 178 (hrH1-coated 4MtG BNCs) 229 (hrH3-coated 4MtG BNCs) (DLS, SEM)	N/A	N/A	N/A	IM immunization in BALB/c mice with a 4-week booster dose to give higher HA domain-specific and M2e-specific immune responses, DC maturation and IL-1β production in BMDCs, as well as an overall increase in survival rate (ELISA, ELISpot, histological analysis, HAI assay, MDCK cell-based neutralization assay, HEK293-based ADCC assay)	Strong cross-reactivity to M2e peptides but the BNCs did not completely protect against all lethal influenza A vira tested hrH3 HA-domain-coated BNCs caused severe weight loss Protection appears to occur through ADCC and ADCP	[23]
	Incubation of the linear epitope with nickel(II) ions and MMPP under stirring, in basic solution and at RT	From 170 to above 3000 (DLS, TEM)	N/A	N/A	Crosslinking did not reduce the antigenic activity of the epitope (ELISA)	Higher immune response of BNCs compared to free peptide in Balb/c mice after IM administration which could be further increased using CpG as adjuvant, with large BNCs (~ 761 nm) being more immunogenic than small BNCs (~ 275 nm) (ELISA)	N/A	[40]
OVA	Desolvation using PBS as solvent and ethanol as antisolvent followed by DTSSP crosslinking and resuspension in PBS For surface coating, the BNCs were resuspended in an OVA solution (PBS) The final BNCs were resuspended in PBS and	271–559 (DLS, SEM)	0.050–0.135 (DLS)	N/A	Enhanced antigen uptake from BNCs compared to free OVA (flow cytometry, confocal microscopy) Upregulation of CD86 and the Fc receptor after treatment with small and medium sized BNCs in JAWS DCs (flow cytometry and ELISA) Increased TNF-α and IL-1β production in BMDCs from BALB/	N/A	Clathrin-mediated uptake in JAWS II cells (flow cytometry)	[41]

(*continued on next page*)

Table 2 (*continued*)

Protein/ peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
	crosslinked by DTSSP				c mice following BNC treatment compared to treatment with free soluble OVA			
	Co-desolvation and crosslinking of SIINFEKL peptides using hexafluoro isopropanol as solvent, diethyl ether as antisolvent, and DTSSP, tri-maleimide, or tri-NHS as crosslinker and resuspension in water	184–233 (DLS)	0.189–0.232 (DLS)	Trithiol-crosslinked BNCs were stable in water for 70 days, whereas tri-maleimide and tri-NHS-crosslinked BNCs showed signs of dissociation into smaller BNCs in water after 0–2 days	N/A	ID immunization in C57BL/6 mice and booster doses (half dose) on day 7 and 14 to induce antigen-specific immune responses (MHC presentation and T cell proliferation) comparable to that of the free peptides as well as improved delivery to lymph nodes (flow cytometry, fluorescence microscopy)	Data suggests a slower processing of BNCs compared to free soluble antigen	[22]
IgG IL-2	Crosslinking of IgG or IL-2 fused to an antibody Fc fragment or the super agonist 15Sa using a reducible carrier-free and reducible crosslinker (similar structure as the DTSSP crosslinker) or BS^3 in PBS	121 90 (IL-2-15Sa) 86 (IL-2-Fc) 121 (IL-2-15Sa with anti-CD45)	N/A N/A	Drug release from reducible BNCs in PBS which could be enhanced further in presence of GSH	N/A	T cell expansion of transferred $CD8^+$ T cells but not T cells in the systemic circulation, tumor-draining lymph nodes, nor tumors (in contrast to treatment with adjuvanted free IL15-Sa) in a syngeneic B16F10 melanoma C57BL/6 mice model (ELISA, flow cytometry, confocal microscopy, bio-luminescence microscopy, WST-1 assay)	The BNCs were referred to as nanogels (NGs) in the study, likely due to a gel-like structure of the formulation Incorporation efficiency of ~94% (IL-2-Fc)	[42]
	10 mol% anti-CD45 was incorporated into the BNCs to allow attachment to T cell surfaces					Lower toxicity of BNC formulations compared to free IL (cytometric bead array mouse inflammation assay)		

2,2′-azinobis-3-ethyl-benzothiozoline-6-sulfonic acid (ABTS). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Antibody-dependent cellular cytotoxicity (ADCC). Antibody-dependent cellular phagocytosis (ADCP). Atomic Force Microscopy (AFM). β-galactosidase (β-GAL). Bis(sulfosuccinimidyl)suberate (BS^3). Bone-marrow-derived DC (BMDC). Circular Dichroism (CD). Cluster of differentiation 8 positive ($CD8^+$). Dendritic cell (DC). Diethyl ether (DEE). Differential Scanning Calorimetry (DSC). Dynamic Light Scattering (DLS). Electrophoretic Light Scattering (ELS). Enhanced green fluorescent protein (eGFP). Enzyme-linked immune absorbent spot (ELISpot). Enzyme-linked immunosorbent assay (ELISA). Extracellular domain of M2 (M2e). Field Emission Scanning Electron Microscopy (FESEM). Fourier-transform IR (FT). Glutaraldehyde (GTA). Glutathione (GSH). Hemagglutinin (HA). Hemagglutination assay (HAI assay). Henriette Lacks (HeLa). Hexafluoroisopropanol (HFIP). Human embryonic kidney 293 (HEK293). Human interleukin 15 super agonist (IL-15Sa). Human Serum Albumin (HSA). Hydrogen peroxide (H_2O_2). Immunoglobulin (Ig). Infrared (IR). Interferon-γ (INF-γ). Interleukin (IL). Intradermal (ID). Intramuscular (IM). Intranasal (IN). Lysozyme (LYS). Madin-Darby Canine Kidney cells (MDCKs). Magnesium monoperoxyphthalic acid (MMPP). Michigan Cancer Foundation-7 (MCF-7). MUTZ-3 (M3). *N*-acetyl-L-cysteine (NAC). N-hydroxysuccinimide (NHS). Not applicable/available (N/A). Oligodeoxynucleotide (ODN). Oncofetal antigen (OFA). Ortho-nitrophenyl-D-galactopyranoside (OPNG). Ovalbumin (OVA). Phosphate-buffered saline (PBS). Polydispersity index (PDI). Reactive Oxygen Species (ROS). Reference (ref). Room temperature (RT). Scanning Electron Microscopy (SEM). Sodium chloride (NaCl). Toll-like receptor 5 (TLR5). Transmission Electron Microscopy (TEM). Tyrosine (Tyr). Tryptophan (Trp). Tumor Necrosis Factor α (TNF-α). Ultra-violet (UV). Zeta potential (ZP).

[a] Specific values not reported. Values listed in this table have been extracted from figures, graphs, images, etc.

dithiobis(sulfosuccinimidyl propionate) (DTSSP) and sulfosuccinimidyl 2-((4,4′-azipentadamido)ethyl)-1,3′-dithiopropionate (sulfo-SDAD) crosslinkers did not give effective peptide crosslinking, likely due to a limited number of reactive sites on the peptides. For the smallest BNCs, a slower peptide uptake was observed compared to the free peptide. The BNCs were not cytotoxic and induced a higher epitope presentation resulting in improved T cell activation. The BNCs were also significantly better retained at the injection site as compared to free peptide. However, the shelf-life stability of the BNCs was less than a week. This study is one of the few examples of crosslinking prior to BNC synthesis [21].

There has been considerable interest in the development of BNC formulations based on the matrix protein 2 ectodomain (M2e) and Hemagglutinin (HA). These are both highly conserved domains in human seasonal influenza A and thus have potential as universal influenza A vaccine therapeutics [18,23,24,40]. Based on data suggesting that the tetrameric conformation of the protein is more immunogenic than other conformations, tetrameric M2e (tM2e) was formed by introduction of the stabilizing GCN4 into M2e followed by crosslinking

Table 3
Overview of biopharmaceuticals nanoclusters (BNCs) prepared via molecular crowding, salting-out, pH-coacervation, mechanical agitation, lipid and emulsion based methods, and by design of self-assembling constructs.

Protein/peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
Molecular Crowding and *Salting-Out*								
BSA	Mixing with a crowding agent (trehalose, PEG 300, NMP or dextran)	~ 250 (DLS)	N/A	Dilution of protein and molecular crowder in phosphate buffer caused dissociation of the BNCs (DLS) Keeping the protein concentration constant and reducing that of the molecular crowding agent also caused BNC dissociation (DLS)	N/A	N/A	N/A	[20]
IgG (pAb)		~ 300 (SEM, DLS)	N/A		N/A	N/A	N/A	
1B7 (mAb)		30 (DLS)	N/A		Full antigen-binding activity of protein in the BNC formulation (indirect PTx ELISA)	IV and SC administration of a standard 1B7 dose in a diluted PEG-NMP suspension, a concentrated BNC suspension or an extremely concentrated BNC suspension in BALB/c mice gave similar pharmacokinetic profiles with a slightly lower C_{max} and slightly delayed t_{max} of the SC groups compared to the IV groups being the only remarkable difference (indirect PTx ELISA, antibody activity assay)	No conformational changes or formation of irreversible aggregates after BNC formation and dilution (CD, SDS-PAGE, SEC, indirect PTx ELISA, thermocycler)	
	Freezing-technique to trap protein in amorphous state followed by dispersion into aqueous solution of crowding agent (NMP, PEG and trehalose)	~ 430 (DLS, SEM)	N/A				Antibodies can be clustered to form non-immunogenic structures and suggest that the BNC structure may allow for smaller injection volumes Lack of improvement in in vitro and in vivo biological/ therapeutic effect of BNCs compared to free antibody	[19]
EXE-4	Calcium ion-based biomineralization (10 mM $CaCl_2$ added to 2 mg/mL EXE-4 or 5 mg/mL INS in DMEM and incubated at 37 °C with 5% CO_2 for 24 h (EXE-4) or 4 h (INS))	125 (DLS, TEM, SEM)	N/A	Stable formulation for at least 7 days (DLS) Dissociation in supersaturated physiological fluid (0.5–2.5 mM) (DLS, TEM, SEM)	N/A	Prolonged retention of 800-CW-cys40-EXE-4 BNCs (> 1 week) compared to free labelled peptide (< 24 h) at injection site after SC administration in C57BL/6 mice (fluorescence microscopy) Similar blood glucose lowering capability of free and BNC EXE-4 in diabetic C57BL/6 db/db mice as healthy mice upon glucose injection 1 h post-EXE-4-treatment (blood glucose meter) Blood glucose lowering effect of BNCs lasted up to 192 h ($t_{½}$ = 43.11 h) while that of free EXE-4 lasted less than 24 h ($t_{½}$ = 1.90 h) after SC administration in C57BL/6 db/db mice (blood glucose meter, ELISA)	Intact native protein structure in BNC formulation (HPLC, CD, FTIR) Relatively low drug load with a weight percentage of 17% organic material, calcium phosphate and water of 17%, 77%, and 6%, respectively (EDS)	[25]
INS		200 (TEM, SEM, DLS)	N/A	Low stability at pH < 7.2 (relevant for controlled drug release, e.g., intracellularly)	Uptake and transport to lysosomes of FITC-labelled INS BNCs in HepG2 cells of which 96% of the cells showed uptake within 30 min and 100% after 2 h (confocal laser scanning microscopy,	Daily IP administration in diabetic KKAy mice and healthy C57BL/6 mice for 2 weeks followed by an oral glucose tolerance test revealed an improved	Intact native protein structure in BNC formulation (FTIR, XRD) Relatively low drug load with a	[43]

(*continued on next page*)

Table 3 (*continued*)

Protein/peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
				BNCs were stable for 30 min of α-chymotrypsin treatment in contrast to free INS which was completely degraded within the timeframe Improved stability of INS in DMEM at 37 °C when formulated as BNCs (SC administration in mice and blood glucose meter)	flow cytometry, TEM) in contrast to free labelled INS which was not taken up by the liver cells Prolonged glucose metabolism of INS-resistant HepG2 (R-HepG2) cells after BNC treatment compared to the free protein (glucose oxidase assay) Increased mRNA levels of IRS-1, IRS-2, GLUT-2, and GLUT-4 in R-HepG2 cells after BNC treatment compared to non-clustered insulin (RT-PCR) Non-cytotoxic BNCs in HepG2 cells (MTT assay)	blood sucrose regulation of the insulin BNCs that was comparable to that of healthy mice and lasted for 3 weeks in contrast to mice that were not treated or treated with free insulin (blood glucose meter) Accumulation of BNCs in liver followed by degradation within 24 h after IP administration in mice (confocal laser scanning microscopy)	weight percentage of INS, calcium phosphate and water of 10%, 85%, and 5%, respectively (TGA/DSC)	
CT	Mixing of 0.1 mM sCT with 1.0 mM Asp-Phe, Ser-Phe, or Ser-Ala, in 5 mM phosphate buffer pH 7.4 at 25 °C	15–40[a] (AFM, TEM)	N/A	Stable BNCs after 14 days of storage (AFM, TEM)	Slow release of sCT from the BNC formulation in phosphate buffer with approximately 30% release within the first 12 h followed by a slower release until reaching 80% after which no more peptide was released (HPLC)	Prolonged decrease in blood calcium levels of BNC formulations compared to free sCT and corresponding higher serum sCT levels after SC administration in male Sprague-Dawley (QuantiChromTM Calcium Assay Kit, ELISA)	Slight increase in β-sheet character of BNC formulations except for that prepared in Asp-Phe which was the most promising formulation in that respect (ThT assay)	[44]
					Much less released from the BNCs in water compared to phosphate buffer (HPLC) Non-cytotoxic when tested in the L929 cell line (MTT assay)	Non-immunogenic as reflected by low levels of IgG, IL-1β, IL-6 and TNF-α following SC administration, as well as low levels of IFN-γ and IL-4 in serum and spleens of SC immunized C57BL/6 mice which had a booster dose at day 14 (H&E staining, immunohistological staining, ELISA)	Intact structure of peptide released from the BNCs (CD)	
pH-coacervation								
BVZ	Titration of BVZ dissolved in HCl using NaOH in presence or absence of different surfactants (Tween-20, Tween-80, or Brij 97) to form BNCs at the antibody pI	334–774 (DLS, SEM)	0.1–0.2 (DLS)	N/A	Similar anti-VEGF activity of BNCs as free BVZ (AlamarBlue anti-VEGF activity assay using HUVECs)	N/A	Synthesis yield ~100% ZP ranging from approximately −5 mV to −26 mV depending on the exact BNC formulation	[45]
					Similar cytotoxic effect of free BVZ and BNCs prepared in 0.1% Tween-80 and higher of surfactant-free BNCs (MTT assay)		Plate-like disrupted protein structure when formulated in absence of surfactants (SEM, CD)	
					Uptake of BNCs via energy-dependent endocytosis and macropinocytosis in A549 cells but not in MRC-5 cells suggesting a specificity towards cancer cells over healthy cells (fluorescence microscopy, TEM, flow cytometry)		Presence of surfactants during BNC formulation protected the protein structure and gave similar numbers of	

(*continued on next page*)

Table 3 (*continued*)

Protein/peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
							monomers and higher-order oligomers as non-clustered mAb upon reconstitution (CD, SE-HPLC)	
INS	Titration of INS in HCl with NaOH followed by GTA crosslinking	200 (NCM, SEM)	N/A	N/A	N/A	Similar blood glucose lowering effect of free and BNC INS after IV injection and a stronger response of BNCs compared to the free protein after IM administration in healthy and alloxan-induced diabetic female Hooded Wister rats (Yellow Spring Glucose Analyzer) Slower and more pronounced blood glucose lowering effect of BNCs compared to free INS after intragastric administration (jejenum) in healthy rats (male or female Wister rats), healthy outbred mice, and diabetic rats (streptozotocin-induced diabetic male or female Wister rats) with a lowering of up to 15–20% of the initial levels 3 h post-administration (Yellow Spring Glucose Analyzer)	N/A	[46]
Mechanical Agitation CAT	Shear stress application in a co-axial cylinder flow cell at low temperature (0.2 mg/mL CAT, 217 s^{-1} shear rate, 5 min shear time)	182 (DLS, SEM)	0.207 (DLS)	Increased resistance to α-chymotrypsin in simulated intestinal fluid Small increase in size and PDI after 3 days at 4 °C Sedimentation ≤12 h when stored at RT Reduced loss of catalytic activity of BNCs compared to free CAT after 4 °C storage (ammonium molybdate-based H_2O_2	90% of the enzymatic activity of free CAT was retained in the BNCs (ammonium molybdate-based H_2O_2 degradation assay)	N/A	BNC synthesis could be optimized to give BNCs with a 90% enzymatic activity retained compared to the free, native protein Small structural changes upon BNC formation (CD, FT-IR, UV, and fluorescence microscopy) Increasing loss of enzymatic activity upon increased shear rate and shear time Synthesis yield ~76%	[35]

(*continued on next page*)

Table 3 (*continued*)

Protein/peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
				degradation assay)				
TRA (Herceptin) (IgG) IgY (chicken)	Agitation or stirring of the proteins at a concentration of 1 mg/mL at pH 2 and 37 °C	Up to 500 nm × 12 nm (AFM)	N/A	N/A	Slower release and binding of IgG and IgY from the BNCs compared to the free mAbs with approximately 75–80% of the total amount restored after 4 and 6 days as observed from binding to HER2-positive SKBR3 cells (FACS, ELISA) Similar binding capacity of mAbs released from the BNCs compared to free mAbs (competition binding assay with FITC-conjugated protein and Herceptin in SKBR3 cells)	N/A	Reversible conformational changes of BNCs (ThT fluorescence, CD, SDS-PAGE, AFM)	[47]
OVA	Shaking of OVA in PBS and 0.01% azide at 180 rpm at RT	100–1000 nm[a]	N/A	N/A	N/A	Higher antibody titers, IgG1, IgG2a, INF-γ, IL-12, and IL-4 of BNCs compared to native OVA after SC immunization in BALB/c mice and C57BL/6 mice (ELISA) The immunogenic responses were higher even without co-delivery of an adjuvant suggesting a self-adjuvating effect of the amyloid BNCs (ELISA)	Amyloid character of the OVA BNCs but native-like structure of OVA released from the BNCs suggesting reversible misfolding of the protein upon BNC synthesis (ThT binding, congo red binding, CD, AFM, TEM) Potential as protein nanocarrier (successful loading and release of DOX)	[48]
Lipid-Based								
myr-FEEERA	Thin-film hydration in presence of PEG2000-DSPE and L-α-PPC and re-suspension in physiological saline or PBS	6–25 (DLS)	N/A	Stable for >18 months when stored at −20 °C and for at least 2 weeks when stored at RT (22 °C)	Cellular uptake, inhibited platelet granule secretion, and secondary platelet aggregation in mouse and human platelets (lumiaggregometer, Western blot, fibrinogen and JON/A binding assay, flow cytometry, fluorescence microscopy)	Reduced risk of excessive bleeding as shown from a reduced bleeding time in C57BL/6 mice and in dogs (hemoglobin concentration and Jorvet Surgicutt time device, respectively) Antithrombotic effect of BNCs alone and synergistic antithrombotic effect when combined with some of the most popular antiplatelet drugs as seen from pharmacokinetic studies based on whole blood and plasma analysis after IV administration in CD-1 mice (mass spectroscopy), platelet thrombus formation in C57BL/6 mice following laser-induced thrombosis (fluorescence and bright-field microscopy), treatment of MI/R-induced thrombosis as studied in a C57BL/6 mouse MI/R model	High drug loading in the BNCs ~70% (mol/mol) Relatively large variation in BNC size with the main batch-to-batch variation being occasional presence of larger particles (50–500 nm) Relatively low potency and in-vivo half-life although improved compared to conventional antiplatelet drugs	[49]

(*continued on next page*)

Table 3 (*continued*)

Protein/peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
						including determination of infarct area/risk area and risk area/total area, reduced thrombosis and neutrophil infiltration in mouse heart, reduced MPO plasma concentration and reduced time of thrombus formation (echocardiography, immunohistochemistry, imaging, ELISA, blood flowmetry) No signs of toxicity observed after IV bolus dose in CD-1 mice nor after bolus or continuous infusion in Sprague-Dawley rats		
Emulsification								
LYZ	Mixing of the mPEGylated protein dissolved in DCM and PBS, ultrasonication, and overnight DCM evaporation	95 (DLS, NTA, TEM)	N/A	BNC size stable for at least 6 months when stored at 4 °C (DLS)	Only 19% of the native enzyme's activity retained upon mPEGylation and only 3% was retained upon formulation of the mPEGylated protein into BNCs (catalytic methylumbelliferyl-based assay) Non-cytotoxic in HeLa cells (MTT assay) BNC uptake in HeLa cells into intracellular compartments (confocal laser scanning microscopy, FACS)	N/A	ZP of BNCs ~ −3 mV Minimum change in secondary protein structure suggesting loss of enzymatic activity being related to mPEGylation at sites important for the biological activity (CD spectroscopy) Reduced stability of DOX-loaded BNCs with slight dissociation after 6 months of storage (DLS)	[50]
OVA		208 (DLS, NTA, TEM)	N/A	N/A	N/A	N/A	N/A	
HSA		173 (DLS, NTA, TEM)	N/A	N/A	N/A	N/A	N/A	
β-LAC		179 (DLS, NTA, TEM)	N/A	N/A	N/A	N/A	N/A	
LYZ	Mixing of epoxy-mPEGylated LYZ dissolved in DCM and PBS, ultrasonication, and overnight DCM evaporation	100–130 (DLS, NTA, TEM)	N/A	BNC size stable for at least 2 months when stored at 4 °C (DLS, NTA)	80% of the native enzyme's activity retained after epoxy-mPEGylation (catalytic methylumbelliferyl-based assay) Other PEGylation types were tested with TFP-mPEG, carbonate-mPEG, and TsT-mPEG preserving 60%, 62%, and 13% of the native protein catalytic activity (catalytic methylumbelliferyl-based assay)	N/A	ZP of BNCs ~ −13 mV Minimum change in secondary protein structure suggesting loss of enzymatic activity being related to mPEGylation at sites important for the biological activity (CD and IR spectroscopy)	[51]

(*continued on next page*)

Table 3 (*continued*)

Protein/peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
LYZ	Ultrasonication of PEGylated protein in DCM and PBS to form a W/O emulsion (single emulsion strategy) and eventually followed by one more round of ultra-sonication with PBS to form a W/O/W emulsion (double emulsion strategy) and overnight DCM evaporation	200 (DLS, NTA, TEM)	N/A	N/A	80% of the native enzyme's activity retained after TFP-mPEGylation (catalytic methylumbelliferyl-based assay) Non-cytotoxic in HeLa cells (MTT assay) No antimicrobial activity and hence lower therapeutic potential of pure LYZ BNCs which may be due to a dense PEGylation layer lowering the protein hydrolase activity (MIC determination, absorbance-based time kill assay using *M. Luteus*)	N/A	ZP of BNCs ~ −10 mV Minimum change in secondary protein structure (CD spectroscopy) The formulation strategy has potential for drug loading and hence as a nanocarrier system but low potential as self-delivery system	[52]
CYTC		140 (DLS, NTA, TEM)	N/A		90% of the native enzyme's activity preserved after PEGylation with an acid-degradable PEG (mP(EG81-co-isoEPB6)) (ABTS assay) Non-cytotoxic in HeLa cells (MTT assay)	N/A	Minimum change in secondary protein structure (CD spectroscopy) ZP of BNCs ~ −2 mV	[53]
Design of Self-Assembling Constructs								
Fusion protein of KGF and ELPs	Heating of KGF-ELP fusion protein to 37 °C (i.e., above its t_t)	500 (DLS, TEM)	N/A	BNCs were stable over a range of temperatures (30–60 °C)[a]	Fusion of KGF to ELP without losing its proliferative effects on epithelial A431 cells and fibroblasts although BNCs of ELP itself had a higher proliferative effect in fibroblasts compared to BNCs of the fusion protein (fluorescence-based growth assay using Hoechst 33258)	Supreme wound-healing properties of the fusion protein BNCs with a reduced risk of fibrosis and scarring as a result of granulation as observed after administration to dorsel wounds in genetically diabetic male B6.BKS(*S*)-*Lepr*^{db}/J mice (histological examination)	Polydispersity reported in terms of the standard deviation on the particle distribution of 20 nm	[54]
INS	Mixing of INS and mPEGylated α-elastin and heating to 37 °C for 24 h	330 (DLS, SEM)	N/A	Low stability of non-PEGylated BNCs (aggregation within a day)	INS-stimulated GLUT-4 translocation in C2C12 mouse skeletal muscle cells (immunostaining with anti-GLUT4 and confocal microscopy)	N/A	Biphasic slow release of INS from BNC formulation Main potential as elastin nanocarrier BNCs as a low INS is expected	[55]
OVA/SIINFEKL	Self-assembling of a self-assembling peptide (two types applied) fused to a SIINFEKL-containing OVA-antigen at concentrations above its CAC	24 (#1) 86 (#2) (DLS, TEM)	0.29 (#1) 0.37 (#2)	N/A	N/A	Stronger T cell expansion as observed from analysis of peripheral blood mononuclear cells after IV injection of splenic and lymphatic CD8+ T cells from CD90.1 OT-I mice into CD90.2 mice and SC vaccination with CpG-adjuvanted SAPE BNCs compared to CpG-adjuvanted free peptide (flow cytometry) Stronger T cell expansion and $CD8^+$ response in C57BL/6 mice after SC vaccination with a booster dose at day 14 and peripheral blood mononuclear cell analysis 5 and 7 days	Secondary protein structure with β-sheet characteristics (CD)	[56]

(*continued on next page*)

Table 3 (*continued*)

Protein/peptide	Synthesis strategy	Size (nm)	PDI	Stability	In-vitro data	In vivo data	Further comments	Ref
						after the booster (flow cytometry) Delayed tumor growth and prolonged survival rate after SC vaccination with CpG-adjuvanted BNC vaccines with a 14-day booster dose compared to control group (tumor growth measurements in a TC-1 tumor mouse model)		
GLP-1	Fusion of GLP-1 to the self-assembling sequence cadyglp1m and titration into phosphate buffer followed by sonication, vortexing and cooling	80	N/A	Improved resistance of GLP-1 derivates towards proteolysis in vitro and in vivo after SC injection in Wister rats (ELISA)	Lack of receptor binding activity in GLP-1 receptor overexpressing INS-1 cells (ELISA) Prolonged insulin stimulation in INS-1 cells (absorbance-based cAMP accumulation assay) Non-cytotoxic in INS-1 cells (MTT assay)	Concentration-dependent stimulation of insulin secretion from islets isolated from Wistar rat pancreas under high-glucose conditions (rat insulin radioimmunoassay) Prolonged blood glucose regulation after SC administration to Wister rats or ZDF rats (blood glucose and HbA_{1c} measurements)	The GLP-1 derivate cadyglp1e did not form BNCs but nanotubes	[57]
IgG	Mixing of IgG with a hexameric fusion protein to form IgG-loaded Hex, H6-SPAB-Hex or Hex-SPAB-H6 BNCs	~25 (DLS, TEM)	N/A	Optimum storage conditions of 25 °C with aggregation when stored at 4 °C	Non-toxic Hex nanocarrier (MTT assay) Increased uptake in HeLa cells compared to the antibody alone by a mechanism that appears to be predominately clathrin-mediated but also occur to some extend via macropinocytosis and caveolae-mediated endocytosis (confocal laser scanning microscopy) Intact red blood cells after treatment with the BNC nanocarrier formulation (confocal laser scanning microscopy) Intracellular trafficking to and accumulation in endosomes followed by escape to the cytosol and dissociation of the Hex-IgG BNC complex after which the antibody can block the nuclear translocation of most STAT3 proteins in living cells (confocal laser scanning microscopy) Slightly different uptake characteristics in different cell types (HeLa, SKBR-3, MDA-MB-231, HUVEC, and J774 cells) (confocal laser scanning microscopy)	N/A	No change in secondary protein structure upon BNC formation (CD) The highest BNC stability achieved with a carrier-to-IgG molar ratio of 1:3 leaving the BNCs on the edge of the BNC definition (SEC)	[58–60]

1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE). 2,2′-azinobis-3-ethyl-benzothiozoline-6-sulfonic acid (ABTS). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Aspartic acid-phenylalanine (Asp-Phe). Atomic Force Microscopy (ATM). β-lactalbumin (β-LAC). Bevacizumab (BVZ). Bovine Serum Albumin (BSA). C57 black 6 (C57BL/6). Calcitonin (CT). Calcium chloride ($CaCl_2$). Catalase (CAT). Cholera Toxin Subunit B (CTB). Circular Dichroism (CD). Critical Aggregation Concentration (CAC). Cytochrome C (CYTC). Diabetic (db/db). Dichloromethane (DCM). Differential scanning calorimetry (DSC). Doxorubicin (DOX). Dulbecco's Modified Eagle Medium (DMEM). Dynamic Light Scattering (DLS). Elastin-like peptides (ELPs). Energy-dispersive X-ray spectroscopy (EDS). Enzyme-linked immunosorbent assay (ELISA). Exendin-4 (EXE-4). Fluorescein isothiocyanate (FITC). Fluorescence-activated Cell Sorting (FACS). Fourier-transform IR (FTIR). Glucagon-like peptide 1 (GLP-1). Glucose transporter type 2 (GLUT-2). Glucose transporter type 4 (GLUT-4). Glutaraldehyde (GTA). Hematoxylin-eosin (H&E). Half-life ($t_{½}$). Half tissue culture infectious dose ($TCID_{50}$). Hemoglobin A1c (HbA_{1c}). Henrietta Lacks (HeLa). High-performance liquid chromatography (HPLC). Human Epidermal Growth Factor Receptor 2 (HER2). Human Serum Albumin (HSA). Human Umbilical Vein Endothelial Cells (HUVECs). Hydrochloric acid (HCl). Hydrogen Peroxide (H_2O_2). Immunoglobulin G (IgG). Immunoglobulin Y (IgY). Infrared (IR). Insulin (INS). Insulin receptor substrate 1 (IRS-1). Insulin receptor substrate 2 (IRS-2). Interferon γ (INF-γ). Interleukin 1 beta (IL-1β). Interleukin- 4 (IL-4). Interleukin 6 (IL-6). Interleukin-12 (IL-12). Intramuscular (IM). Intraperitoneal (IP). Intravenous (IV). Isoelectric point (pI). Keratinocyte growth factor (KGF). KK.Cg-$A^{y/J}$ (KKay). Lysozyme (LYZ). Maximum concentration (c_{max}). Messenger ribonucleic acid (mRNA). Methoxy-PEGylation (mPEGylation). Minimum Inhibitory Concentration (MIC). Monoclonal antibody (mAb). Myeloperoxidase (MPO). Myocardial infarction/ischemia reperfusion (MI/R). *N*-methylpyrrolidone (NMP). Nepho-colorimeter (NCM). NeuN antibody/anti-FOX3 antibody (1B7). Not applicable/available (N/

A). Ovalbumin (OVA). Polyclonal antibody (pAb). Polydispersity index (PDI). Polyethylene glycol (PEG). Polymerase Chain Reaction (PCR). Pentraxin ELISA (PTX-ELISA). Phosphatidylcholine (PPC). Protein A domain B (SPAB). Real-time PCR (RT-PCR). Reference (ref). Room temperature (RT). Rotavirus inner capsid protein 6 (VP6). Rounds per minute (rpm). Salmon calcitonin (sCT). Scanning Electron Microscopy (SEM). Serine-Alanine (Ser-Ala). Serine-phenylalanine (Ser-Phe). Size Exclusion Chromatography (SEC). Size Exclusion HPLC (SE-HPLC). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Sodium Hydroxide (NaOH). Subcutaneous (SC). Thermogravimetric analysis (TGA). Thioflavin T (ThT). Time to c_{max} (t_{max}). Transmission Electron Microscopy (TEM). Transition temperature (t_t). Trastuzumab (TRA). Tumor Necrosis Factor α (TNF-α). Ultra-violet (UV). Vascular Endothelial Growth Factor (VEGF). Water-in-oil (W/O). Water-in-oil-in-water (W/O/W). X-ray Powder Diffraction (XRD). Zeta Potential (ZP). Zucker Diabetic Fatty (ZDF).

[a] Specific values not reported. Values listed in this table have been extracted from figures, graphs, images, etc.

by the BS^3 crosslinker. tM2e BNCs were synthesized via ethanol-induced desolvation of tM2e followed by GTA crosslinking, had a mean size of ~227 nm, a relatively high size polydispersity index (PDI) of ~0.42, and were stable for 1 month in PBS at 4 °C. In vivo intranasal administration of the BNCs in mice resulted in significantly higher immune responses and reduced mortality upon virus exposure compared to groups treated with free tM2e [18]. In an attempt to reduce the immunogenicity of the BNCs, BNCs were synthesized based on constructs of flagellin where its immunogenic region was replaced by four tandem copies of M2e (f4M2e), the H1 HA2 domain (fHApr8) or the H3 HA2 domain (fHAaichi). The BNCs were prepared by DTSSP-crosslinking of f4M2e, a mixture f4M2e/fHApr8, or a mixture of f4M2e/fHAaichi. The resulting BNCs were 47–82 nm and therapeutically active; they showed agonist activity in a Toll-like receptor 5 bioactivity assay and provided efficient protection against lethal doses of the H3N2 influenza A strain. Also, specific immunoglobulin G (IgG) antibodies could be detected in mice 2 weeks post-administration. The effect of f4M2e BNCs were lower than that of the two other BNCs suggesting that the HA epitope may be a more promising antigen for vaccination against influenza virus A and that the HA domain may be responsible for immunogenicity [24]. Based on these findings, a M2e-based BNC vaccine coated by HA derivates was developed. The BNCs had a core of the stable tetramer of M2e (4MtG) comprising four different M2e types and were coated by derivates of HA1 and HA3, which had their heads removed to prevent formation of a HA tetramer (hrH1 and hrH3). The BNCs was prepared by ethanol-induced desolvation of 4MtG and some of them were crosslinked by DTSSP in presence of either hrH1, hrH3 or a mixture of both to give an additional surface-coating. The BNC sizes were 178–229 nm. The BNC antigens were more efficiently taken up by phagocytes compared to soluble antigens, and intramuscular immunization of mice using the BNCs with hrH3 and both hrHA domains gave very high immune responses in contrast to the uncoated BNCs comprising only the 4MtG core. The survival rate was higher for mice immunized with the 4MtG BNCs compared to the control, but mice immunized with the hrH3 coated and the hrH1/hrH3 coated BNCs suffered severe weight loss [23]. Another study prepared BNCs of the linear epitope of the M2e. By simple stirring at room temperature and in basic solution, the peptide was incubated with nickel(II) ions and magnesium monoperoxyphthalic acid (MMPP) to form BNCs. By taking advantage of the common histidine tag of proteins and peptides, the histidine residues would complex with nickel (II) ions and be oxidized by the MMPP. The oxidation reaction would withdraw an electron from a nearby tyrosine to create a tyrosyl radical and form a dityrosine bond to another tyrosine residue. By changing and optimizing the synthesis conditions BNCs with sizes ranging from approximately 170 nm to above 3000 nm were synthesized. Intramuscular administration induced a higher immunogenic response by the BNCs compared to the free non-clustered peptide, and the response could be further enhanced using the CpG adjuvant. Large BNCs (~ 761 nm) were more immunogenic than smaller BNCs (~ 275 nm), likely due to a slower processing by the immune system [40].

The immunogenic and commonly-used model protein ovalbumin (OVA) as well as OVA-derived peptides have also been of interest within the field of immunotherapeutic BNCs. Double-layered OVA BNCs were prepared via ethanol-induced desolvation and DTSSP-crosslinking, followed by resuspension in an OVA solution for coating. DTSSP was added as a final step to crosslink the outer OVA layer and the final BNCs were observed to have different coating thicknesses and sizes (270, 350, and 560 nm). There was an improved cellular uptake, DC maturation and production of the pro-inflammatory marker interleukin 1β (IL-1β) of the BNCs compared to the free protein, which had a limited inflammatory response in the assays applied in the study. The improvement was most significant for the thin-coated BNCs, likely due to an improved cellular uptake of smaller BNCs combined with a greater release from a weaker surface coat [41]. BNCs of the SIINFEKL-containing peptide construct GKCSIINFEKLCKG have been prepared using different homo-trifunctional crosslinkers (trithiol, tri-maleimide, and tris-NHS) prior to desolvation. The disulfide, thioether, and amide crosslinked BNCs had mean sizes of 233 nm, 184 nm, and 205 nm, respectively, and PDI values of approximately 0.2. The shelf-life size stability varied from 2 days to at least 70 days, and the BNCs possessed slow degradation kinetics under reducing conditions in vitro. Irrespective of crosslinker type, the peptide BNCs induced antigen-specific immune responses comparable to that of soluble SIINFEKL *co*-delivered with the cytotoxic T-cell inducing poly(I:C) adjuvant. In lymphatic dendritic cells (DCs), the amide BNCs induced higher major histocompatibility complex (MHC) I presentation of SIINFEKL compared to the free epitope, while the disulfide and thioether BNCs had this effect on MHC II presentation in lymphatic DCs. Interestingly, ex vivo re-stimulation of the T cells with SIINFEKL resulted in a higher number of cells producing the pro-inflammatory cytokine TNF after administration of the thioether BNCs compared to the other groups. Internalization and biodistribution studies showed significant uptake of all BNCs and a relatively high delivery of the amide-crosslinked BNCs to the lymph nodes compared to that of the adjuvanted free peptide. The clear differences in the processing of the BNCs illustrates very well that the type of crosslinker matters. The formulation of the epitope as BNCs improved the delivery to the site of interest compared to the adjuvanted peptide, which may explain the similar immune response of soluble and BNC SIINFEKL. In this study, the amide crosslinked BNCs showed the most promising immune responses [22].

Using a reducible 'carrier-free' crosslinker (i.e., no structural crosslinker remnant after crosslinker cleavage), BNCs of a fusion protein between IL-2 and an antibody Fc fragment (IL-2Fc), a human IL-15 super-agonist complex (ALT-803/IL-15Sa), bovine serum albumin (BSA) or IgG have been prepared. These BNCs were designed to release their cargo upon T cell activation and utilize the higher levels of free thiols on the T cell surface as when compared to other cell types. The BNCs were 90–131 nm in size and loaded into nanogels comprising CD45 to stimulate the attachment of the BNCs to T cells. A 16-fold higher T cell expansion was observed from the BNCs compared to the free cytokines, and the BNCs could be administered in 8-fold higher doses than the free cytokines without causing toxic side effects. The BNCs expanded the transferred $CD8^+$ T cells in tumors but not in distal lymph nodes. This was an improvement compared to systemic IL-Sa15 treatment, which resulted in T cell expansion of all T cells. Unfortunately, the formulation induced toxicity in mice after repeated doses of the IL-15Sa BNC formulation [42].

2.2. Structural considerations regarding the desolvation method

α-lactalbumin (α-LAC) is an example of a protein with potential

therapeutic effect in the treatment of GI-tract-related conditions, different neurological conditions as well as cancer, of which BNCs have been prepared [32–34,69]. Conjugation of α-LAC to oleic acid to form the complex named Human α-LAC Made Lethal to Tumor cells (HAMLET) potentiates its anti-cancer effect [70–72]. Using deionized water as solvent, ethanol as antisolvent and GTA as crosslinking agent post-desolvation, BNCs with a size of approximately 150 nm have been prepared. The BNCs were enzymatically degraded in presence of gastric and pancreatic enzymes, but their antioxidative effects were reduced compared to free α-LAC [32]. The same researchers later synthesized α-LAC BNCs using 10 mM NaCl in deionized water as solvent and 80% acetone as desolvating agent. Using this slightly changed synthesis protocol, BNCs of 150 nm were successfully formed. However, this method suffered from a low yield (54%) as well as large conformational changes in the protein secondary and tertiary structure [33]. Based on these observations, it is reasonable to hypothesize that BNCs in the first study were of low activity due to structural protein damage. Another plausible explanation is poor dissociation of protein from the formulation, or a combination of both. Finally, the change in solvent and hence presence of salt ions in the process may have prevented assembly of the α-LAC molecules and therefore caused the low yield. It should be noted that only 4–6% of the final formulation was estimated to consist of protein meaning that these particles do not meet the BNC definition presented here. Other studies confirm how organic solvents may introduce structural protein changes but also show how biopharmaceutical activity may be preserved despite the changed conformation. Ethanol-based desolvation followed by GTA crosslinking resulted in α-LAC BNCs (diameters approx. 300 nm), which exhibited improved thermal, pH, and enzymatic stability despite changes in the secondary protein structure. The BNCs showed cytotoxic effects in three different cancer cell lines via production of reactive oxygen species (ROS). This effect was not observed in healthy cells, and the total anti-cancer effect of the BNCs appeared to be higher than that of HAMLET [34]. Other studies confirm how biopharmaceutical activity may be preserved despite a disrupted secondary protein structure. In a study, crosslinking α-LAC using the tyrosine-crosslinking enzyme polyphenol oxidase resulted in formation of dimers, trimers, and higher-order polymers. The secondary protein structure in the higher polymers changed drastically; the α-helical content decreased from around 29 to 10% and the β-sheet content increased from 20 to 32%. Furthermore, protein absorbance changed from the native protein to the crosslinked one with increased exposure of chromophores and hydrophobic groups on the protein surface. Crosslinked α-LAC was more resistant towards enzymatic degradation and induced a smaller immune response in mice compared to the native protein [69].

Likewise, lysozyme (LYZ) which has potential as a therapeutic for treatment of bacterial infections and cancer has been clustered into nanosized particles [37,52]. The LYZ BNCs were made from desolvation using water as solvent and ethanol as antisolvent followed by GTA crosslinking. Despite a disrupted LYZ structure after desolvation and crosslinking, the BNCs had improved stability at varying temperatures, pH values, and in the presence of degrading enzymes. They further showed cellular uptake via a non-hemolytic mechanism and showed the desired cytotoxic effect against a breast cancer cell line. The cytotoxicity appeared to involve ROS formation as addition of an anti-oxidant increased the cell proliferation [37]. Interestingly, the structural changes induced by organic solvents or the clustering process itself may also have a positive effect and in some cases make the active site of biopharmaceuticals more accessible. The potential anti-cancer enzyme cholesterol oxidase (CHOX) was formulated as BNCs with the aim of increasing the sensitivity of a cholesterol biosensor. The BNCs were synthesized via desolvation with ethanol as antisolvent and crosslinking by GTA and had sizes ranging from 100 to 200 nm. There was a significant increase in CHOX activity when formulated as BNCs compared to the free protein [36]. Nevertheless, conformation protein changes may not always be trivial nor favored from a therapeutic point of view as exemplified by the catalase enzyme, which has therapeutic potential in the treatment of various diseases affected or caused by formation of ROS. Synthesis of catalase BNCs using acetonitrile as antisolvent strongly affected the protein's secondary structure causing a reduction of ~50% and ~ 90% of its native enzymatic activity when desolvated with 20% and 75% acetonitrile, respectively [35].

2.3. Co-formulation and crosslinking for protection of the therapeutic protein

It remains a challenge to synthesize BNCs comprising only the biopharmaceutical of interest with an acceptable stability and therapeutic effect. Alternatively, biopharmaceuticals may be co-formulated with protein nanocarriers as these have potential to protect the therapeutic. Although not pure BNCs, these systems can have relatively high loading of the therapeutic peptide or protein, and they sit close in concept to BNCs. We highlight some examples here as the mixing of different proteins can have beneficial effects, which may be highly pertinent to future BNC design with multiple biopharmaceuticals. The β-Galactosidase (β-GAL) enzyme with therapeutic potential for treatment of lactose intolerance has successfully been co-formulated with Human Serum Albumin (HSA) and the enzyme stabilizing and cell-penetrating protein 30Kc19. The BNCs with a size ranging from 170 to 350 nm were prepared via ethanol-based desolvation and GTA crosslinking and had a loading efficiency of β-GAL of 80–95%. Increasing 30Kc19 concentration increased particle size as well as β-GAL activity. Since more than 50% 30Kc19 (wt%) resulted in deformed particles, the maximum enzyme activity for therapeutically active particles was achieved using 50% 30Kc19 (wt%). β-GAL particles using only HSA as nanocarrier improved the enzyme uptake and activity too, however, the improvement was much greater when 30Kc19 was included in the nanocarrier system [38]. Other researchers have prepared BNCs of β-GAL both in its pure form and co-formulated with enhanced green fluorescent protein (eGFP). The BNC synthesis was ethanol-induced desolvation followed by crosslinking with DTSSP, BS^3 or GTA. Only 25% of the native enzyme activity was retained in pure β-GAL BNC, whereas co-formulation with eGFP retained 75% of its activity. The β-GAL-eGFP BNC showed an increased cellular uptake compared to the free protein. The cellular uptake was higher at 37 °C compared to 4 °C suggesting an active transport mechanism. No drug loading was reported, however, assuming a molecular weight of the enzyme of 465 kDa and of the nanocarrier of 33 kDa, the applied enzyme:nanocarrier ratio of 1:24 corresponds to a 59% protein loading [39]. Thus, this study presents a nanocarrier system of relatively high loading and protection of the biopharmaceutical.

2.4. Summary of desolvation as synthesis method for BNCs

The studies assessed here illustrate a main challenge of structural protein damage using the desolvation method and stresses the importance of investigating the cause to the lack of biopharmaceutical effect if noticed. Proteins are expected to change conformation during the desolvation process to reduce the thermodynamic costs. However, whether this misfolding is reversible or enhances a desired biopharmaceutical activity depends on the BNC in question. Most peptide-based BNCs have not included direct data on the protein structure, and it is relevant to know if smaller biopharmaceuticals are less prone to structural damage due to their small sizes and limited flexibility. Most studies use circular dichroism (CD) to determine changes to protein secondary structure in BNCs. However, CD spectra of BNCs may be affected by light scattering effects and absorption flattening, which challenges the interpretation of data [73,74]. Alternative experimental methods including spectroscopy methods such as X-ray scattering, Nuclear Magnetic Resonance (NMR), Infrared (IR), and Raman spectroscopy, as well as thermal analyses such as Differential Scanning Calorimetry (DSC), however, these techniques may also be associated with a risk of BNC structure interference with the readout [75,76]. Indeed, some proteins and peptides may misfold in a

reversible manner upon clustering, thereby providing data that is not reflective of the reality.

Protein nanocarriers seems to have potential to protect biopharmaceuticals in BNC formulations although their use may significantly reduce drug loading [39,38]. From the studies reviewed here, another explanation to a reduced biopharmaceutical activity is a lack of release of the biopharmaceuticals (e.g., due to a strong surface crosslinking) or a decreased access to the active site(s) (e.g., due to binding to the nanocarrier, or an undesired orientation of the biopharmaceutical in the final formulation). An excess of crosslinker is usually applied to ensure sufficient crosslinking. However, unreacted crosslinker may be incorporated into the final formulation, thereby decreasing the biopharmaceutical content to an extent that does not meet the BNC definition [33].

A very limited number of studies crosslink the biopharmaceutical prior to desolvation [21,22]. Others rely only on the process of crosslinking to form BNCs without desolvation [24,42]. The reason and potential advantages of protein crosslinking may be improved control over the desolvation process or protection of the secondary protein structure (i.e., crosslinking will likely decrease the conformational freedom to prevent misfolding). Depending on the type of crosslinker, protein crosslinking may also increase total hydrophobicity to enhance the displacement of water molecules and promote protein clustering. More studies are needed to reveal the true advantages of how protein crosslinking can optimally be used, either alone or in combination with desolvation, for the formation of BNCs.

Immunotherapeutic BNC formulations have shown enhanced immunogenic performance compared to the free protein or peptide epitope and potentially have self-adjuvanting properties [18,21–24,40,42]. This may increase the number of antigen-presenting cells (APCs) at the site of administration or enhance the delivery of antigens to the APCs, as well as prevent that co-administered epitope and adjuvant do not reach the same cells. The high success of immunotherapeutic BNCs may be related to the use of epitopes since small peptides could be less prone to structural damage during the synthesis, or the immunogenic responses which might develop from nanoparticular structures in general [77].

3. Molecular crowding and *Salting-out*

Clustering of proteins and peptides can be introduced in the presence of cluster-promoting agents and occurs in nature in highly crowded environments, e.g., *intracellularly* [19,20]. This synthesis strategy relies on addition of a crowding agent to a solution of the biopharmaceutical and represents the method used in the first studies on protein BNCs [27–30]. The crowding agent must be added in a high concentration to occupy a large volume of the bulk solution and promote short-range interactions between proteins and peptides. The BNCs are formed by depletion forces where the clustering of proteins creates free space for co-solutes, thereby increasing the entropy of the surroundings. Optimal clustering of proteins requires attraction of protein molecules to one another in a controlled fashion to induce cluster formation while limiting cluster size. Studies predict that high protein concentrations stabilize proteins in their native folded state although proteins may become more susceptible to irreversible aggregation, gelation, and precipitation in extremely crowded environments [20].

3.1. *Antibody nanoclustering using diverse molecular crowding agents*

Johnston and colleagues synthesized reversible BNCs of monoclonal antibody (mAb) 1B7, polyclonal sheep IgG, and BSA by simple mixing of lyophilized powder of the protein and a molecular crowder (trehalose, polyethylene glycol (PEG) 300, *N*-methylpyrrolidone (NMP), or dextran). Dissolution resulted in formation of nanosized protein BNCs (mAb B17 ~ 300 nm, IgG ~ 250 nm, BSA ~ 30 nm) that dissociated into native-state monomers upon dilution (mAb B17 ~ 11 nm, IgG ~ 11 nm, BSA ~ 4–5 nm). The in vitro and in vivo behavior of the mAb 1B7 BNCs after subcutaneous administration was not significantly different to that of the non-clustered monomers suggesting dissociation upon injection [20]. In another study, the researchers prepared reversible mAb 1B7 BNCs by a slightly modified method using a new spiral-wound-in-situ freezing technique (SWIFT) followed by dispersion into aqueous solutions of crowding agents (NMP, PEG, and trehalose). SWIFT is based on rapid freezing of a solution in liquid nitrogen under rotation to trap proteins in their amorphous state while minimizing the exposure to liquid-air interfaces normally present in conventional freeze-drying procedures, thereby preventing protein aggregation. The BNCs were formed using a protein concentration of 190 mg/mL, had a mean size of approximately 430 nm and dissociated upon dilution [19]. The slight increase in 1B7 BNC size compared to their first study may lie in the slightly changed preparation method and/or the use of different crowding agents. In vivo experiments revealed similar pharmacokinetic profiles when a standard 1B7 dose was administered intravenously or subcutaneously as a diluted PEG-NMP BNC suspension, a concentrated BNC suspension, or a highly concentrated 1B7 BNC suspension (10-fold higher than the standard dose). The only difference was a slightly lower maximum concentration of the four subcutaneous groups compared to intravenous administration. The authors hypothesized this result to be due to incomplete cluster dissociation and/or increased clearance.

3.2. *Salt and small peptides as molecular crowding agents*

Calcium ions have been used to cluster insulin and the glucagon-like-peptide-1 (GLP-1) receptor agonist Exendin 4 (EXE-4). EXE-4 is a 39 amino acid peptide that has a 53% conserved amino acid sequence with GLP-1 and similar potency with respect to stimulation of glucose-induced insulin secretion from pancreatic β cells. Although EXE-4 is less prone to degradation compared to GLP-1, subcutaneous injections of EXE-4 are still needed twice daily for optimum blood glucose regulation [78]. EXE-4 BNCs and insulin BNCs with mean size 125 nm and 200 nm, respectively, were formed by calcium ion-based mineralization. Chelation of the protein amino acid residues with calcium ions resulted in nuclei formation, which in turn grew to form nanosized structures at equilibrium. Single-dose subcutaneous administration of EXE-4 resulted in complete clearance from the blood and normalization of the blood glucose levels within 24 h, whereas clustered EXE-4 could still be detected and gave slightly reduced blood glucose levels after 192 h. Clustering of EXE-4 thus gave a 22.7-fold increase in its half-life (from 1.9 h to 43.1 h). Similarly, subcutaneous administration of free and BNC insulin to diabetic mice over a period of 2 weeks resulted in an improved ability to regulate the blood glucose levels (i.e., lower the blood glucose levels after glucose administration) in the BNC-treated mice. This effect was observed for 3 weeks and was hypothesized to be due to cellular uptake and intracellular processing of the BNCs. Data suggested complete BNCs degradation within 24 h of administration while an increase in the glucose metabolism and upregulation of IRS-1, IRS-2, Glut-2, and Glut-4 mRNA was observed in cells treated with the insulin BNCs. This effect lasted for at least 72 h in BNC-treated cells and up to 48 h in cells treated with free insulin. Using thermal gravimetric analysis, the studies reported the weight percentage (wt%) of EXE-4 and insulin in the BNC formulations to be 17% and 10%, respectively. Whereas the insulin BNCs dissociated at pH values less than 7.2, the EXE-4 BNCs dissociated upon dissolution in a supersaturated physiological fluid [25,43].

Finally, the calcium bone-resorption inhibitor calcitonin self-assembled into BNCs using dipeptides as cluster-promoting agents. Using salmon calcitonin (sCT) which is positively charged at physiological pH and the aspartic acid-phenylalanine (Asp-Phe) dipeptide containing a negatively charged and a hydrophobic residue, sCT self-assembled at physiological pH in low-concentration phosphate buffer. The observation was supported by docking studies showing a strong electrostatic interaction between a lysine residue in sCT and Asp, as well as a hydrophobic interaction between the dipeptide and sCT. Asp-Phe

decreased the Critical Association Constant (CAC) from 1 mM for sCT to 0.1 mM in presence of the dipeptide. Other dipeptides (Asp-Ala, Ser-Phe, and Ser-Ala) having a negatively charged amino acid, a hydrophobic amino acid, or none of the two promoted self-assembly to a lesser extent; Asp-Ala decreased the CAC to 0.4 mM, whereas the other dipeptides had no effect on this factor. The native protein structure of sCT was preserved in the BNCs and protected against fibrillation in contrast to free sCT. sCT was released from the nanoformulation when diluted to a concentration below the CAC or in the presence of ions. The BNCs showed no signs of cytotoxicity nor immunogenicity with an improved duration of action. The serum calcium reducing effect of the BNCs lasted up to a month after a single subcutaneous dose in contrast to free sCT, which lasted for a day [44].

3.3. Summary of biopharmaceutical nanoclusters prepared via molecular crowding and Salting-out

The high concentration of cluster-promoting agents in the studies presented raises the question as to whether the formulations as a whole are actual BNCs. Nonetheless, a central advantage of using molecular crowders such as sugars or salts to form BNCs is the exclusion of agents that may have harsh effects on protein structure as seen in the desolvation method. In fact, many of the common molecular crowding agents such as sugar can have protective effect on protein structure. This is reflected by their use in many manufacturing processes such as freeze-drying. Based on our literature search, the number of studies on biopharmaceutical BNCs prepared through molecular crowding and *salting out* is quite limited. Literature reflects a potential for subcutaneous delivery of mAbs as BNCs using reduced dose volumes [19,20]. In the clinic, mAbs are normally administered intravenously at relatively low concentrations to prevent protein aggregation. Higher mAb concentrations requires larger injection volumes, which is often associated with discomfort, pain, tissue hardening, and edemas [79–81]. Thus, BNCs hold potential for improving the patient administration experience. However, formulation of the mAb 1B7 as BNCs did not enhance the general therapeutic effect [19,20]. To reveal the true potential of mAb BNCs, the lack of improved therapeutic effect using large doses must be addressed. A general challenge of BNCs prepared via molecular crowding is the instability caused by a high sensitivity towards the surrounding macromolecules. This not only limits their application to other administration routes, but it will also make it difficult to predict the in vivo fate. Blood and intracellular fluid are complex non-static environments, which can affect the stability, behavior, and efficacy of BNCs synthesized by molecular crowding. In contrast to the studies on mAb BNCs, formulation of EXE-4 and sCT as BNCs resulted in a significant improvement in the pharmacokinetics [25,44]. These promising findings could indeed be supported by more studies using this type of formulation strategy.

4. Clustering via pH-coacervation

Coacervation is a method based on phase separation, usually induced by changing solvent polarity, pH, or ionic strength. The phase separation can be either liquid-liquid (molecules in suspension) or liquid-solid (particle synthesis) in systems comprising one (simple coacervation) or more macromolecules (complex coacervation). Coacervation is an entropically driven process based on delocalization of counter ions or molecules from the protein surface. pH-coacervation is a type of simple coacervation carried out at or near the isoelectric point (pI) where the protein or peptide has no net electrical charge. As pH moves away from the pI, its absolute charge will increase, thereby introducing repulsive intermolecular forces between protein or peptide molecules of the same composition. This will reduce agglomeration following classical electrostatic colloidal stability theory, and the Debye screening length will be dependent on the salt concentration [7,10,11]. pH-coacervation is not a common BNC synthesis method as reflected by the limited number of studies. Although the studies mentioned here do not report a drug loading efficiency, it is reasonable to conclude that the BNCs mainly comprises the therapeutic protein based on the synthesis formulas.

Self-assembling bevacizumab BNCs have been developed for treatment of non-small cell lung cancer and to overcome the challenge of limited passive uptake of mAbs in cells due their relatively large sizes. The researchers behind the study observed a protective effect of the surfactants on protein structure during the pI nanoprecipitation when they titrated lyophilized bevacizumab in hydrochloric acid using sodium hydroxide. At its pI, bevacizumab clustered into BNCs, which after centrifugation, re-suspension in deionized water and freeze-drying had mean sizes of 334–774 nm depending on type and concentration of the surfactant (Tween-80, Tween-20, or Brij-97). The antibody and BNCs prepared in absence of surfactants had plate-like structures and a disrupted secondary protein structure. Presence of surfactants during the nanoprecipitation made the BNCs uniform in shape, protected the antibody structure, and reduced the tendency to agglomerate. Tween-80 mAb BNCs had anti-Vascular Endothelial Growth Factor (VEGF) activity, anti-proliferation, and cytotoxicity similar to that of the free antibody, whereas the surfactant-free BNCs had decreased cytotoxicity. There was an increased accumulation of the BNCs in A549 cancer cells as compared with healthy MRC-5 cells. Cellular uptake of the BNCs appeared to be non-specific and involve both energy-dependent endocytosis and micropinocytosis [45]. Oppenheim et al. prepared insulin BNCs using pI nanoprecipitation and GTA crosslinking. Fast-acting insulin Actrapid was dissolved in hydrochloric acid, the pH was increased using sodium hydroxide, and GTA was used for crosslinking of the resulting BNCs giving them a size of approximately 200 nm. They BNCs were absorbed from the GI tract of diabetic and non-diabetic rats and reduced blood glucose concentrations to 15–20% of the initial levels 3 h after administration at doses of 30–70 mg BNC/100 g body weight. Since less than 2% of dosed insulin is normally absorbed in the GI tract, this was a significant improvement. The response was slower but more pronounced compared to that of Actrapid [3,46]. No drug loading was reported. As previously discussed, crosslinkers may be the main component of crosslinked BNCs, meaning that no true classification can be made with respect to whether it is a BNC formulation.

5. Clustering via mechanical agitation

Proteins and peptides are often exposed to agitation during manufacturing (e.g., during shaking, centrifugation, sonication, mixing, vortexing, and flow) as well as in many biological processes. This is usually of high concern because the sensitive nature of biopharmaceuticals makes them prone to aggregation and misfolding under such conditions. Mechanical deformation can be utilized to form BNCs. This will require a tightly controlled application of agitation to prevent maturation and growth of the destabilized protein or peptide structure into large irreversible aggregates. The use of agitation to form BNCs is an interesting approach, and in contrast to many other methods, it can be carried out without use of any type of additive.

Catalase BNCs were successfully synthesized by applying shear stress to the protein in a co-axial cylinder flow cell in an ice-water bath. The authors observed that the catalytic activity of catalase decreased with increasing shear rate and shear time. Thus, although the lowest protein concentration, highest shear rate and longest shear time gave the smallest BNCs, these clusters only retained 20% of the original enzymatic activity as compared to the monomer. The final and biologically-active catalase BNCs were synthesized from a protein concentration of 0.2 mg/mL, shear rate 217 s^{-1} and shear time of 5 min to have a mean size of 182 nm and PDI 0.21. CD data suggested a slightly conformational change in the enzyme structure with a decrease in its α-helical content. This slight structural change had little effect of the resulting activity as more than 90% of the original activity was preserved in the final BNCs [35]. Other researchers prepared self-assembled nanostructures of the first line breast cancer therapeutic anti-HER2

humanized monoclonal IgG Trastuzumab (Herceptin) and of chicken IgY. The self-assembly was induced by agitation or stirring at pH 2 and at 37 °C causing the mAbs to go from a tri-nodular monomeric structure to a rounded state with little free monomeric mAb left. Upon dilution, the mAbs were released and pharmaceutically active. The mAbs bound to HER2-positive SKBR3 human breast cancer cells or Newcastle disease virus antigen in a time-dependent manner. Storing under harsh conditions (high temperature and shaking) for 2 months showed loss of binding capacity of the mAbs after dilution [47]. Lastly, researchers have successfully developed an adjuvant-free nanosheet formulation of OVA by continuous shaking at 180 rpm. The nanosheet structures were not fibrillar but rather had amyloid characteristics. They observed a much stronger antibody response in mice compared to free OVA [48]. These observations suggest a self-adjuvanting effect of the nanostructures. A self-adjuvating effect of BNCs has also been observed in other cases [22,82].

The impact of mechanical forces on protein structure is a well-studied area, and research has shown that shear stress can cause unfolding, aggregation, degradation, and loss of activity of several therapeutically relevant proteins including insulin, β-lactoglobulin, lysozyme, fibrinogen, and catalase [83]. Even so, many studies are at variance with each other with respect to defining the type of biopharmaceuticals that are prone to mechanical aggregation as well as the conditions under which it occurs. A previously reported example is the inactivation of catalase at a shear rate of 1155 s^{-1} observed by some researchers, while others did not observe any damage on the enzyme at shear rates up to 10^6 s^{-1} [84,85]. Finally, researchers have hypothesized that extremely high shear rates alone will not have promoted structural protein damage and aggregation, and that only conditions where air-liquid interfaces are formed (e.g., cavitation) should be of concern. This statement was based on their findings where the structure of nine proteins (α-lactalbumin, fibroblast growth factor 2, granulocyte colony stimulating factor (GCSF), GFP, hemoglobin, albumin, lysozyme, and two antibodies) were tested under different types of mechanical stress (shear stress, dimensionless shear, a combination of cavitation and shear stress, or a combination of shear stress, cavitation, and foaming) [86]. Potential explanations to the observed interstudy differences appear to lie in factors such as protein concentration, batch purity, and laboratory materials, and reflects how even subtle conditional changes may affect protein structure and function as well as our interpretation of their stability. Identification of the point of mechanically induced aggregation is interesting and of great importance. Keeping the mechanical stress below this threshold may prevent formation of larger aggregates and instead result in formation of smaller reversible and pharmaceutically active aggregates (i.e., BNCs). Another remaining question of concern regards the potential immunogenic effects of protein and peptide aggregates, including how much this depends on protein structure (primary, secondary, etc.), aggregate size, or other factors. In that respect, the in vitro activation of human monocyte-derived dendritic cells and T cell responses have been observed to differ between aggregates of different IgGs. When having the same stimulatory potential, this suggests a critical role of the primary protein sequence [87]. However, most protein aggregates are in the low micrometer range, which is considerably larger than the optimal BNC size. This limits the conclusions that can be drawn with respect to immunogenic effects of BNCs synthesized by mechanical forces. Only few studies use the mechanical approach to synthesize BNCs. This is likely due to uncontrolled growth and maturation of protein aggregates, which hinders obtaining BNCs of an appropriate size. Altogether, more studies are needed to conclude on whether application of mechanical stress is an ideal synthesis method of BNCs. This will require identification of controlled mechanical stress conditions under which therapeutic proteins and peptides form BNCs with retained biological activity.

6. Lipid-based biopharmaceutical nanoclusters

Popular lipid-based formulation methods for delivery of therapeutic proteins and peptides include liposomes and solid lipid nanoparticles (SLNs). In these formulations, loading is usually done by mixing of the biopharmaceutical with melted lipids, while homogenization is done via mechanical agitation or ultrasonication. Protein denaturation from high temperatures in the lipid melts and harsh homogenization methods are major factors limiting the use of lipid-based particle systems for delivery of biopharmaceuticals. Although more gentle methods such as cold high-pressure homogenization may be applied to prevent protein structure damage, lipid-based nanodrugs suffer from a low drug loading [7,88].

Interestingly, researchers have managed to design lipid-stabilized particles loaded with an antithrombotic peptide to overcome the challenge with low drug loading in conventional lipid nanostructures. The therapeutic peptide constituted up to 70% of the final molarity, allowing more than 10 mM of the drug to be delivered by intravenous injection in humans. The mP6 peptide (myr-FEEERA) is derived from the $G\alpha_{13}$-binding ExE motif of the cytoplasmic integrin β_3 domain found on platelets and involved in platelet aggregation. The peptide was myristoylated in its N-terminal and had alanine changed to leucine in the C-terminal to become M3mP6. M3mP6 assembled into micellar nanostructures in presence of phosphatidylcholine and 1.2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-attached polyethylene glycol (PEG) using the thin-film hydration technique where a dry thin lipid layer is formed and re-suspended in an aqueous solvent. The BNCs had mean sizes of 6–25 nm reflecting that the synthesis was challenged by a relatively large variation in the mean particle size. In vitro, the BNCs were taken up by platelets and inhibited human platelet granule secretion and secretion-dependent secondary platelet aggregation induced by low-dose thrombin. Furthermore, the particles partially inhibited collagen-induced and U46619-induced platelet aggregation but did not affect adenosine diphosphate (ADP)-induced platelet aggregation. In vivo, M3mP6 was not very potent and had a relatively short half-life in the blood. However, the peptide was fast-acting and had a longer plasma half-life than other standard antiplatelet drugs. Interestingly, the peptide had a synergistic antithrombotic effect in combination with some of the commonly used antiplatelet drugs (clopidogrel, ticagrelor, and cangrelor) with or without aspirin. There was also a reduced risk of excessive bleeding in animals treated with the BNCs compared to some of the standard antiplatelet drugs. Altogether, data suggested the optimal anti-thrombotic treatment to be a combination of the BNCs and low-dose standard antiplatelet drug. Finally, injection of the same peptide concentrations using BNCs for which M3mP6 comprised 36% of the total mass and nanoparticles where this mass percentage was 50 caused similar anti-thrombotic effects suggesting that the effect is related to the peptide concentration and not formulation changes [49].

7. Emulsion-based methodologies for biopharmaceutical nanoclusters

In contrast to the conventional lipid-based nanoformulations, therapeutic drugs can be dissolved in an aqueous phase when loaded into emulsified nanosystems. Emulsions are dispersions of two or more immiscible liquids such as an oil phase and a water phase, usually stabilized by an interfacial layer of surfactant molecules. Emulsified systems can be prepared without high temperatures but normally require use of ultra-sonication or mechanical agitation [52,50,51,53].

A group of researchers have modified the solubility of several proteins to allow synthesis of oil-in-water (O/W) nano emulsions via single and double emulsification methods. For example, BNCs of m-polyethylene glycolated (mPEGylated) LYZ, β-lactalbumin (β-LAC), OVA, and HSA were prepared alongside with a careful characterization of the LYZ BNCs. mPEGylation increased the protein solubility in dichloromethane (DCM) making it possible to prepare water-dispersed BNCs of

the protein. mPEGylated protein was dissolved in DCM and mixed with PBS followed by 45 s of ultra-sonication and overnight DCM evaporation. The resulting BNCs were 95 nm, 208 nm, 173 nm, and 179 nm for LYZ, β-LAC, OVA, and HSA, respectively. LYZ was only released from the particles under acidic or reducing conditions and in the presence of proteases. mPEGylated LYZ had only 19% of the native protein activity, while only 3% enzymatic activity was retained in the final formulation. Since CD spectra showed a preserved secondary protein structure after mPEGylation and sonication, the most obvious reason for the reduced biopharmaceutical activity is mPEGylation at sites important for the enzymatic activity of LYZ [50]. Using a range of PEG analogues, the investigators prepared BNCs of PEGylated LYZ with sizes 100–130 nm using the single emulsion method. As in their previous study, CD spectra revealed a preserved secondary protein structure, although only epoxy-mPEGylated LYZ did not significantly suffer loss of biopharmaceutical activity [51]. Altogether, the system has potential for delivery of hydrophobic drugs that can be co-formulated with mPEGylated LYZ. However, the BNC synthesis method does not appear suitable for self-delivery of an mPEGylated protein in general. Switching to a double emulsion strategy, 200 nm LYZ BNCs were prepared in a stable W/O/W emulsion using a water-soluble PEG to keep the protein in the aqueous phase during synthesis and protecting it from DCM. Despite a promising 81% preserved antibacterial activity of the PEGylated LYZ compared to the native protein, there was no antibacterial effect of the empty BNCs in the final formulation. Instead, the formulation was found to have potential as a protein nanocarrier system as LYZ and fluorophore-labelled lysozyme (Cy5-LYZ) could be encapsulated in the BNCs with a drug loading of around 5%. Free and encapsulated fluorophore-labelled protein had similar Minimum Inhibitory Concentrations (MICs), but the effect was induced slower and lasted longer in the BNC formulation [52]. In continuation hereof, the authors used the double-emulsion method to prepare BNCs using cytochrome *c* (CYTC) as a model protein. CYTC was modified with acid-degradable PEG and the resulting emulsified BNCs had an average size of 140 nm. PEGylated CYTC had 91% of its native activity, and CD confirmed minor structural alterations upon PEGylation. As expected, the BNC stability decreased with decreasing pH, and in contrast to native CYTC and PEGylated CYTC, which cannot penetrate cell membranes, increasing BNC concentration decreased cell survival suggesting endosomal cellular uptake. In contrast to the single emulsification method, the double-emulsification approach had potential for loading of hydrophilic drug, although the potential is limited by a drug loading of 1.7% using Oregon-Green 488 dextran [53]. Altogether, the double emulsification method where the biopharmaceutical is dispersed in the aqueous phase and protected from DCM might hold potential for self-delivery of proteins. Contrary to the single emulsification method, the increased biopharmaceutical activity of LYZ using double-emulsification suggests that DCM may induce structural damage in the proteins during the synthesis, similar to what was discussed on using organic solvents in desolvation.

8. Designing self-assembling constructs

Many proteins naturally self-assemble into highly ordered structures under specific conditions. Examples include collagen, elastin, silk, and keratin. Some proteins will have self-assembling properties under specific conditions, while others may achieve self-assembling properties from chemical modification such as insertion of self-assembling amino acid sequences (e.g., π stacking motifs coupled with cationic motifs). Unfortunately, modification of native proteins and peptides to introduce or alter self-assembling properties can be challenging because even a subtle modification may introduce major consequences, e.g., a reduction in therapeutic efficacy. Theoretically, all methodologies for the synthesis of BNCs are based on self-assembly, however, in this section, focus is on self-assembling constructs where non-biopharmaceutical constructs have been attached to the therapeutic protein or peptide to enhance or introduce self-assembling properties.

8.1. Elastin as self-assembling biomaterial

Elastin and elastin-like peptides (ELPs) have successfully been used in the design of self-assembling constructs owing to their hydrophobicity and mechanical properties. Examples include micellar particles and combinations with other functional proteins [54,55,89]. Keratinocyte growth factor (KGF), which promotes re-epithelialization, has been fused to ELP V40C2, which promotes wound healing and has self-assembling properties. At physiological temperatures, the fusion protein and the ELP itself self-assembled into spherical BNCs with diameters of approximately 500 nm. The proliferative properties of the BNCs on KGF-responsive epithelial cells measured 2 days after administration were similar to that of free KGF (2.3-fold versus 2.0-fold, respectively), whereas ELP itself had no effect. On the other hand, the proliferative effects on fibroblasts increased 1.6-fold for the KGF-ELP BNCs and 4.9-fold for the ELP BNCs compared to the control. Administration of the BNCs to diabetic mice and histological examination showed enhanced re-epithelialization and granulation of the BNCs compared to the controls. Compared to the ELP BNCs administered either alone or in combination with free KGF, the KGF-ELP BNCs had a slightly smaller effect on the granulation but a larger effect of the re-epithelialization. It seems that fusion of KGF to ELP diminishes some of its biological activity, but that KGF and ELP may work synergistically with respect to wound healing properties. The fused BNCs likely hold the greatest therapeutic potential since excessive granulation can result in fibrosis and scarring [54]. Self-assembling BNCs based on mPEGylated human α-elastin has been developed for delivery of insulin. BNCs of non-PEGylated α-elastin grew overnight due to aggregation, while BNCs of mPEGylated α-elastin were stable and had limited self-assembling properties due to the hydrophilic nature of PEG. The resulting BNCs were approximately 330 nm and had a narrow size distribution. Insulin was suspended in mPEGylated human α-elastin and heated to 37 °C for spontaneous encapsulation of the protein. Based on the elastin BNC and insulin concentration listed in the article, the protein loading is expected to be too low to meet the BNC definition. However, the biphasic slow release over 72 h and prolonged stimulatory effect on glucose transporter type 4 (GLUT4) translocation from intracellular stores reflect the potential of elastin as a protein nanocarrier [55]. In contrast to the ELP and KGF, where the two fused components work synergistically to form BNCs and give a therapeutic effect, this formulation appears do not meet the BNC definition based on the synthesis protocol. Thermo-responsive self-assembly may be a promising methodology for synthesis of protein-based BNCs and reflect a potential of α-elastin as protein nanocarrier. Furthermore, PEGylation may be necessary for improved stability and controlled particle size although it increases the proportion of non-therapeutic in the BNC.

8.2. Limited drug loading capacity due to size of self-assembling construct

Self-assembling cluster of differentiation 8 ($CD8^+$) epitope BNCs for anticancer vaccine delivery have been developed by fusion of a self-assembling peptide (Ac-AAVVLLLW-COOH) (SA) to the N terminus of the $OVA_{250–264}$ antigen (SGLEQLESIINFEKL) and Human Papilloma Virus (HPV) antigen (GQAEPDRAHYNIVTF). Above their critical aggregation concentration (~ 15 μM), the two fusion proteins OVA-SA and HPV-SA self-assembled into spherical BNCs with a mean size of 24 nm and 86 nm, respectively. The BNCs were administered subcutaneously with or without unmethylated cytosine-guanine dinucleotide (CpG) as an adjuvant. Subcutaneous administration of the OVA-SA BNCs after injection of OVA-specific $CD8^+$ T cells resulted in a strong T cell expansion. Subcutaneous administration of the HPV-SA BNCs to naïve C57BL/6 mice and mice that had TC-1 tumor cells injected resulted in T cell expansion and significantly higher specific CD8+ responses compared to the control. The results showed that a booster vaccination was required to induce a strong immune response and that the effect of the two epitope vaccines was improved using the CpG adjuvant. The

effect was larger for the BNCs compared to the free peptide to illustrate the importance and potential of the BNC structure [56]. In another study, BNCs of glucagon-like peptide 1 (GLP-1) analogues were synthesized by inserting different self-assembling peptides into its sequence. GLP-1 is a gut hormone released after oral glucose administration, which stimulates insulin secretion and inhibits glucagon secretion resulting in lowered blood glucose levels. In the study, the receptor binding affinity decreased for all tested analogues. However, the stability of the analogues was improved as seen from their slower in vitro proteolysis by dipeptidyl peptidase (DPP IV) and slower in vivo degradation. In contrast to GLP-1, which was cleared in less than 4 h in vivo, the GLP-1 analogues were still present 70 h post injection. Some of the GLP-1 derivates formed nanotubes, whereas other formed nanospheres. Neither GLP-1 nor the analogues stimulated insulin secretion in pancreatic rat islets ex vivo, but subcutaneous administration in rats showed a longer as well as 2.8-fold and 3.1-fold higher insulin secretion for two of the GLP-1 peptides compared to native GLP-1. Interestingly, male and female rats responded differently to the treatment; blood glucose decreased faster in female rats than in male ones. The study illustrates how the in vivo biopharmaceutical response of peptides significantly can be improved when formulated into an BNC structure despite the self-assembling analogues themselves having a much lower binding affinity towards their target [57]. The OVA/HPV and GLP-1 systems are both close to being BNCs, however, due to the size of the self-assembling structure, a drug loading closer to 50% is expected. Hence, designing self-assembling constructs are challenged by the size of the biopharmaceutical compared to that of the self-assembling modifications. A potential strategy to overcome this is use of self-assembling proteins that can accommodate several protein or peptide molecules.

8.3. Increased drug loading using cage-like structures

Champion and colleagues loaded IgG into a self-assembling hexameric nanocarrier, H6-SPAB-Hex, which has a theoretic potential of carrying up to six drug molecules [58]. Combination of a self-assembling α-helical peptide with Protein A domain B (SPAB) by a flexible glycine-serine linker allowed formation of BNCs, and the high affinity of SPAB for the Fc region of IgG allowed for a final BNC formulation where the bulk mass consisted of therapeutic. Two nanocarrier systems with different conformations were designed and synthesized (H6-SPAB-Hex and Hex-SPAB-H6) with size 25–35 nm. The BNCs were not cytotoxic and were actively taken up by HeLa cells to a much higher degree compared to the free, soluble antibody. However, the BNC uptake was much faster when loaded with rabbit IgG compared to mouse IgG (mice IgG subclass not stated). Introduction of the endosomolytic domain Aurein 1.2 peptide in the two Hex nanocarrier sequences retained its self-assembling properties while increasing the degree and rate of cellular uptake of IgG [59]. However, despite this optimization, the study reflects how changes in the protein, e.g., due to its source, may have serious effects on its pharmacokinetic and hence therapeutic potential. As a follow-up study, the researchers characterized the Hex carrier system and observed that the large molecular weight of the mAb (150 kDa) compared to that of the loaded Hex carrier (duplicate of 503 and 510 kDa) limited the loading to three IgG molecules per SPAB domain [58]. They also observed that the most stable IgG-loaded Hex BNCs with the fewest aggregates were formed upon mixing of Hex and IgG in a ratio 1:3, whereas other ratios resulted in unstable systems. The system was most stable at 37 °C and less stable at lower temperatures. This is advantageous from a drug administration point of view but challenges the shelf-life storage. The IgG secondary protein structure was retained in the final BNC formulation. A figure from the study indicates the size of the self-assembling carrier to correspond to that of two H6 molecules. Hence, this is another example of a system not completely meeting the definition of a BNC as presented here. The Hex-IgG formulation was internalized by varying degrees and kinetics in HeLa, SKBR-3, MDA-MB-231, HUVEC, and J774 cells with clathrin-mediated internalization being the main uptake mechanism, and macropinocytosis and caveolae-mediated endocytosis representing secondary mechanisms. BNC trafficking to endosomes was followed by escape to the cytosol and dissociation of the biologically-active antibody and the Hex nanocarrier [60].

One advantage of self-assembling proteins is that the process is usually spontaneous under gentle conditions, thereby decreasing the risk of conformational damage of the nanocarrier and the biopharmaceutical. Despite progression in the design of self-assembling proteins and peptides, several challenges remain before these systems can be rationally designed. The studies presented here show how drug delivery can be significantly improved when biopharmaceuticals are formulated as BNCs compared to being in the free, soluble form. They further reflect how even a subtle change may impair the self-assembling properties of a protein. Finally, the limited number of studies on self-assembly of larger proteins reflect that it may be more complicated to make larger biopharmaceuticals self-assemble into BNC structures, likely because the self-assembling sequences comprise a relatively small part of the entire system which weakens their self-assembling forces.

9. Conclusion

As of today, the most successful methods for synthesis of BNCs include desolvation, pH-coacervation, molecular crowding, salting out and physical methods such as application of shear stress. Other methods including lipidation, emulsification, and design of self-assembling constructs have shown promising results but have not yet led to synthesis of BNCs where a biopharmaceutical represents the main component of the nanoparticle and/or is biologically active.

Desolvation followed by crosslinking appears to be the most used method for synthesis of BNCs. The method has successfully been applied to form stable BNCs with appropriate sizes for cellular uptake and drug delivery. The method's applicability to therapeutic proteins and peptides is limited by the potential damaging effect of anti-solvents applied in the synthesis process and the addition of crosslinker in the final BNC formulation [39,32,33,36]. To overcome the challenges of a damaged protein structure, the therapeutic may be co-formulated with a protein nanocarrier or non-toxic additives. Co-formulation with a protein nanocarrier has shown potential to increase the therapeutic effect of some biopharmaceuticals [39,38]. On the other hand, co-formulation of a biopharmaceutical with a protein nanocarrier may result in a low drug loading capacity similar to what has been observed for crosslinked BNCs. A high crosslinker concentration in the final BNC formulation does not only reduce drug loading, it also increases the risk of potential side effects related to the crosslinker. Emulsification is another synthesis method that often uses harsh chemical and is therefore associated with many of the same risks as the desolvation method [52,35,50,51,53]. Both molecular crowding and *salting-out* have potential to overcome damages to the biotherapeutic as the formulation normally involves the use of non-toxic and structure-protecting crowders [19,20,25,43,44]. Independent of synthesis method, BNC stability may be challenged by fast dissociation upon a change in the surrounding environment or dilution. Because the structure of BNCs prepared from molecular crowding or *salting-out* are highly dependent on the surrounding molecules, this type of BNC formulation is particularly at risk of possessing low stability [19,20,25]. We have seen examples on BNCs that dissociate upon simple dilution (e.g., in physiological media) and BNCs of low shelf-life stability as seen from either dissociation or aggregation upon storage in common formulation solvents [19–22,25]. Altogether, these examples underline the structural fragility by which BNCs may be characterized. BNCs prepared by application of some type of mechanical force is the only synthesis strategy that completely fulfills the requirement of a BNC, as no additives are needed. Successful application of this method requires identification of stressful conditions at which formation of larger aggregates is prevented [37,47,48]. Conflicting studies in the field signify the challenges and long-standing process of identifying the

point of aggregation of proteins [83–87]. Possibly the discrepancies are related to small differences in batch purity and temperature, but a consistent approach needs to be developed for this method to be generally applicable. For the same reason, the use of a controlled mechanical destabilization to form smaller-sized BNCs is limited. Challenges associated with the design of self-assembling BNC constructs include detrimental effects of single amino acid substitution with respect to the self-assembling properties and the minimum size of self-assembling sequences for successful formation of BNC structures [57–59]. From the minimal number of studies identified, we conclude that lipid-based BNCs is not a reality at the moment [49].

The limited number of studies as well as literature search challenges to find studies on BNCs is expectedly a consequence of the rather recent introduction of the NC term. From the even more limited number of in vivo studies, it is not possible to correlate the type of synthesis strategy to the most relevant route of administration. Nonetheless, almost all types of BNCs appear to have potential for increased cellular uptake compared to the free biopharmaceutical (Table 1). Additionally, many BNC systems seem to give a stronger biological response and/or extended duration of action (Table 1). Thus, the main advantages of BNC formulations are concluded to be higher cellular uptake, stronger biological activity, and extended duration of action. The main disadvantages of the BNCs are evaluated to be the risk of misfolding, aggregation and high polydispersity independent of the formulation strategy (Table 1).

10. Perspectives

The need for more convenient administration methods of therapeutic proteins and peptides requires the development of new drug delivery systems [3,6]. The development of self-delivery systems where the therapeutic protein or peptide comprise the bulk of the particular system will allow for maximum drug loading and cellular uptake efficiency whilst preventing side effects from the vehicle and co-formulants [16]. Studies on BNCs further contribute to our general understanding of opportunities and limitations within the field of protein and peptide formulation. The ideal BNC formulation for delivery of therapeutic proteins and peptides is characterized by:

i) Reproducible formulation strategy to give a high-yield manufacturing process to avoid loss of expensive therapeutic and increase cost-effectiveness.
ii) Optimal size and charge to ensure maximum bioavailability and shelf-life stability.
iii) High loading efficiency with the protein therapeutic being the main component of the formulation to prevent side effects from additives and a limited absorption below the minimum effective dose.
iv) Stable protein structure and BNC formulation to ensure an adequate shelf life and biological activity as well as prevent toxic and/or undesired immunogenic in vivo responses from misfolded or aggregated proteins. Biopharmaceutical BNCs for oral administration must furthermore be somewhat resistant to low pH and proteolytic degradation.
v) Composed of balanced matrix density with a stability that prevents undesired degradation while preserving a slow and sustained release of the therapeutic. Ideally, the intermolecular forces of BNCs are strong enough to prevent fast dissociation upon dilution and weak enough to allow dissociation on a practical time scale and for example in the presence of certain biological molecules. Improved pharmacokinetics will furthermore ensure controllable dosing and avoid toxicity of biopharmaceuticals with small therapeutic windows.

Independently of the synthesis strategy, some of the consistent limitations on the studies of biopharmaceutical BNCs is drug loading, BNC stability, polydispersity, and missing mechanistic and biological studies (Table 3).

We have seen examples on how the presence of non-therapeutic in BNC formulations can significantly decrease drug loading to not meet the definition of a true BNC. This may be more critical in some cases (e.g., high concentrations of crosslinking agents or other types of additives) compared to others (e.g., use of a protein nanocarrier or use of high sugar concentrations). Few studies report the drug loading of BNCs, probably due to the difficulties associated with experimental determination of this factor. Many BNC formulations are highly polydisperse as reflected by the reported PDI values (Tables 1 and 2). Most of the reported PDI values are in the range of 0.15–0.45, and only few are below 0.1. PDI values below 0.3 are usually accepted for nanoparticle formulations, while only PDI values below 0.05 are considered monodisperse. Formulations characterized by a high polydispersity may encounter dosing difficulties [90].

The relatively high structural sensitivity of proteins and peptides among other therapeutics encounters their conformation, biological activity as well as the stability of the final BNC formulation. Studies have unambiguously demonstrated that BNCs can improve both the therapeutic efficacy and cellular uptake of biopharmaceuticals significantly compared to the free, soluble molecule (Fig. 1 and Table 1). However, we have also shown examples where minute changes in the BNC formulation impair a therapeutic effect and the cause is rarely identified. Neither have studies focused on determining the mechanism of BNC action (e.g., does it dissociate to the free molecule or does the BNC interact with multiple targets that stimulates response). In continuation hereof, most studies on BNCs continue to focus on in vitro experiments. This overreliance on in vitro data limits BNCs translating to the clinic.

The majority of the in vivo studies observing an improved therapeutic effect (e.g., stronger biological response or longer duration of action,) of the BNCs in comparison with the free biopharmaceutical are based on subcutaneous, intranasal, and intraperitoneal administration. Among the few studies using IV injection, a remarkable therapeutic improvement is generally not observed. The single study on lipid-based BNCs is an exception as the developed BNC system had a faster onset of action and longer duration of action compared to similar therapeutics on the market (Table 3). Based on these studies and general observations related to the stability of the BNCs, it is fair to hypothesize that the main potential of BNC formulations lies in the invasive routes of administration.

Numerous studies on non-invasive formulations of biopharmaceuticals have been published [1,2,91,92]. The most popular formulation methods in these systems are loading into carrier systems (e.g., liposomes or polymeric nanoparticles), co-formulation with absorption enhancers, as well as protein modifications [1,3–6]. The clinical translation of these delivery systems is not impressive, which is likely attributed to a low drug loading, low bioavailability, toxic side effects and/or the inconsistencies often observed in the translation from in vitro experiment to in vivo studies and finally clinical studies. An example of a biopharmaceutical of low oral bioavailability that has reached the market is desmopressin (DDAVP®). Desmopressin is a modified and much more potent peptide analogue of vasopressin, which is delivered nasally or orally. With a bioavailability of less than 0.16%, the main reason for the successful clinical translation of oral desmopressin is its high potency [1,91,93]. Many of the successful in vivo findings are based on buccal or nasal administration, which is similar to the trend we see in the clinic as many of the approved non-invasive biopharmaceutical formulations are based on these two delivery routes [1]. An example to this is the two insulin dry powder formulations for inhalation Afrezza® and Exubera®. Exubera® was discontinued in 2017 because of difficulties related to handling of the device and correct dosing, the risk of side effects, and high costs [1]. In the meantime, most diabetics hold on to SC insulin formulations, and insulin continues to dominate in the increasing number of oral biopharmaceutical formulations that enter clinical trials [1]. All things considered, there is a need

for the development of non-invasive biopharmaceutical formulations as well as improved invasive biopharmaceutical formulations to improve patient compliance and treatment of disease.

BNCs have potential to give a stronger therapeutic response and a prolonged duration of action (Table 3). This can result in reduced dosing, dosing volume, and dosing frequency, and ultimately an improved patient compliance. The conjugation of polyethylene glycol (PEG) to biotherapeutics and nanocarriers (i.e., PEGylation) is used to extend the circulation time of drugs. After FDA-approval of the first PEGylated biopharmaceutical in 1990 (i.e., PEGylated deaminase, Adagen®), PEGylation has been applied to other biopharmaceuticals including certolizumab pegol (Cimzia®) and naloxegol (Movantik®) [2,94]. Disadvantages of PEGylation include a reduced biological activity of the drug and immunogenic responses including the production of anti-PEG antibodies [52,50,51,95]. The biocompatibility and structural reversibility of BNCs will likely reduce the risk of immunogenic responses compared to many of the current strategies used for prolonging the therapeutic response. Examples on biopharmaceuticals that must be delivered in high doses include antibodies for the treatment of migraine, rheumatoid arthritis, psoriasis, and dyslipidemia among others, and insulin for insulin-resistant diabetics. Consequently, high-dose antibody formulations (e.g., Sarilumab® and Canakinumab®) and high-dose insulin formulations (e.g., Humulin® R U-500) have reached the market [96,97]. Likewise, the potential of BNCs having a higher biological response compared to the free monomer and the structural compactness of the BNC structure can allow for delivery of higher doses without increasing the dosing volume significantly, which is not always feasible.

Finally, it is worth nothing that more knowledge is needed for determining the protein or peptide concentration of BNCs. Some studies do not report protein or peptide concentration, and most studies do not report the method on how protein or peptide concentration in the BNCs was determined. A recent study on the challenges of determining the correct protein concentration in nanoparticles synthesized by the desolvation method highlighted the issues in using colorimetric assays, the need for baseline correction using ultra-violet (UV) spectroscopy, and the importance of investigating the effect of additives (e.g., crosslinkers) on the protein properties using these methods [98]. Of the studies reporting the concentration method, most relied on UV spectroscopy with only few mentioning the use of baseline correction [23,25,33,43,58]. There are also a few reported examples on the use of the BCA assay as a colorimetric method [41,34]. More recently, ^{1}H NMR has been used with an internal standard reference to calculate the concentration of peptide BNCs [22]. Finally, there are examples on analysis of byproduct material from the formulation process to calculate the amount loaded [42]. However, it should be noted that some BNC concentrations can possibly be correctly determined from the starting material without the need for analyzing the final BNC formulation. This is for example expected to be the case for BNCs prepared via molecular crowding and *salting-out* where the initial protein or peptide concentration is not expected to change during BNC synthesis. Reporting the protein or peptide concentration of BNCs in particularly important with respect to the overall evaluation of therapeutic advantages and disadvantages. For example, intramuscular injection of insulin BNCs prepared from pH coacervation and crosslinking appeared to give a stronger blood sugar response compared to free insulin when administered at a remarkably higher dose than that of the free protein [46]. While such observations may be explained by local degradation or rapid clearance, they still leave questions with respect to evaluation of therapeutic efficacy and cost-effectiveness of the BNC formulation over the free molecule.

Altogether, BNCs represents a relatively new type of formulation for the delivery of protein and peptide therapeutics. Recently, there has been a growing interest in formulations meeting or approaching the definition of true biopharmaceutical BNCs. The studies presented here reflect the potential of BNCs with respect to improved and prolonged therapeutic efficacy. However, more studies on the stability, drug loading, and mechanism of action as well as more in vivo studies are needed. This will fill the gaps of our current understanding of this type of drug formulation and hence reveal the true therapeutic potential of BNCs.

Conflicts of interest

None.

Acknowledgements

This work was supported by the Novo Nordisk Foundation [Grant. No. NNF16OC0022166].

References

[1] A.C. Anselmo, Y. Gokarn, S. Mitragotri, Non-invasive delivery strategies for biologics, Nat. Rev. Drug Discov. 18 (1) (2018) 19–40, https://doi.org/10.1038/nrd.2018.183.
[2] A.M. Vargason, A.C. Anselmo, S. Mitragotri, The evolution of commercial drug delivery technologies, Nat. Biomed. Eng. 5 (9) (2021) 951–967, https://doi.org/10.1038/s41551-021-00698-w.
[3] B. Homayun, X. Lin, H.J. Choi, Challenges and recent progress in oral drug delivery systems for biopharmaceuticals, Pharmaceutics 11 (3) (2019), https://doi.org/10.3390/pharmaceutics11030129.
[4] S.B. van Witteloostuijn, S.L. Pedersen, K.J. Jensen, Half-life extension of biopharmaceuticals using chemical methods: alternatives to PEGylation, ChemMedChem. 11 (22) (2016) 2474–2495, https://doi.org/10.1002/cmdc.201600374.
[5] W.R. Strohl, Fusion proteins for half-life extension of biologics as a strategy to make biobetters, BioDrugs. 29 (4) (2015) 215–239, https://doi.org/10.1007/s40259-015-0133-6.
[6] M. Kristensen, H.M. Nielsen, Cell-penetrating peptides as carriers for oral delivery of biopharmaceuticals, Basic Clin. Pharmacol. Toxicol. 118 (2) (2016) 99–106, https://doi.org/10.1111/bcpt.12515.
[7] S. Hong, D.W. Choi, H.N. Kim, C.G. Park, W. Lee, H.H. Park, Protein-based nanoparticles as drug delivery systems, Pharmaceutics 12 (7) (2020) 1–28, https://doi.org/10.3390/pharmaceutics12070604.
[8] P. Foroozandeh, A.A. Aziz, Insight into cellular uptake and intracellular trafficking of nanoparticles, Nanoscale Res. Lett. 13 (2018), https://doi.org/10.1186/s11671-018-2728-6.
[9] Ventola C. Lee, Progress in nanomedicine: approved and investigational nanodrugs, Pharm. Ther. (P&T) 42 (12) (2017).
[10] D. Verma, N. Gulati, S. Kaul, S. Mukherjee, U. Nagaich, Protein based nanostructures for drug delivery, J. Pharm. 2018 (2018) 1–18, https://doi.org/10.1155/2018/9285854.
[11] W. Lohcharoenkal, L. Wang, Y.C. Chen, Y. Rojanasakul, Protein nanoparticles as drug delivery carriers for cancer therapy, Biomed. Res. Int. 2014 (2014), https://doi.org/10.1155/2014/180549.
[12] N. Rathore, R.S. Rajan, Current perspectives on stability of protein drug products during formulation, fill and finish operations, Biotechnol. Prog. 24 (2008) 504–514, https://doi.org/10.1021/bp070462h.
[13] J. Wang, K. Liu, R. Xing, X. Yan, Peptide self-assembly: thermodynamics and kinetics, Chem. Soc. Rev. 45 (20) (2016) 5589–5604, https://doi.org/10.1039/c6cs00176a.
[14] Y. Yan, J. Huang, B.Z. Tang, Kinetic trapping-a strategy for directing the self-assembly of unique functional nanostructures, Chem. Commun. 52 (80) (2016) 11870–11884, https://doi.org/10.1039/c6cc03620a.
[15] D.S. Pisal, M.P. Kosloski, S. Balu-Iyer, v., Delivery of therapeutic proteins, J. Pharm. Sci. 99 (6) (2010) 2557–2575, https://doi.org/10.1002/jps.22054.
[16] J. Shen, J. Wolfram, M. Ferrari, H. Shen, Taking the vehicle out of drug delivery, Mater. Today 20 (3) (2017) 95–97, https://doi.org/10.1016/j.mattod.2017.01.013.
[17] H. Schellekens, W.E. Hennink, V. Brinks, The immunogenicity of polyethylene glycol: facts and fiction, Pharm. Res. 30 (7) (2013) 1729–1734, https://doi.org/10.1007/s11095-013-1067-7.
[18] L. Wang, A. Hess, T.Z. Chang, et al., Nanoclusters self-assembled from conformation-stabilized influenza M2e as broadly cross-protective influenza vaccines, Nanomedicine 10 (2) (2014) 473–482, https://doi.org/10.1016/j.nano.2013.08.005.
[19] M.A. Miller, T.A. Khan, K.J. Kaczorowski, et al., Antibody nanoparticle dispersions formed with mixtures of crowding molecules retain activity and In Vivo bioavailability, J. Pharm. Sci. 101 (10) (2012) 3763–3778, https://doi.org/10.1002/jps.23256.
[20] K.P. Johnston, J.A. Maynard, T.M. Truskett, et al., Concentrated dispersions of equilibrium protein nanoclusters that reversibly dissociate into active monomers, ACS Nano 6 (2) (2012) 1357–1369, https://doi.org/10.1021/nn204166z.
[21] A.N. Tsoras, J.A. Champion, Cross-linked peptide nanoclusters for delivery of oncofetal antigen as a cancer vaccine, Bioconjug. Chem. 29 (3) (2018) 776–785, https://doi.org/10.1021/acs.bioconjchem.8b00079.

[22] A.N. Tsoras, K.M. Wong, A.K. Paravastu, J.A. Champion, Rational design of antigen incorporation into subunit vaccine biomaterials can enhance antigen-specific immune responses, Front. Immunol. 11 (2020), https://doi.org/10.3389/fimmu.2020.01547.
[23] L. Deng, T. Mohan, T.Z. Chang, et al., Double-layered protein nanoparticles induce broad protection against divergent influenza A viruses, Nat. Commun. 9 (1) (2018), https://doi.org/10.1038/s41467-017-02725-4.
[24] L. Deng, J.R. Kim, T.Z. Chang, et al., Protein nanoparticle vaccine based on flagellin carrier fused to influenza conserved epitopes confers full protection against influenza A virus challenge, Virology. 509 (2017) 82–89, https://doi.org/10.1016/j.virol.2017.06.001.
[25] W. Chen, G. Wang, B.C. Yung, G. Liu, Z. Qian, X. Chen, Long-acting release formulation of exendin-4 based on biomimetic mineralization for type 2 diabetes therapy, ACS Nano 11 (5) (2017) 5062–5069, https://doi.org/10.1021/acsnano.7b01809.
[26] H.J. Park, D.J. Shin, J. Yu, Categorization of quantum dots, clusters, nanoclusters, and nanodots, J. Chem. Educ. 9 (2021), https://doi.org/10.1021/acs.jchemed.0c01403. Published online March.
[27] E.J. Yearley, P.D. Godfrin, T. Perevozchikova, et al., Observation of small cluster formation in concentrated monoclonal antibody solutions and its implications to solution viscosity, Biophys. J. 106 (8) (2014) 1763–1770, https://doi.org/10.1016/j.bpj.2014.02.036.
[28] A. Stradner, H. Sedgwick, F. Cardinaux, W.C.K. Poon, S.U. Egelhaaf, P. Schurtenberger, Equilibrium cluster formation in concentrated protein solutions and colloids, Nature. 432 (7016) (2004) 492–495, https://doi.org/10.1038/nature03109.
[29] T.M. Scherer, J. Liu, S.J. Shire, A.P. Minton, Intermolecular interactions of IgG1 monoclonal antibodies at high concentrations characterized by light scattering, J. Phys. Chem. B 114 (40) (2010) 12948–12957, https://doi.org/10.1021/jp1028646.
[30] L. Porcar, P. Falus, W.R. Chen, et al., Formation of the dynamic clusters in concentrated lysozyme protein solutions, J. Phys. Chem. Lett. 1 (1) (2010) 126–129, https://doi.org/10.1021/jz900127c.
[31] W. Wang, Advanced protein formulations, Protein Sci. 24 (7) (2015) 1031–1039, https://doi.org/10.1002/pro.2684.
[32] I.J. Arroyo-Maya, J.O. Rodiles-López, M. Cornejo-Mazón, et al., Effect of different treatments on the ability of α-lactalbumin to form nanoparticles, J. Dairy Sci. 95 (11) (2012) 6204–6214, https://doi.org/10.3168/jds.2011-5103.
[33] I.J. Arroyo-Maya, H. Hernández-Sánchez, E. Jiménez-Cruz, M. Camarillo-Cadena, A. Hernández-Arana, α-Lactalbumin nanoparticles prepared by desolvation and cross-linking: structure and stability of the assembled protein, Biophys. Chem. 193–194 (2014) 27–34, https://doi.org/10.1016/j.bpc.2014.07.003.
[34] S. Mahanta, S. Paul, Stable self-assembly of bovine α-lactalbumin exhibits target-specific antiproliferative activity in multiple cancer cells, ACS Appl. Mater. Interfaces 7 (51) (2015) 28177–28187, https://doi.org/10.1021/acsami.5b06076.
[35] X.N. Huang, X.Y. Du, J.F. Xing, Z.Q. Ge, Catalase-only nanoparticles prepared by shear alone: characteristics, activity and stability evaluation, Int. J. Biol. Macromol. 90 (2016) 81–88, https://doi.org/10.1016/j.ijbiomac.2015.08.056.
[36] S. Chawla, R. Rawal, Sonia, Ramrati, C.S. Pundir, Preparation of cholesterol oxidase nanoparticles and their application in amperometric determination of cholesterol, J. Nanopart. Res. 15 (9) (2013), https://doi.org/10.1007/s11051-013-1934-5.
[37] S. Mahanta, S. Paul, A. Srivastava, A. Pastor, B. Kundu, T.K. Chaudhuri, Stable self-assembled nanostructured hen egg white lysozyme exhibits strong anti-proliferative activity against breast cancer cells, Colloids Surf. B: Biointerfaces 130 (2015) 237–245, https://doi.org/10.1016/j.colsurfb.2015.04.017.
[38] H.J. Lee, H.H. Park, J.A. Kim, et al., Enzyme delivery using the 30Kc19 protein and human serum albumin nanoparticles, Biomaterials. 35 (5) (2014) 1696–1704, https://doi.org/10.1016/j.biomaterials.2013.11.001.
[39] L.H. Estrada, S. Chu, J.A. Champion, Protein nanoparticles for intracellular delivery of therapeutic enzymes, J. Pharm. Sci. 103 (6) (2014) 1863–1871, https://doi.org/10.1002/jps.23974.
[40] L.R. Wilks, G. Joshi, M.R. Grisham, H.S. Gill, Tyrosine-based cross-linking of peptide antigens to generate nanoclusters with enhanced immunogenicity: demonstration using the conserved M2e peptide of influenza A, ACS Infect. Dis. 7 (9) (2021) 2723–2735, https://doi.org/10.1021/acsinfecdis.1c00219.
[41] T.Z. Chang, S.S. Stadmiller, E. Staskevicius, J.A. Champion, Effects of ovalbumin protein nanoparticle vaccine size and coating on dendritic cell processing, Biomater. Sci. 5 (2) (2017) 223–233, https://doi.org/10.1039/c6bm00500d.
[42] L. Tang, Y. Zheng, M.B. Melo, et al., Enhancing T cell therapy through TCR-signaling-responsive nanoparticle drug delivery, Nat. Biotechnol. 36 (8) (2018), https://doi.org/10.1038/nbt.4181.
[43] Y. Xiao, X. Wang, B. Wang, X. Liu, X. Xu, R. Tang, Long-term effect of biomineralized insulin nanoparticles on type 2 diabetes treatment, Theranostics. 7 (17) (2017) 4301–4312, https://doi.org/10.7150/thno.21450.
[44] S. Cao, Y. Liu, H. Shang, et al., Supramolecular nanoparticles of calcitonin and dipeptide for long-term controlled release, J. Control. Release 256 (2017) 182–192, https://doi.org/10.1016/j.jconrel.2017.04.014.
[45] A.R. Srinivasan, A. Lakshmikuttyamma, S.A. Shoyele, Investigation of the stability and cellular uptake of self-associated monoclonal antibody (MAb) nanoparticles by non-small lung cancer cells, Mol. Pharm. 10 (9) (2013) 3275–3284, https://doi.org/10.1021/mp3005935.
[46] R.C. Oppenheim, N.F. Stewart, L. Gordon, H.M. Patel, The production and evaluation of orally administered insulin nanoparticles, Drug Dev. Ind. Pharm. 8 (4) (1982) 531–546, https://doi.org/10.3109/03639048209022119.
[47] Y. Dekel, Y. Machluf, T. Gefen, et al., Formation of multimeric antibodies for self-delivery of active monomers, Drug Delivery 24 (1) (2017) 199–208, https://doi.org/10.1080/10717544.2016.1242179.
[48] S. Tufail, M.O.H.D. Asif Sherwani, S. Shoaib, S. Azmi, M. Owais, N. Islam, Ovalbumin self-assembles into amyloid nanosheets that elicit immune responses and facilitate sustained drug release, J. Biol. Chem. 293 (29) (2018) 11310–11324, https://doi.org/10.1074/jbc.RA118.002550.
[49] A. Pang, N. Cheng, Y. Cui, et al., High-loading Gα 13-binding EXE peptide nanoparticles prevent thrombosis and protect mice from cardiac ischemia/reperfusion injury, Sci. Transl. Med. 12 (2020) 7287, https://doi.org/10.1126/scitranslmed.aaz7287.
[50] M. Fach, L. Radi, P.R. Wich, Nanoparticle assembly of surface-modified proteins, J. Am. Chem. Soc. 138 (45) (2016) 14820–14823, https://doi.org/10.1021/jacs.6b06243.
[51] L. Radi, M. Fach, M. Montigny, E. Berger-Nicoletti, W. Tremel, P.R. Wich, Methods of protein surface PEGylation under structure preservation for the emulsion-based formation of stable nanoparticles, Medchemcomm. 7 (9) (2016) 1738–1744, https://doi.org/10.1039/c5md00475f.
[52] E. Steiert, L. Radi, M. Fach, P.R. Wich, Protein-based nanoparticles for the delivery of enzymes with antibacterial activity, Macromol. Rapid Commun. 39 (14) (2018), https://doi.org/10.1002/marc.201800186.
[53] E. Steiert, J. Ewald, A. Wagner, U.A. Hellmich, H. Frey, P.R. Wich, PH-responsive protein nanoparticles: via conjugation of degradable PEG to the surface of cytochrome c, Polym. Chem. 11 (2) (2020) 551–559, https://doi.org/10.1039/c9py01162e.
[54] Koria P, Yagi H, Kitagawa Y, et al. Self-assembling elastin-like peptides growth factor chimeric nanoparticles for the treatment of chronic wounds. doi:https://doi.org/10.1073/pnas.1009881108/-/DCSupplemental.
[55] J.D. Kim, Y.J. Jung, C.H. Woo, Y.C. Choi, J.S. Choi, Y.W. Cho, Thermo-responsive human α-elastin self-assembled nanoparticles for protein delivery, Colloids Surf. B: Biointerfaces 149 (2017) 122–129, https://doi.org/10.1016/j.colsurfb.2016.10.012.
[56] M. Rad-Malekshahi, M.F. Fransen, M. Krawczyk, et al., Self-assembling peptide epitopes as novel platform for anticancer vaccination, Mol. Pharm. 14 (5) (2017) 1482–1493, https://doi.org/10.1021/acs.molpharmaceut.6b01003.
[57] Y. Li, T. Cui, X. Kong, et al., Nanoparticles induced by embedding self-assembling cassette into glucagon-like peptide 1 for improving in vivo stability, FASEB J. 32 (6) (2018) 2992–3004, https://doi.org/10.1096/fj.201701033RRR.
[58] A. Dhankher, M.E. Hernandez, H.C. Howard, J.A. Champion, Characterization and control of dynamic rearrangement in a self-assembled antibody carrier, ACS Appl. Mater. Interfaces (2020), https://doi.org/10.1021/acs.biomac.9b01712. Published online.
[59] S.I. Lim, C.I. Lukianov, J.A. Champion, Self-assembled protein nanocarrier for intracellular delivery of antibody, J. Control. Release 249 (2017) 1–10, https://doi.org/10.1016/j.jconrel.2017.01.007.
[60] W. Lv, J.A. Champion, Demonstration of intracellular trafficking, cytosolic bioavailability, and target manipulation of an antibody delivery platform, Nanomedicine 32 (2021), https://doi.org/10.1016/j.nano.2020.102315.
[61] I.J. Joye, D.J. McClements, Production of nanoparticles by anti-solvent precipitation for use in food systems, Trends Food Sci. Technol. 34 (2) (2013) 109–123, https://doi.org/10.1016/j.tifs.2013.10.002.
[62] S.M. Ahsan, C.M. Rao, The role of surface charge in the desolvation process of gelatin: implications in nanoparticle synthesis and modulation of drug release, Int. J. Nanomed. 12 (2017) 795–808, https://doi.org/10.2147/IJN.S124938.
[63] J.J. Marty, R.C. Oppenheim, P. Speiser, Nanoparticles - a new colloidal drug delivery system, Pharm. Acta Helv. 53 (1) (1978) 17–23.
[64] T. Takigawa, Y. Endo, Effects of glutaraldehyde exposure on human health, J. Occup. Health 48 (2006) 75–87, https://doi.org/10.1539/joh.48.75.
[65] S. Gaber, S. Abdel-Latif, Effect of education and health locus of control on safe use of pesticides: a cross sectional random study, J. Occup. Med. Toxic. 7 (1) (2012), https://doi.org/10.1186/1745-6673-7-3.
[66] Y. Saricay, P.A. Wierenga, R. de Vries, Changes in protein conformation and surface hydrophobicity upon peroxidase-catalyzed cross-linking of apo-α-lactalbumin, J. Agric. Food Chem. 62 (38) (2014) 9345–9352, https://doi.org/10.1021/jf502664q.
[67] J. Seaberg, N. Flynn, A. Cai, J.D. Ramsey, Effect of redox-responsive DTSSP crosslinking on poly(l-lysine)-grafted-poly(ethylene glycol) nanoparticles for delivery of proteins, Biotechnol. Bioeng. 117 (8) (2020) 2504–2515, https://doi.org/10.1002/bit.27369.
[68] H. Niknejad, R. Mahmoudzadeh, Comparison of different crosslinking methods for preparation of docetaxel-loaded albumin nanoparticles, Iran J. Pharm. Res. 14 (2) (2015) 385–394.
[69] X. Li, H. Bai, Y. Wu, et al., Structural analysis and allergenicity assessment of an enzymatically cross-linked bovine α-lactalbumin polymer, Food Funct. 11 (1) (2020) 628–639, https://doi.org/10.1039/c9fo02238d.
[70] D.K. Layman, B. Lönnerdal, J.D. Fernstrom, Applications for a-lactalbumin in human nutrition, Nutr. Rev. 76 (6) (2018) 444–460, https://doi.org/10.1093/nutrit/nuy004.
[71] M. Svensson, A. Håkansson, A.K. Mossberg, S. Linse, C. Svanborg, Conversion of-lactalbumin to a protein inducing apoptosis, Proc. Natl. Acad. Sci. U. S. A. (2000), https://doi.org/10.1073/pnas.97.8.4221.
[72] C. Svanborg, H. Agerstam, A. Aronson, et al., HAMLET kills tumor cells by an apoptosis-like mechanism cellular, molecular, and therapeutic aspects, Adv. Cancer Res. (2003), https://doi.org/10.1016/s0065-230x(03)88302-1. Published online.
[73] A.J. Miles, B.A. Wallace, Circular dichroism spectroscopy of membrane proteins, Chem. Soc. Rev. 45 (18) (2016) 4859–4872, https://doi.org/10.1039/c5cs00084j.

[74] N.J. Greenfield, Using circular dichroism spectra to estimate protein secondary structure, Nat. Protoc. 1 (6) (2007) 2876–2890, https://doi.org/10.1038/nprot.2006.202.
[75] O.C. Tatford, P.T. Gomme, J. Bertolini, Analytical techniques for the evaluation of liquid protein therapeutics, Biotechnol. Appl. Biochem. 40 (1) (2004) 67, https://doi.org/10.1042/ba20030183.
[76] A. Bolje, S. Gobec, Analytical techniques for structural characterization of proteins in solid pharmaceutical forms: an overview, Pharmaceutics. 13 (4) (2021), https://doi.org/10.3390/pharmaceutics13040534.
[77] A.N. Ilinskaya, M.A. Dobrovolskaia, Understanding the immunogenicity and antigenicity of nanomaterials: past, present and future, Toxicol. Appl. Pharmacol. 299 (2016) 70–77, https://doi.org/10.1016/j.taap.2016.01.005.
[78] A.L. Parkes, D. Parkes, C. Jodka, et al., Pharmacokinetic actions of exendin-4 in the rat: comparison with glucagon-like peptide-1, Drug Dev. Res. 53 (4) (2001) 260–267, https://doi.org/10.1002/ddr.1195.
[79] B. Bittner, W. Richter, J. Schmidt, Subcutaneous Administration of Biotherapeutics: an overview of current challenges and opportunities, BioDrugs. 32 (5) (2018) 425–440, https://doi.org/10.1007/s40259-018-0295-0.
[80] M.X. Yang, B. Shenoy, M. Disttler, et al., Crystalline monoclonal antibodies for subcutaneous delivery, Proc. Natl. Acad. Sci. U. S. A. 100 (12) (2003) 6934–6939, https://doi.org/10.1073/pnas.1131899100.
[81] S. Frokjaer, D.E. Otzen, Protein drug stability: a formulation challenge, Nat. Rev. Drug Discov. 4 (4) (2005) 298–306, https://doi.org/10.1038/nrd1695.
[82] B. Sun, T. Xia, Nanomaterial-based vaccine adjuvants, J. Mater. Chem. B 4 (33) (2016) 5496–5509, https://doi.org/10.1039/c6tb01131d.
[83] I.B. Bekard, P. Asimakis, J. Bertolini, D.E. Dunstan, The effects of shear flow on protein structure and function, Biopolymers. 95 (11) (2011) 733–745, https://doi.org/10.1002/bip.21646.
[84] C.R. Thomas, P. Dunnill, Action of shear on enzymes: studies with catalase and urease, Biotechnol. Bioeng. 21 (12) (1979) 2279–2302, https://doi.org/10.1002/bit.260211209.
[85] S.E. Charm, B.L. Wong, Shear effects on enzymes, Enzym. Microb. Technol. 3 (2) (1981) 111–118, https://doi.org/10.1016/0141-0229(81)90068-5.
[86] M. Duerkop, E. Berger, A. Dürauer, A. Jungbauer, Impact of cavitation, high shear stress and air/liquid interfaces on protein aggregation, Biotechnol. J. 13 (7) (2018), https://doi.org/10.1002/biot.201800062.
[87] V. Rombach-Riegraf, A.C. Karle, B. Wolf, et al., Aggregation of human recombinant monoclonal antibodies influences the capacity of dendritic cells to stimulate adaptive T-cell responses in vitro, PLoS One 9 (1) (2014), https://doi.org/10.1371/journal.pone.0086322.
[88] S. Martins, B. Sarmento, D.C. Ferreira, E.B. Souto, Lipid-based colloidal carriers for peptide and protein delivery-liposomes versus lipid nanoparticles, Int. J. Nanomedicine 2 (4) (2007) 595–607.
[89] P. Katyal, M. Meleties, J.K. Montclare, Self-assembled protein-and peptide-based nanomaterials, ACS Biomater. Sci. Eng. 5 (9) (2019) 4132–4147, https://doi.org/10.1021/acsbiomaterials.9b00408.
[90] M. Danaei, M. Dehghankhold, S. Ataei, et al., Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems, Pharmaceutics 10 (2) (2018), https://doi.org/10.3390/pharmaceutics10020057.
[91] D.J. Drucker, Advances in oral peptide therapeutics, Nat. Rev. Drug Discov. 19 (4) (2020) 277–289, https://doi.org/10.1038/s41573-019-0053-0.
[92] S. Mitragotri, P.A. Burke, R. Langer, Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies, Nat. Rev. Drug Discov. 13 (9) (2014) 655–672, https://doi.org/10.1038/nrd4363.
[93] M. Glavaš, A. Gitlin-Domagalska, D. Dębowski, N. Ptaszyńska, A. Łęgowska, K. Rolka, Vasopressin and its analogues: from natural hormones to multitasking peptides, Int. J. Mol. Sci. 23 (6) (2022) 3068, https://doi.org/10.3390/ijms23063068.
[94] F. Moncalvo, M.I. Martinez Espinoza, F. Cellesi, Nanosized delivery systems for therapeutic proteins: clinically validated technologies and advanced development strategies, Front. Bioeng. Biotechnol. 8 (2020), https://doi.org/10.3389/fbioe.2020.00089.
[95] J.R. Mora, J.T. White, S.L. DeWall, Immunogenicity risk assessment for PEGylated therapeutics, AAPS J. 22 (2) (2020), https://doi.org/10.1208/s12248-020-0420-0.
[96] I.B. Hirsch, R. Juneja, J.M. Beals, C.J. Antalis, E.E. Wright, The evolution of insulin and how it informs therapy and treatment choices, Endocr. Rev. 41 (5) (2021) 733–755, https://doi.org/10.1210/ENDREV/BNAA015.
[97] S.S. Wang, Y. Yan, K. Ho, US FDA-approved therapeutic antibodies with high-concentration formulation: summaries and perspectives, Antibody Therapeutics. 4 (4) (2021) 262–273, https://doi.org/10.1093/abt/tbab027.
[98] P. Khramtsov, T. Kalashnikova, M. Bochkova, et al., Measuring the concentration of protein nanoparticles synthesized by desolvation method: comparison of Bradford assay, BCA assay, hydrolysis/UV spectroscopy and gravimetric analysis, Int. J. Pharm. 599 (2021), https://doi.org/10.1016/j.ijpharm.2021.120422.