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Rational Design and Development of Polymeric Nanogels as Protein Carriers

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Proteins have gained significant attention as potential therapeutic agents owing to their high specificity and reduced toxicity. Nevertheless, their clinical utility is hindered by inherent challenges associated with stability during storage and after in vivo administration. To overcome these limitations, polymeric nanogels (NGs) have emerged as promising carriers. These colloidal systems are capable of efficient encapsulation and stabilization of protein cargoes while improving their bioavailability and targeted delivery. The design of such delivery systems requires a comprehensive understanding of how the synthesis and formulation processes affect the final performance of the protein. This review highlights critical aspects involved in the development of NGs for protein delivery, with specific emphasis on loading strategies and evaluation techniques. For example, factors influencing loading efficiency and release kinetics are discussed, along with strategies to optimize protein encapsulation through protein-carrier interactions to achieve the desired therapeutic outcomes. The discussion is based on recent literature examples and aims to provide valuable insights for researchers working toward the advancement of protein-based therapeutics.

1. INTRODUCTION

In the last decades, advances in molecular medicine have helped untangle the fundamental mechanisms involved in multiple diseases. This understanding is leading therapeutic options to increasingly evolve toward the field of biopharmaceuticals instead of small-molecule drugs.[1] Among these, proteins are seeing an exponential boost in clinical potential, being more than 40% of the biopharmaceuticals under clinical development. Their success is linked to their high specificity and potency, reduced toxicity, and long action due to slow clearance.[2] The protein therapeutics market was estimated at almost $284 Billion in 2020, and is expected to reach $566 Billion by 2030.[3]

The use of therapeutic proteins is, however, limited by their challenging formulation. Proteins can be unstable and suffer from plasma proteolysis, aggregation, immunogenicity, and can exhibit difficulties to cross biological membranes.[4] Their degradation under acidic pH in the stomach also limits the convenient oral administration route.[5] With the rise of nanotechnology, different strategies have been proposed to overcome these limitations, such as polymeric, lipid-based, or inorganic nanoparticles.[6] Despite the efforts, controlled release systems still find difficulties to reach the clinical market, and most approved formulations still rely on repeated injections.[1]

Nanogels (NGs) are swollen crosslinked hydrogels with nanometric size and high water content. They are promising carriers for drug delivery due to their colloidal properties and flexible structure. For specific chemical compositions, these properties can be combined with biocompatibility and prolonged circulation time in the bloodstream.[6–8] Furthermore, the networks of NGs can be finely tuned to enable a triggered release. For this, responsive networks can be used to control the diffusion of compounds by adjusting the swelling of the mesh in response to external stimuli (pH, temperature). Also, cleavable crosslinks can be introduced to induce drug release by corresponding network degradation.[9] In addition to the tunable release characteristics, NGs can be designed to cross different biological barriers,[10] e.g., biological membranes, both at the cellular level and through different administration routes.[11] Up to date, these properties have been utilized mostly in cancer therapy, but they also find applications in inflammatory and infectious diseases, tissue engineering, and metabolic disorders.[9,12–14]

The use of nanogels as carriers for small molecule drugs has been thoroughly studied in the last decades. More recently, the delivery of proteins has become similarly important.[15–17] Here the described unique stimuli-responsive properties of NGs are very promising, since the highly swollen polymeric network can efficiently entrap proteins due to their comparably large size

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(comparable to small molecules and the mesh size). A release can be induced by network swelling or degradation. Most importantly, the hydrogel matrix can stabilize the proteins' quaternary structure while reducing aggregation phenomena. Compared to other types of nanoparticles, NGs often provide higher loading capacity, improved biocompatibility, stability, targeted delivery, and the possibility to control the release through external stimuli.[4]

It becomes evident that NGs are highly interesting materials for protein delivery. However, up to now, no specific review exists that could guide the design of NGs as protein carriers. Current reviews either focus on the chemistry involved in NG synthesis or the final clinical use. For example, chemistry-oriented reviews focus on the structural aspects of NGs for delivery applications (e.g, stimuli-responsiveness, synthetic methods, etc.).[15–20] In these articles, the encapsulation of proteins is mostly used as an example to show the versatility of these carriers, and little attention is given to the interaction between NGs and proteins or the intrinsic differences of each type of protein/application. Regarding the clinical aspects, protein delivery is mostly used in cancer therapy and vaccines.[13,21]

With this review article, we aim to guide the rational design of NGs as carriers for therapeutic proteins, thus supporting new researchers in entering the growing field of therapeutic protein delivery. To address this, we will discuss how NG design influences the therapeutic performance through different interactions between polymer and proteins, which key parameters must be considered when developing such carriers, and how to evaluate protein content and stability in the NGs.

The main points of this review will be the following:

- Introduction of NG synthesis and characterization.
- Encapsulation of therapeutic proteins in the NGs: mechanisms used for encapsulation, factors affecting encapsulation efficiency, and strategies to optimize encapsulation. Advantages and disadvantages of using physical entrapment toward covalent bonding/bioconjugation will be also stated.
- Release of proteins from NGs: main release mechanisms, factors affecting release, strategies to control the release.
- Stability of the complexes: how to determine protein activity and perform the in vitro and in vivo evaluation methods (if applicable), factors affecting protein activity, and stability.
- Outlook on the clinical translation and future perspectives of NGs as protein carriers.

2. NG Synthesis and Classification

Although NGs have been classified based on their stimuli-responsiveness capacity or the type of polymer in their structure (natural/synthetic), the most common classification pays attention to their synthesis and crosslinking method, with two main categories: NGs crosslinked by physical methods, and covalently-bound NGs.[16]

2.1. Physical Crosslinking

Physically cross-linked NGs are self-assembled by (supramolecular) interactions between the same or different polymer chains[19] These interactions are reversible and include hydrogen bonding, van der Waals forces, electrostatic and hydrophobic interactions.[22] The crosslinking process usually takes place in an aqueous medium and under mild conditions, and the lack of covalent crosslinking agents/catalysts results in reduced side products and improved biocompatibility.[16] The size of the NG can be modulated by modification of physical conditions during formulation, such as polymer concentration, pH, ionic strength, and temperature.[23] In general, the stability of these assemblies is lower than that of covalently crosslinked NGs, as the interactions are more susceptible to environmental changes. However, their ease of manufacture and good biocompatibility are promising to facilitate clinical translation of these NG-containing formulations. One example is the cholesteryl-modified pullulan (CHP) self-assembled by hydrophobic interactions, which is widely used in formulations for cancer therapy and vaccines, some of them already in clinical trials.[21]

2.2. Chemical Crosslinking

NGs can also be formed by connecting polymer chains through covalent chemical interactions. These bonds are more stable than physical interactions, since they are stronger and mostly irreversible.[21,24] In general, we can divide covalent NG synthetic procedures into 1) polymerization of monomers in homogeneous or heterogeneous environment, 2) crosslinking of preformed monomers or polymers, and 3) template-assisted nanofabrication.[25] NG synthesis methods have been thoroughly described in the literature and are not the main subject of this review; for more details we would like to refer the readers to existing literature.[18,26,27] However, some key aspects of polymerization processes used in works described in this review are listed below.

NG synthesis during radical polymerization of monomers is widely used (most prevalent in the studies reviewed in this article). In this method, formation of the network-forming polymer, crosslinking, and formation of the colloidal NG all occur simultaneously.[28] Bi- or multi-functional crosslinkers are used in such polymerization processes to generate the covalent network. Their amount and molecular structure allow control over final particle size and stability.[18] Particle size, size distribution, and morphology can be further controlled through modification of the colloidal environment.

On one hand, polymerization can be conducted in heterogeneous systems, e.g., in emulsion, mini emulsion, microemulsion, or aqueous suspension of the monomers. On the other hand, polymerization can start from a homogeneous phase, where the monomers are dissolved in water and growing oligomer chains precipitate upon polymerization.[16] Heterogeneous phase reactions involving organic solvents, such as o/w or w/o emulsions, require additional steps for solvent removal.[18]

In emulsion polymerization, a monomer of low water solubility is emulsified in water containing a water-soluble initiator and a surfactant.[29] Additional surfactant micelles incorporate monomers that diffuse from the monomer droplet, leading to particle formation inside the surfactant micelles. This statistical process results in narrow particle size distributions (PSDs). However, it is difficult to use this method in copolymerization, since different monomers have different diffusion coefficients

that may lead to core-shell morphologies.[30] In miniemulsion polymerization, polymerization takes place inside droplet sizes of smaller diameter. By the addition of osmotic pressure agents (or ultrahydrophobes) the net diffusion between droplets is prevented. It is therefore better suited for copolymerizations and can be transferred to inverse w/o systems. However, miniemulsions are thermodynamically unstable systems which require high shear forces to maintain the stability, and usually lead to broad PSDs.[31] Microemulsions are thermodynamically stable, however, they are only formed for a very specific ratio of monomer, solvent, and surfactants, which limits their versatility.[32]

Precipitation polymerization is mostly used for temperature-sensitive systems, where the monomer is solubilized in the solvent (frequently water), but precipitates upon polymerization.[33] Reactions are carried out at high temperatures (above the lower critical solution temperature, LCST) that cause the precipitation and phase separation of the forming oligomer chains. The reaction can be carried out in the absence of colloidal stabilizers, making it simple, straightforward, clean, and efficient.[34] However, the addition of small amounts of surfactant helps increasing particle homogeneity, and decreasing particle size.[35] Besides its versatility and simplicity, the use of precipitation polymerization is limited by the choice of polymers, as they need to have a specific temperature-responsive behavior.[36]

Besides combining the polymerization, crosslinking, and particle formation in one step, it is also possible to decouple the particle preparation from the polymer synthesis.[37] For this, preformed polymers are crosslinked to form covalent networks in the nanoscale. This approach simplifies NG preparation, as there is no need to remove unreacted monomers, initiators, or surfactant molecules.[38] Particle gelation is carried out by adding multifunctional external crosslinking agents, or by crosslinking the polymer chains internally. The second option usually implies a prior functionalization of the polymer with reactive groups, that will form a covalent bond upon activation.[39] Compared to polymerization methods, the crosslinking density is relatively low, allowing for larger mesh sizes.[40] Since these groups are often not fully reacted after NG crosslinking, they can be further exploited to covalently attach additional functionalities, e.g., protein cargoes. Usually, orthogonal reactions are employed, such as click chemistry or Schiff-base reactions.[13,19] Cleavable crosslinkers will help induce NG degradation upon specific stimuli, such as enzyme activity or reductive environment.[37,41]

Finally, microfluidics approaches are gaining importance in the manufacture of delivery systems, including NGs.[42] A fluid flow is manipulated with precise control at microscopic scale, with minimized reagent consumption, decreased reaction time, and enhanced process accuracy and efficiency. In the end, they help reducing batch-to-batch variation and increasing manufacture throughput.[43] Nevertheless, a successful formulation requires a precise understanding and adjustment of the formulation conditions, which can be done with machine learning approaches.[44]

3. Protein Encapsulation and Release

Stabilization of protein therapeutics is needed to protect their structure against certain physiological events (e.g., enzymatic or acidic degradation in the stomach, aggregation in the bloodstream),[45] thus enhancing their bioavailability. In addition, protection against external environmental conditions is important to reduce the reliance on refrigeration for storage and cold chain transportation.[46,47] To address these requirements, several strategies have been proposed, such as the addition of stabilization agents (sugar, salts, and polyols),[48] surface immobilization of proteins, and encapsulation into hydrated matrices.[49] Among such matrices, polymeric NGs as colloidal hydrogels can entrap high molecular weight proteins in their water swollen crosslinked networks. Here, the network structure controls the physical environment around the protein. For example, if the polymer matrix contains functional moieties that interact with the protein (by opposite charge, hydrophobic interactions, or affinity binders), protein affinity and protection are increased. Ultimately, the colloidal size of NGs offer extended advantages over macro- and microscale hydrogels. Their high surface area allows for high cargo loading and enhanced targeted delivery. Also, the nanoscopic size guarantees a low viscosity of the suspensions, allowing extrusion through small gauge needles and facilitating intravenous delivery.[50,51] Through slight variations in the NG synthesis, stiffness, size, and degradability can be modulated, allowing for their adaption to the specific requirements of each therapeutic application (i.e., pass through biological barriers, extra- or intracellular protein delivery).[52,53]

A rational NG design, as well as the choice of encapsulation method, will determine the overall performance of the protein drug carrier. In general, the objective is to enhance protein entrapment in the network and prevent undesired and premature passive release (i.e., leakage) of the drug from the NG until the desired time point or location is reached. To address this objective, protein size is a key parameter to consider.[54] For high molecular weight proteins (w.r.t. to small molecule cargoes), changes in the NGs' mesh size can be used to adjust entrapment and control release kinetics. However, it is necessary to balance entrapment and leakage. For example, NGs with big mesh sizes allow for a better diffusion of the proteins in the network. While this favors high loading contents, it can also result in a faster diffusion-based release. To prevent such a scenario, additional responsiveness is often introduced into the NGs to adjust interpolymer interactions and interactions between the network and the cargo. This can be used to control the release through external stimuli (i.e., pH, enzymes, T) that, i.e., change the swelling state, lead to NG degradation, or cleave interactions between protein and polymer (physical or covalent).[15] Additionally, a combination of multiple loading and release mechanisms in a single NG can be employed to achieve the controlled release of multiple proteins. For instance, NGs can incorporate a protein A during the crosslinking process, leading to a loaded NG that can subsequently encapsulate another protein B through a different mechanism.[55]

3.1. Mechanisms for Encapsulation

As stated above, an enhanced entrapment of the protein in the NG equals to a successful encapsulation. The overall performance of the encapsulation process is determined by two parameters: encapsulation efficiency (EE), which is the amount of protein incorporated to the NGs with respect to the initial protein fed, and loading content (LC), which is the amount of protein

incorporated with respect to the dry weight of the NGs.[56,57] A high value of these two parameters indicates an adequate interaction protein-carrier, and a proper encapsulation method.

Several methods and strategies can be used to incorporate proteins into (colloidal) hydrogels. Physical entrapment or adsorption relies on (specific) protein-network interactions, and reversible non-covalent or covalent binding can be achieved through degradable linkers. In general, the loading of biomacromolecules, e.g. proteins, into NGs can be carried out via different processes described below:

- Chemical conjugation to the polymer (covalent binding)
- Physical entrapment (physical immobilization of the enzyme within the polymer network, with or without extra stabilization by ionic/other affinity interactions between polymer matrix and protein)

These processes can be carried out by diffusion, where proteins are entrapped into previously formed NGs, or in situ, where protein is loaded during NG synthesis.[15] In diffusion-based encapsulation, a high protein feed ratio might be needed to increase the concentration gradient and favor protein diffusion through the NG. However, using such an excess of protein may lead to lower entrapment efficiencies.[58] In situ loading generally leads to higher encapsulation efficiency and homogeneous distribution of the protein throughout the polymer.[28] However, this type of encapsulation is rarely compatible with the chemical crosslinking process, since it requires conditions that can compromise protein structure and activity. To avoid protein denaturation in such processes, they must be carried out in water and under mild temperature conditions.

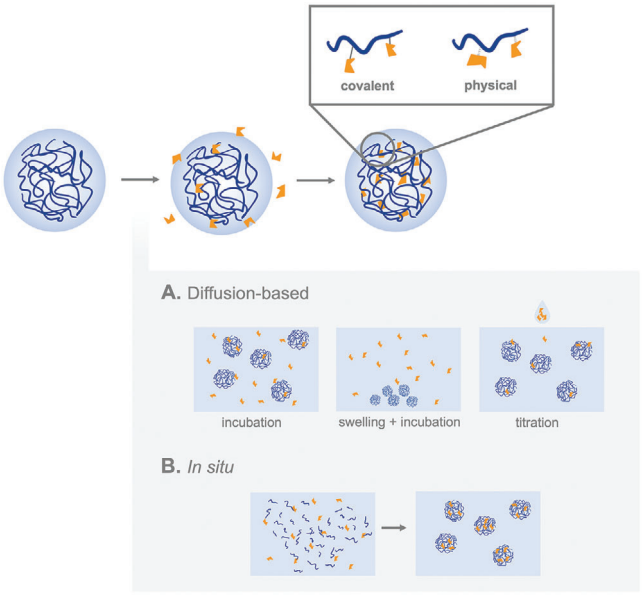

Figure 1. A) Protein entrapment can be carried out by diffusion of the protein inside the NGs, which can be done by A1) incubation, A2) swelling+incubation, or A3) titration A3, or B) in situ during NG synthesis, where the protein is put in contact with the monomer/polymer prior to particle formation.

3.1.1. Covalent Binding

Proteins can be attached to preformed polymer matrices by making use of the proteins' reactive thiol and amino groups in a bioconjugation approach (**Figure 1**). These groups can react with functional hydroxy, amine, or carboxyl groups from the polymer matrix (introduced through functionalization reactions, blending, or co-polymerization during particle preparation). Direct protein-network conjugation can be done through reactive amino acid residues naturally present in the protein. For example, thiol groups in cysteine residues can form redox-sensitive disulfide bonds with thiol groups in the NGs.[59] Amide bonds can also be formed by a reaction between amines and activated carboxylic acids (N-hydroxysuccinimide, NHS esters). Indirect protein-polymer coupling can be performed through the introduction of covalent linkers. For example, proteins can be modified through amide formation with a carboxylic acid-spacer-azide molecule. The installed azides can be reacted with a polymer containing alkyne groups.[60] Such "click reactions" have attracted a lot of attention since they allow for particle functionalization due to their (bio-)orthogonality, since they help preserving particle properties and function.[61] The addition of spacers between the protein and the polymer can also introduce stimuli-responsiveness not achievable through the natural aminoacids of the protein (e.g., addition of a redox-responsive disulfide linker to cysteine-free proteins).[62] Depending on the resulting covalent connection between polymer and protein, hydrolysis, reduction reactions, or enzymatic cleavage can be employed to induce a release.[63] This method enables to control the release of the protein by taking advantage of specific enzymatic activities in targeted areas, or different environments inside the cell.[16] It is also a promising strategy to optimize encapsulation efficiency and prevent leakage. However, there may be challenges with maintaining the stability and biological activity of the protein through the loading process.[64]

Covalent binding can be performed after physical entrapment in preformed nanogels. This strategy enables to increase the loading capacity of the protein while avoiding protein damage during NG formation and leakage in the final formulation. In an example, Li et al. developed cationic dextran NGs to load them with an oppositely charged protein antigen by electrostatic interactions.[65,66] After this physically driven encapsulation of the protein inside the NGs, the protein was covalently immobilized in the network via thiol-disulfide exchange reactions. This approach provided reduction-responsiveness to the complex. In addition, it allowed to maintain the protein activity during the loading process, to achieve both high loading capacity and encapsulation efficiencies, and to provide a controlled release inside the cells. A similar strategy was followed by Kordalivand et al. to develop polyethyleneimine coated nanogels for the intracellular delivery of RNase A for cancer therapy.[67] More recently, the same authors followed the same procedure to load cationic NGs with synthetic long peptides containing the cytotoxic T lymphocyte and $CD4^+$ T-helper epitopes for cancer vaccination.[68]

Besides conjugating the protein to a preformed NG, it can also be already connected to the monomer/polymer before or during particle formation. An example for this was proposed by Boehnke, who investigated the stabilization of the

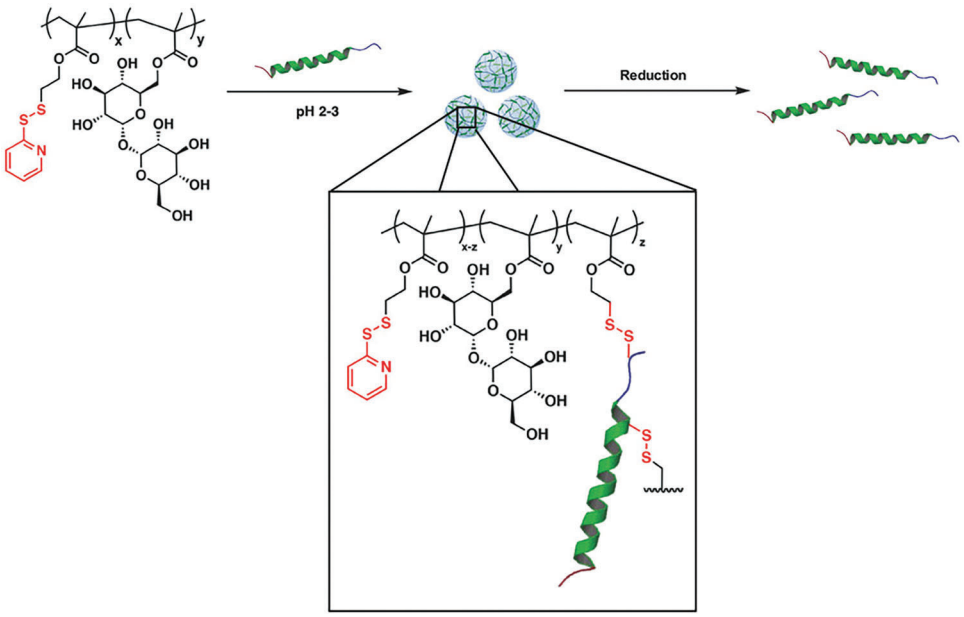

Figure 2. Synthesis of PDSMA-*co*-TrMA NGs using thiolated glucagon as crosslinker, which allowed for NG disintegration and release upon reduction. Reproduced with permission.[69] Copyright 2018, Wiley-VCH.

hormone glucagon using trehalose glycopolymer NGs (**Figure 2**).[69] Here, a thiolated glucagon was used as reduction-responsive crosslinker in the formation of pyridyl disulfide ethyl methacrylate (PDSMA)-*co*-trehalosemethacrylate(TrMA) NGs. Using the protein as crosslinker prevented from using additional crosslinking agents and resulted in simultaneous release of the protein and degradation of the NGs under reductive conditions.

The main purpose of covalently conjugating a protein to a NG Is to avoid unwanted passive diffusional release ("leakage") once it is applied in a biological environment. This will increase circulation time and can enable delivery at a specific target site. For this, the conjugation should be sensitive to specific environmental changes as stated above. However, it is possible to use covalent binding exclusively during NG preparation to favor subsequent physical protein entrapment. The Maynard group investigated this approach and used a triggered cleavage of the covalent linkage after particle formation to induce a high physically entrapment of the cargo rather than induce a local release.[70] This was realized by conjugating the lysine residues of a model enzyme phenylalanine ammonia lyase (PAL, used in enzyme replacement therapy) to an acrylamide-nitrobenzyl-carbonate (**Figure 3**). Free radical polymerization was performed with poly(ethylene glycol) methacrylate (PEGMA) and ethylene glycol methacrylate (EGDMA) as crosslinker, leading to a NG-PAL conjugate. NGs were subsequently irradiated with UV light to break the covalent bond between polymer and enzyme, leaving the enzyme only physically entrapped within the NG. However, even after this bond was broken, this strategy led to irreversible

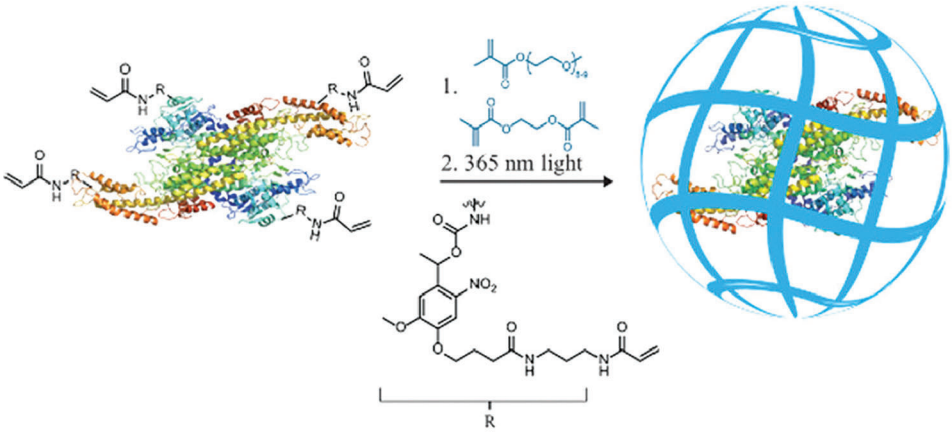

Figure 3. Covalent bonding through cleavable links can be used to entrap more efficiently the protein in the NG and subsequently cleave the interactions, leaving the enzyme physically entrapped in the final formulation. Reproduced under the terms of a Creative Commons CC BY 4.0 license.[70] Copyright 2022, The Authors, published by American Chemical Society.

modification of the enzyme, reducing its enzymatic activity to ≈50%.

3.1.2. Physical Entrapment

In contrast to the covalent functionalization, physical entrapment avoids harsh experimental conditions. As a result, these methods can lead to improved biocompatibility and better preservation of the protein activity. In this process, the protein is immobilized within the 3D polymer network through physical interactions such as hydrogen bonds, electrostatic interactions, van der Waals forces, and hydrophobic interactions. This is complementary with protein engineering, as the immobilized protein does not require further modification.[71] In physical entrapment, the loading process is optimized through (a) a rational design of the carriers' network chemistry, including the insertion of groups that enhance protein-polymer interaction (i.e., groups of opposite charges, hydrophobic groups or affinity binders), and (b) a well-adjusted network structure, i.e., ratio of mesh size to protein size. It is worth mentioning that strong interactions may cause denaturation of the protein due to modification of their tertiary and quaternary structure, while weak interactions decrease the encapsulation efficiency and can lead to fast release. Thus, for both cases, the encapsulation method and the respective conditions need to be adjusted carefully. In general, we can classify the loading procedures in diffusion-based entrapment, where NGs are loaded after they are formed, or in situ entrapment, where loading is carried out during NG formation.

Diffusion-Based Entrapment: During diffusion-based entrapment, a protein solution is put in contact with preformed NGs (Figure 1A). Proteins are usually incorporated in the NGs following Fickian diffusion, therefore, their movement through the NG network will depend on a concentration gradient and their diffusivity through the gel.[72] The latter is highly influenced by the gel mesh size, which is mainly dependent on crosslinking density, crosslinking efficiency, and crosslinker length.[73] Proteins are then absorbed into the NG polymer network and onto the NG surface. The amount of protein loaded will essentially depend on its ability to diffuse through the pores, which is a function of several parameters such as protein and pore size (see Section 3.3.1.), and the specific interactions between protein and polymer. It is worth mentioning that if these interactions are strong, a layer with high density of protein adsorbed may be formed at the surface of the NGs, hindering the penetration of further molecules to the inside ("skin effect").

Following the abovementioned Fick's diffusion law, increased concentration in the NG surroundings will favor NG entrapment.[74] To achieve this, the loading process can be carried out by incubating the NGs with an excess of protein solution. In this case, the protein solution can be added either to a suspension of NGs in their swollen state, or to a powder of freeze-dried NGs. In the latter, swelling of the NGs actively promotes the concomitant pull of proteins into the network. While these methods can enable high loading contents of the proteins, the required excess of cargo can often lead to reduced encapsulation efficiencies, i.e., large amounts of protein might not be entrapped.[46] To circumvent this, a NG suspension can be titrated with precise amounts of protein solutions. Although this helps adjusting the feed ratio, it is also more time-consuming, and the lower concentration gradient of the protein might reduce its diffusion through the NGs resulting in reduced LCs.[75]

During incubation of NG suspensions with protein solutions, incubation time and stirring rate are important parameters to control. This was emphasized recently by Mudassir and co-workers, who examined the loading of insulin into polymeric NGs for protection in oral administration.[76] For this, pH-sensitive electrolyte methyl methacrylate (MMA)/itaconic acid NGs were loaded with insulin by electrostatic interactions in an incubation process. The formation of the insulin/nanogels polyelectrolyte complex (PEC) was confirmed by a change of the isoelectric point and zeta potential. Interestingly, they observed an improvement of the EE from 4 to 6 h of incubation time, but no further improvement when extending the incubation time to 8 h. Optimization of the conditions led to EEs as high as ≈85%. They also concluded that proper stirring rate is needed to optimize EE: very low (100 rpm) or no agitation leads to low EE due to poor interaction between the protein and the NG, while increasing stirring rate to 200 rpm caused detachment of the insulin from the NGs and lowered the EE.

Controlling the release of cargoes in response to stimuli is one of the main advantages of NGs and has been widely described in the literature.[4] Although less described, this stimuli-responsive character can also be convenient to modulate the loading process upon incubation. For example, Elshaarani et al. used the thermo-responsive behavior of NGs for controlling the release, but also the loading process. For this, they prepared N-isopropylacrylamide (NIPAM) and phenylboronic acid (PBA)-based NGs crosslinked with dextran-maleic acid to control the delivery of insulin.[77] Loading was carried out by incubation of the NGs with a solution of insulin at 4 °C, way below the volume phase transition temperature (VPTT) of the NGs to ensure maximum and higher interaction between the insulin molecules and the phenylboronic acid derivative. Higher EEs were achieved by increasing the ratio of the phenylboronic acid residues.

Incubation of a protein solution with freeze-dried NGs might reduce the preparation steps: Since most NGs are suspended in water after their synthesis, they usually need to be transferred to an appropriate buffer for the loading with proteins (e.g., phosphate buffered saline, PBS of pH 7.4). This is required to ensure stability of the proteins in a suitable medium and enables determination of LC and EE under conditions that are closer to those encountered in the final application. Consequently, adding protein solutions in buffer to freeze-dried NGs will circumvent additional transfer steps from water to buffer for the NGs. Charbaji and co-workers followed this strategy to load pNIPMAM-based nanogels with etanercept (ETN), a fusion protein that is used as anti-tumor necrosis factor α (TNFα) in the treatment of inflammatory diseases of the gastrointestinal tract.[22] In this case, ETN was loaded by swelling the dry NGs in a protein solution (in PBS pH 7.4) for at least 24 h at 6–8 °C. The use of disulfide containing polyglycerol as a biodegradable crosslinker slightly reduced the EE (79% vs 88% for controls without a disulfide bond). This effect could potentially be assigned probably to differences in the zeta potential or mesh size (ξ). Encapsulation in the NGs stabilized the ETN during a 14 day storage in solution, as was observed in the binding affinity to TNFα (**Figure 4**).

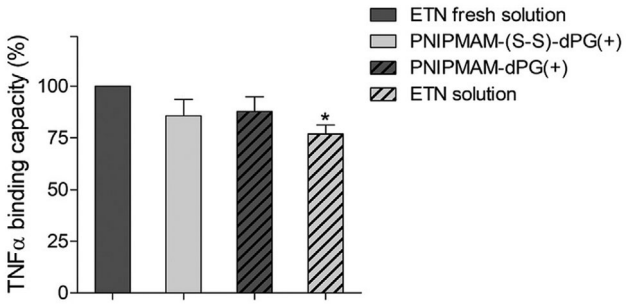

Figure 4. Stability of ETN loaded in the NGs based on their TNFα binding capacity. Both NGs containing ETN retained better the activity of the protein with respect to the free protein solution. Adapted with permission.[78] Copyright 2021, Wiley-VCH.

Another diffusion entrapment strategy is the titration of NG suspensions with a protein solution. This process can be monitored, for example, by DLS to check for changes in particle size that could indicate protein aggregation. Alternatively, zeta potential measurements can detect the formation of electrostatic interactions. With this, it is possible to control the feed ratio of protein very precisely, thus favoring maximum loading capacity and entrapment efficiency. This was demonstrated in a study by Zhou, who used the titration method to establish a relationship between protein content and particle aggregation.[75] They loaded bovine serum albumin (BSA) into nanogels consisting of a cationic core and a neutral shell (PEG) (**Figure 5**A). The loading process was monitored by dynamic light scattering (DLS) and the authors observed that low loading contents of the protein affected particle density (Figure 5B) without altering particle size (Figure 5C). However, higher protein contents triggered interparticle aggregation. They determined a critical protein concentration and estimated that NGs can load 1.6 mg of protein per mg of NG. This high LC (160%) was attributed to the high net charge density and stability of the NGs, as provided by the neutral outer shell. Such high values for LC are difficult to achieve with neutral NGs or polyion complex NGs without the outer PEG shell, thus demonstrating the importance of structural design in carriers.[79,80] In addition, this work highlighted the importance of an optimal pH during loading to maximize charge interactions between cationic NG and anionic protein. Importantly, the NGs preserved the secondary structure of the enzyme, and provided enhanced thermal stability. Additionally combining DLS measurements with electrophoresis analysis can reinforce the loading optimization, as proved by Cai and co-workers.[81] They loaded cationic poly (dimethyl aminoethyl methacrylate) NGs with lipase by titration. Besides increasing scattering intensity, when the enzyme is properly encapsulated in the NGs (and able to diffuse to the pores), the electrophoretic mobility of loaded NGs is not affected. However, after optimal loading is achieved, further protein addition leads to deposition on the surface of the NG and two effects are observed: increase in particle size and PDI, and drop in the electrophoretic mobility.

In Situ Entrapment: In this method, protein loading is carried out during NG formation (Figure 1B). For NG preparation via chemical crosslinking, harsh synthetic conditions and hazardous chemicals are often required. Since these can damage the proteins, this route is used only sparsely. However, for mild polymerization conditions, protein structure and activity can be maintained. As an example, Qi et al. loaded poly acrylamide (PAAM) nanogels with the enzyme catalase during particle formation via radical polymerization of acrylamide in PBS at 4 °C.[82] The mild temperature and lack of organic solvents during the preparation process led to preservation of the original enzymatic activity (**Figure 6**A). Furthermore, the resulting nanogels had a protective effect against treatment with different solvents (acetone, Figure 6B, dichloromethane or ethylacetate), which may be useful for further processing of nanogel formulations in coatings or bioprinting. In this sense, the in situ entrapment allowed for a tight entrapment of the protein with high control on the diffusion, which resulted in a controlled release with improved enzymatic stability from the NGs with respect to a polymeric coating.

In contrast to chemical crosslinking, the most prevalent strategy for in situ loading is based on NG preparation via physical crosslinking in a self-assembly process. Prominent examples for such materials are CHP nanogels that have been examined for vaccination applications. For example, Miura and colleagues demonstrated successful in situ entrapment of the antigen OVA during the self-assembly of CHP through hydrophobic interactions (in PBS and PBS containing urea) (**Figure 7**).[83] The polymer-protein complexes remained stable after complexation and urea removal, increasing the size of the nanogels from 30 to 63 nm with respect to the unloaded nanogels. Hydrophobic interactions between the cholesteryl group in CHP and the hydrophobic domains in OVA formed and stabilized the complex. These interactions were stronger when formed in presence of urea due to reversible denaturation of the OVA, which increases the hydrophobic interactions. When incubated for 24 h with BSA, the initial complexes immediately released OVA due to protein exchange. However, this fast release was not observed for the nanogels formed in urea.

Proteins can also be loaded in situ by host-guest interactions. As example, the cytokine interferon α (IFN-α) was loaded in cyclodextrin (CD)-based NGs by Zhang and co-workers.[84] They recently developed a pH-responsive NG that was formed by host-guest interactions between a β-CD functionalized 6-arm PEG and a similar star-shaped PEG that was end functionalized with a near-infrared IR825 dye. These NGs were designed for the delivery of IFN-α (**Figure 8**). The β-CD hydrophobic inner cavity hosted the lipophilic guest molecule IR825 by hydrophobic and van der Waals interactions. Cytokine IFN-α could benefit from these host-guest interactions and was encapsulated in the NGs during the self-assembly process with high loading efficiency (91%). Furthermore, the NGs provided enhanced pH-responsive programmable controlled protein release triggered by remote NIR due to a photothermal effect.

3.2. Release Mechanisms

In drug delivery, release rates and release profiles need to be carefully adjusted to the desired application. Two main strategies can be distinguished: 1) A passive slow and controlled release is desired to prolong the overall therapeutic effective concentration. 2) An active triggered (or stimuli-responsive) release is desired to restrict the release to the site of the therapeutic target, thus enhancing the efficacy of the protein drug.[85] The second strategy was

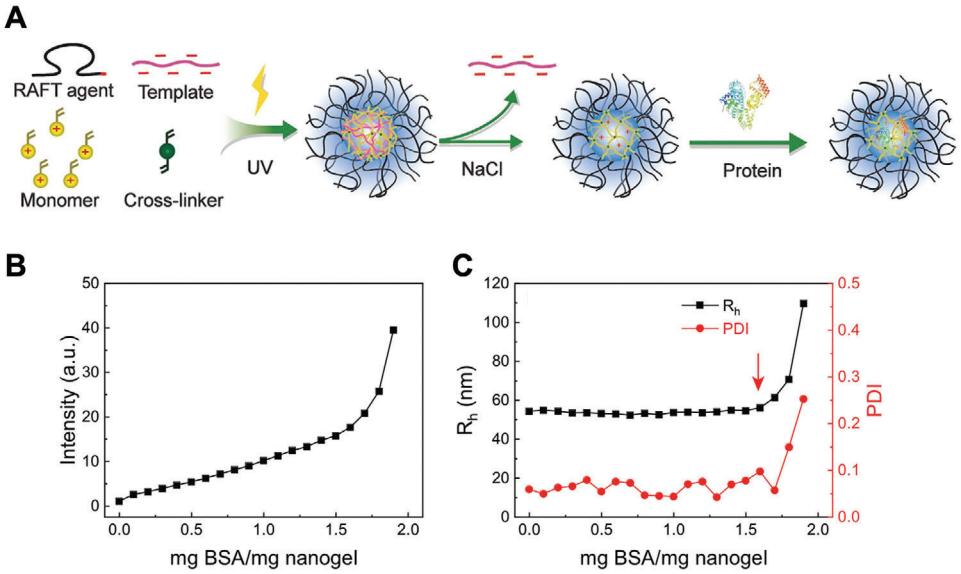

Figure 5. Synthesis and protein loading of NGs with cationic core (A). Light scattering intensity (B), hydrodynamic radius (R_h) and polydispersity index (PDI) C) of NGs at increasing concentration of BSA added by titration. Adapted with permission.[75] Copyright 2022, Elsevier.

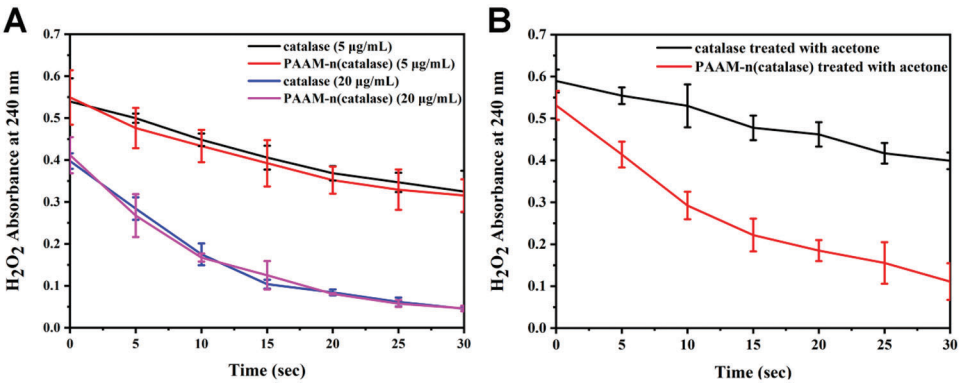

Figure 6. PAAM NGs preserved the activity of catalase after in situ polymerization and protein entrapment A). The NGs also provided a protective effect on the enzyme toward the action of organic solvents B). Reproduced under the terms of the Creative Commons CC BY 4.0 license.[82] Copyright 2020, The Authors, published by Royal Pharmaceutical Society.

reviewed for the specific case of intracellular delivery of proteins and other biotherapeutics by Li et al.[86] However, the hydrophilic character of both NGs and proteins often leads to a premature initial burst release once administered. As a result, only residual amounts of protein-loaded NGs reach the target site.[87,88]

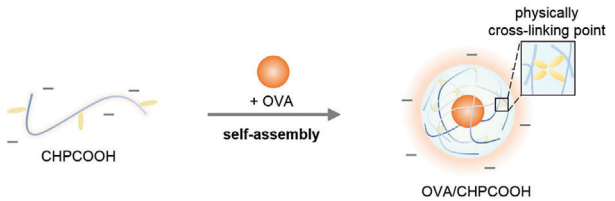

Figure 7. CHP forms NGs in contact with the antigen OVA through a self-assembly/in situ loading process. Adapted with permission.[83] Copyright 2020, American Chemical Society.

Controlling the release rate from bioconjugated proteins is possible by using covalent bonds/linkers between NG network and protein that can be cleaved as response to biological stimuli (enzymes, pH changes, reductive and oxidative environment). Since bioconjugation approaches are not the subject of this review, we refer the readers to existing reviews on the linkers and reactions that can be used.[89,90]

In the field of physically entrapped proteins, drug release is mainly dependent on the diffusion of the proteins through the polymer network. Since this is similar to the loading process, parameters to control the release rate are the same as those discussed for the loading (Section 3.3.1.): (1) structural influences, i.e., mesh size, versus protein diameter (**Figure 9**A) and (2) interactions between network and protein (covalent or physical). Consequently, an optimized carrier system needs to carefully balance these parameters to maximize loading, minimize burst release, and control the (triggered) release. This can be achieved

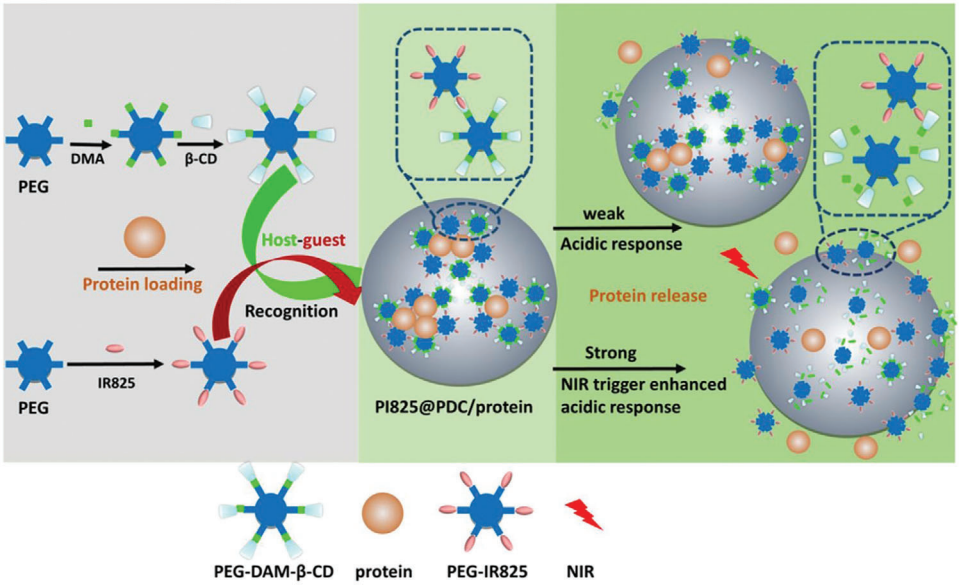

Figure 8. Nanogels formed by host-guest recognition between PEGylated β-CD (PDC) and PEGylated IR825 dye (PI825) were able to load protein through host-guest interactions. Reproduced with permission.[84] Copyright 2021, Elsevier.

through various strategies, that correspond to different release mechanisms.[20]

Regardless of the mechanism, the main driving force for the release is protein diffusion following a concentration gradient (Fickian diffusion). Several kinetic models can be applied to describe cargo release from a hydrogel matrix (**Table 1**). Adjusting release data to specific release profiles helps understanding the main mechanism involved and provides kinetic parameters that allows comparison between different carriers. To the best of our knowledge, in most works of NGs for protein delivery model fitting is not applied. However, Morgulchik and Kamaly have observed that most redox-responsive NGs adapt to Higuchi's or Peppas-Sahlin's model based on a meta-analysis of published data.[91] Higuchi's model describes the diffusion of a drug from an insoluble matrix, considering diffusion as the main limiting step. In contrast, the Peppas-Sahlin model, which integrates the relative contribution of the Fickian diffusion mechanism and macromolecular relaxation of the polymer chains from the NGs, would define better protein release from NGs, from a theoretical point of view.[92]

An important strategy to control protein diffusion in the NGs is based on adjusting the network structure. Especially, dynamic changes in the mesh size can induce a triggered release. This

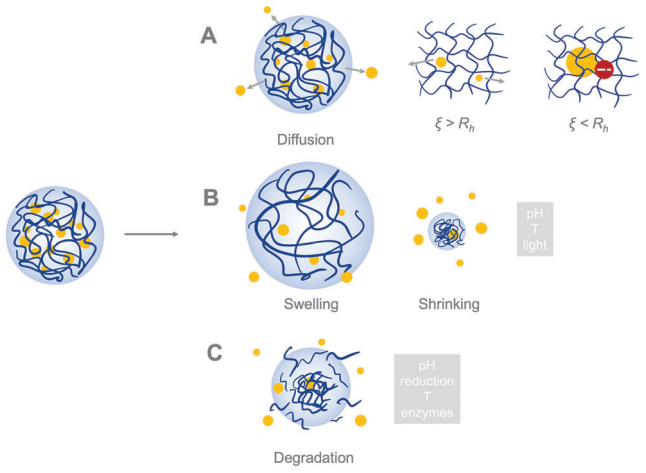

Figure 9. Release mechanisms of proteins from NGs can be based on diffusion mechanisms, which mainly depend on mesh size (ξ) and protein hydrodynamic diameter A), changes in NG swelling state B), and NG degradation C).

Table 1. Kinetic models of drug release. Adapted with permission from ACS Applied Nano Materials 2021, 4, 5, 4256–4268. Copyright 2021 American Chemical Society.

Model	Equation	Description and type of system
zero order	$Q_t = k_0 t$	Ideal delivery system, the drug is released at constant rate with no burst effect
Korsmeyer-Peppas	$Q_t = k_{KP} t^n$	Drug release from a polymer matrix (only $Q_t \leq 60\%$)
Ritger-Peppas[92]	$Q_t = kt^n$	Release considering geometry ($n = 1$ in thin sheets, 0.89 in cylinders and 0.85 in spheres)
Peppas-Sahlin	$Q_t = k_1 t^m + k_2 t^{2m}$	Relative contribution of Fickian diffusion and macromolecular relaxation
Higuchi	$Q_t = k_H t^{1/2}$	Fickian diffusion of a drug from insoluble matrix
Hixson-Crowell	$(100\text{-}Q_t)^{1/3} = k_{HC} t$	Erosion-controlled release systems with surface area changes

Q_t = fraction of drug released at time t; k_0 = zero order release constant; k_{KP} = Korsmeyer-Peppas constant; n = release exponent; k_1 = Fickian diffusion constant; k_2 = macromolecular relaxation constant; m = diffusional exponent; k_H = Higuchi dissolution constant; k_{HC} = Hixson-Crowell constant.

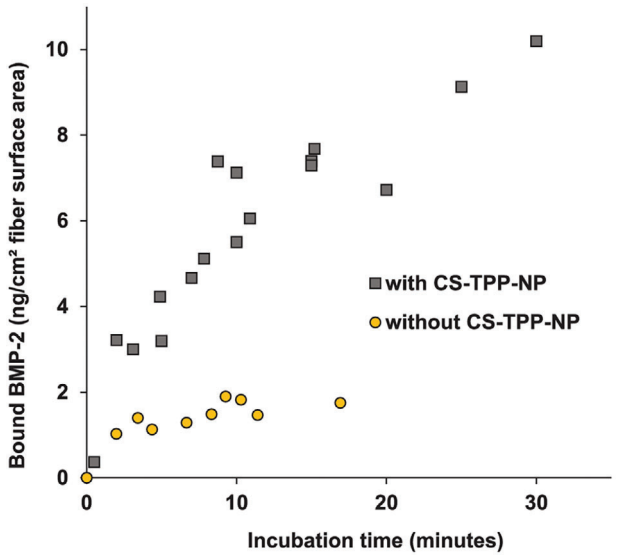

Figure 10. Improvement on BMP-2 loading in PCL fiber mats with respect to uncoated samples.[97] The NGs provided a combination of enhanced loading of the protein and controlled release. Reproduced under the terms of the Creative Commons CC BY license.[97] Copyright 2021, The Authors, published by Wiley Periodicals.

can be achieved through controlling the interactions between network polymer and the aqueous medium by chemical or biological triggers, e.g., pH, enzymes, reductive or oxidative species etc.[93] A corresponding change in the swelling/deswelling of the NG is accompanied by different mesh sizes. In addition, degradable NGs offer the possibility to break down the mesh structure into linear polymers by using cleavable crosslinking points or polymers containing degradable groups.[37,94] In both cases, release should only occur upon stimulation. However, most NGs suffer a residual release in the absence of such stimuli, i.e., a slight burst effect. This is mainly due to the proteins bound to the surface of the NGs, which can easily diffuse to the surroundings.[95]

A different strategy to control the release rate is based on adjusting the interactions between polymer network and loaded protein (physical or covalent). To reduce burst release effects, strong interactions between NG network and the protein cargo need to be introduced, e.g., ionic interactions, hydrophobic interactions, or affinity binders.[96] While these interactions can significantly slow down the free diffusion of proteins from the network, they also provide the potential for triggered release. In this case, the interactions are broken or reduced by changes in the NGs' environment. For example, ionic interactions crucially depend on pH and ionic strength.[46]

Besides release from the NGs themselves, the combination of NGs with other materials can also help tuning their respective release profile. This was demonstrated for combinations of NGs with polymer coatings or fiber mats. Sundermann and co-workers investigated the loading and release of bone morphogenic factor 2 (BMP-2) onto polycaprolactone (PCL) fiber mats with different surface modifications.[97] Coating PCL with a chitosan/alginate layer increased protein adsorption and a correct release profile, however a low loading content prevented therapeutic concentrations (**Figure 10**). Functionalizing with polydopamine led to a huge increase in protein loading, but the strong interactions limited the release below therapeutically efficient concentrations, i.e., less than 30% of the bound protein was released after 50 days. By combining surface hydrophilization with an extra coating of chitosan-tripolyphosphate (CS-TPP) nanogels (crosslinked in presence of the BMP-2) an optimal balance between protein adsorption and release profile was obtained. BMP-2 was loaded both in the fiber mats and inside the negatively charged core of the positively charged NGs, providing an initial burst release followed by a controlled release that maintained therapeutic concentrations for up to 7 weeks.

3.3. Factors Affecting Encapsulation Efficiency and Release Profile

3.3.1. Network Structure: Mesh Size and Degree of Swelling, Degradation

Nanogels are crosslinked polymeric networks, and open spaces (meshes) between polymeric chains allow for water diffusion. Considering the structure of the network, it is the difference between the NGs' mesh size and the proteins' hydrodynamic diameter that determines the release profile.[98] Typical mesh size values for nanogels range between a few and up to ten nm.[99,100] If the hydrodynamic diameter of the protein is smaller than the mesh size, the protein is able to diffuse through the gel. In this case, release will be controlled by diffusion, and the protein can be loaded in the NG by a diffusion process.[54,101] Here, a smaller mesh size will result in a slower release.[64] However, as stated before, it will also reduce the encapsulation efficiency since it hinders the protein from both diffusing in and diffusing out of the network.

One way to circumvent this problem is to build a network with small mesh size around the protein. For this, the crosslinking density can be increased in an in situ encapsulation process, or by adding an additional mesh of polyelectrolyte layers on the NG surface, for example, by layer by layer (LbL) assembly.[102] However, some authors have suggested that particle size has a stronger effect on LC and release than crosslinking density/mesh size.[81] It can be expected that larger particles are able to load more protein due to their higher volume. Similarly, since more proteins can be entrapped in the interior of the NG, release from large particles should be slower due to higher diffusion pathway of the entrapped proteins. However, if the protein is loaded on the surface of the NGs, a larger particle size will result in stronger burst release.

Another way to balance a maximized loading capacity with a minimized burst release is using dynamic changes in the mesh size.[54] This can be realized by controlling the NGs' degree of swelling as response to external stimuli. In such systems, a highly swollen network can be used to enhance diffusion of proteins into the NGs. Changes in the NG environment (or the application of an external stimulus) can be used to change the polymer-solvent interaction, which can lead to a deswelling of the network.[103] Since this is accompanied by a reduction in mesh size, proteins can be entrapped in the collapsed network – especially if there is an additional physical polymer-protein interaction.[104] A subsequent (triggered) increase in swelling (and mesh size) can then induce a release again.

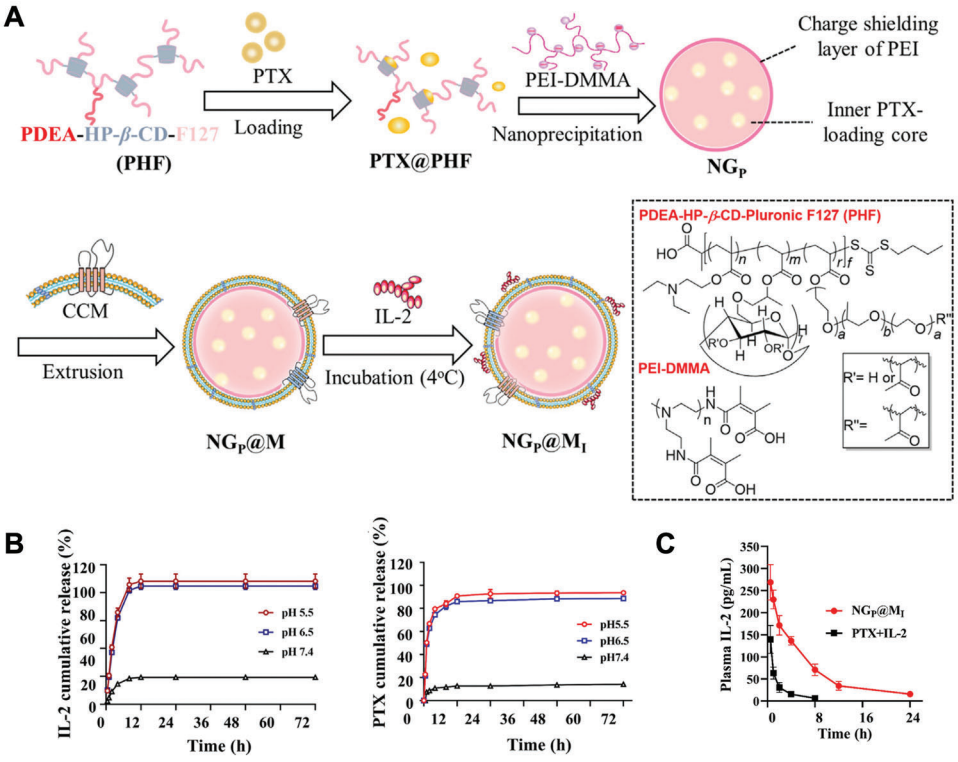

Figure 11. Core-shell NGs containing a pH responsive core swellable at acidic pH A). Protein IL-2 was loaded in the shell consisting of biomimetic cancer cell membrane. NGs were able to release loaded IL-2 in slightly acidic pH due to swelling of the core and disruption of the shell B). NGs improved bioavailability of IL-2 in vivo C). Reproduced under the terms of the Creative Commons CC BY-NC-ND 4.0 license.[106] Copyright 2022, The Authors, published by Elsevier.

Prominent examples for such a swelling-induced release mechanism are pH responsive NGs. These are colloidal gels that are formed from crosslinked polyelectrolytes, i.e., polymers with acidic or basic moieties. At specific pH values, these groups are charged (cationic or anionic) which results in an electrostatic repulsion and an increased osmotic pressure that causes network swelling and mesh size increase.[105] Thus, small variations in the environmental pH (different targeted sites) can be used to induce NG swelling with corresponding release of cargo. Since charges on the network also generate interactions with the charged groups in the proteins, these interactions can also be used to further tune the release profile. By carefully adjusting network swelling and network-protein interactions, specific pH variations can be used to enhance loading capacity (through protein-polymer interactions – see Section 3.3.2.) and induce protein release (through swelling). Although release by swelling is less explored in the field of NGs, where usually the release of the compound is triggered by gel shrinkage, this strategy has been explored for micro- and macrogels.[104]

Besides changes in mesh size, a combination of this effect with more complex NG structures can be used to implement alternative release mechanisms. For example, in core-shell NGs, a volume increase of the NG core can serve as osmotic trigger to release proteins loaded in the shell. Following this strategy, Shang et al. developed NGs from pH-responsive poly-2-(diethylamino)-ethyl acrylate (PDEA).[106] The cationic polymeric NGs were loaded with the chemotherapeutic agent paclitaxel (PTX) and coated with a biomimetic cancer cell membrane, which was further loaded with interleukin 2 (IL-2). This aimed to promote activation of cytotoxic T lymphocytes in triple-negative breast cancer (**Figure 11**A). EE of IL-2 depended on the feeding amount, with optimum loading capacity of 78.3 ± 3.9%. In acidic media, the core of the NGs was protonated and swelled due to the corresponding osmotic pressure. The resulting volume increase caused the disruption of the cancer cell membrane, thus triggering the release of IL-2. Results showed that at physiological pH of 7.4, less than 20% of the interleukin was released after 12 h (Figure 11B). However, at acidic pH, the amount released reached 85% over the same time period. Release of PTX followed a similar trend. The nanoplatform improved the biodistribution and bioavailability of both paclitaxel and IL-2 (Figure 11C), and achieved a controlled drug release of both drugs exclusively in the tumor microenvironment.

In contrast to swelling-induced release, the deswelling (or collapsing) of NG networks is also reported to result in protein release. This effect occurs mainly in NGs that do not show strong interactions between polymer network and protein. In such cases, a collapse of the network results in the expulsion of water and protein, as suggested by Theune et al.[107] An example for this mechanism are temperature responsive NGs. These systems exhibit a change in particle size at a temperature named volume phase transition temperature (VPTT). The most studied materials in drug delivery, that exhibit this effect, are based on polymers that show a lower critical solution temperature (LCST), e.g.,

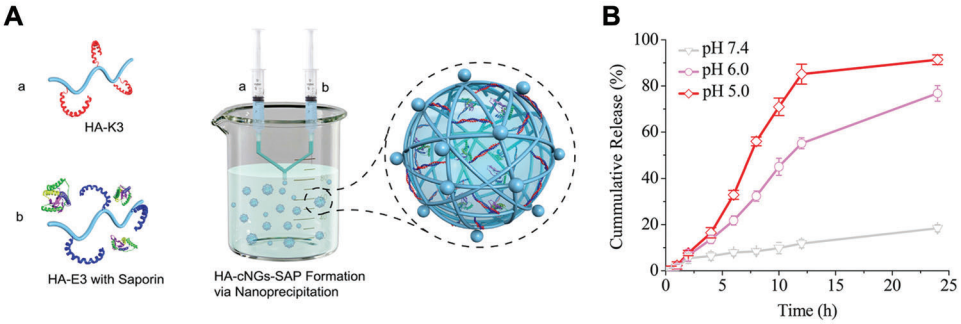

Figure 12. pH-responsive coiled-coil peptide-crosslinked HA NGs A). Release of CytC from acid NGs at different pH B). NGs barely released the protein at neutral pH, but at acidic pH they released the cargo fast upon degradation. Adapted with permission.[81] Copyright 2018, American Chemical Society.

poly[oligo(ethylene glycol) methyl ether methacrylates] (POEGMAs), PNIPAM, poly(N-vinylcaprolactam) (PVCL) etc. Here, at $T >$ LCST, unfavorable polymer-water interactions and favorable polymer-polymer interactions cause network de-swelling and a decrease in particle size.[108] Depending on the LCST, NGs can shrink at body temperature, immediately releasing the cargo once administered.[109] However, if the VPTT is slightly higher than body temperature, an external thermal stimulus needs to be applied such as in thermal therapy.[110] This allows for a spatio-temporal control of the release. In this field, Ji and co-workers explored the potential of using thermo-responsive nanogels in thermal magnetic resonance to induce the release of BSA-FITC from PNIPAM-PNIPMAM NGs.[110] This approach would help combining thermal therapy with diagnostic tools in cancer treatment. Another way to induce deswelling in thermo-responsive NGs is to include NIR photothermal agents such as gold nanoparticles or nanorods, which heat up upon irradiation. This causes a local increase in the temperature and induces shrinkage of the NGs at local site.[111] NIR-range wavelengths are preferred since they have better tissue penetration and compatibility with healthy tissue than UV light.[112]

Besides changes in swelling state, NGs can also be designed to degrade upon changes in the biological or chemical environment. For this, either the crosslinking points or the polymeric backbone can be rendered cleavable under specific conditions. As example, NGs crosslinked with pH-sensitive molecules, such as hydrazone, *cis*-aconityl, and acetal bonds can break in acidic environments.[113,114] This property can be exploited to release cargoes in tumor microenvironments. Tumor tissue often exhibits poor blood diffusion and subsequent local hypoxia, which induces a shift in glucose metabolism of the cells from oxidative phosphorylation to glycolysis.[115] This metabolic change leads to an increase in the production of lactic and carbonic acid, creating an acidic pH gradient with respect to healthy tissue. Something to consider when designing such degradable systems, is that changes in the environment upon NG degradation (i.e., acidification) might compromise protein activity.[116] To provide an overview of the synthesis of such cleavable NGs, we refer the readers to the review by Zhang.[37] Combined with an adequate encapsulation method, the release can be precisely controlled even in the absence of covalent bonding between protein and polymer. As an example, Ding and co-workers loaded pH-responsive coiled-coil peptide-crosslinked hyaluronic acid (HA) NGs with cytochrome C (CytC) in situ during polymerization, achieving high entrapment of the protein (close to 100% for the optimal concentration) (**Figure 12**A).[114] Release at physiological pH was minimized due to very low diffusion of the protein through the gels (Figure 12B). However, at acidic pH the NGs were cleaved due to protonation of amino acids that play a key role in coil-coil stabilization. This caused a controlled release of the loaded protein, with more than 80% of the protein payload released after ten hours.

Redox-responsive NGs use redox-responsive bonds (both in the polymer structure and in the crosslinker) that can be degraded in the presence of glutathione (GSH). This approach is frequently used when designing NGs for the intracellular delivery of anti-cancerous agents, since GSH concentration is highly increased inside cells subjected to oxidative stress. Common crosslinkers include disulfide, diselenide and maleimide tioether bonds.[78,117,118] For instance, Huang and co-workers incorporated disulfide-based cystamine residues in HA-methacrylate (MA) based NGs to induce cleavage in tumor cells. The NGs were fabricated via microfluidics by crosslinking using a tetrazole-alkene click reaction (**Figure 13**A).[119] Saporin was efficiently loaded in NGs presenting different particle sizes (80 and 150 nm) and crosslinking densities (normal and high, HX). Less than 20% of the protein payload was released in the absence of GSH, however, in presence of GSH around 80% of the protein was released due to cleavage of cystamine (Figure 13B). This effect was barely dependent on the crosslinking density, with a slightly lower release observed for the higher crosslinking density. Interestingly, NG size did not influence the release profile.

Finally, degradation of the NGs can also be triggered by enzyme activity using substrate-like crosslinkers.[120] In this case, short peptide sequences provide more specificity toward specific enzyme types. An example is the work by Massi et al., who developed NGs responsive to matrix metalloprotease (MMP)−7.[121] They produced block copolymers containing a PEG block and a block with tunable LCST around body temperature (33 to 44 °C) by copolymerizing NIPAM and N-cyclopropylacrylamide (NCPAM). The preformed polymers contained a middle section with alkyne groups that were crosslinked via copper-catalysed azide-alkyne cycloaddiction (CuAAC) by a bifunctional MMP-7-sensitive crosslinker ($K(N_3)PLELRAK(N_3)$ at slightly elevated temperatures (**Figure 14**A). The mild crosslinking strategy enabled the in situ loading of BSA. For this, BSA was introduced during the self-assembly process before crosslinking. In addition, tuning the ratio of NIPAM and NCPAM allowed to fine-tune the

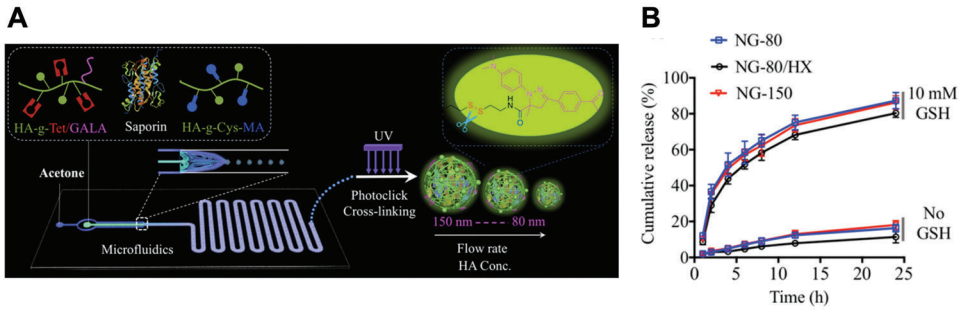

Figure 13. HA-Cys-MA NGs fabricated by microfluidics for saporin delivery A) Release of saporin from in presence and absence of GSH, which cleaves the cystamine moieties B). NGs with higher crosslinking density (NG-80/HX) showed a slightly slower release profile, whereas no difference was observed for NGs of higher particle size (NG-150). Adapted with permission.[119] Copyright 2019, American Chemical Society.

self-assembly behavior of the NGs prior to crosslinking. The release of BSA was performed at physiologically relevant MMP-7 concentrations, allowing to compare diffusion/non-specific release with enzymatic induced release (Figure 14B). While in the absence of the enzymatic trigger the NGs only partially released the protein after 14 h due to diffusion effects, high MMP-7 concentrations led to fast release due to enzymatic degradation of the NGs.

Besides introducing the cleavable groups through the crosslinkers, it is possible to use biodegradable polymers that will be degraded by enzymes in vivo. A common choice is hyaluronic acid (HA), since can be degraded by the hyaluronidase overexpressed in tumor cells.[122] Yang and co-workers loaded CytC in photo-crosslinked NGs containing HA, PEG and polyaspartamide derivatives (which improve protein loading and promote endosomal escape) and obtained a very similar release profile to Massi in the presence of hyaluronidase, with less than 20% of release in absence of the enzyme and fast release at high enzyme concentrations.[123]

The degradable systems mentioned above lack of chemical fixation of the protein to the polymer network, making it possible for the protein to diffuse through the nanogel even in the absence of the degradation trigger (which leads to initial passive release of the protein). To avoid this, Li et al. proposed a coated NG system where OVA was physically encapsulated inside a dextran nanogel, which was coated by LbL technique with different types of reduction-responsive polymeric coatings.[124] First, they adjusted the polymer composition and molecular weight of the external layers, which avoided protein leaking during the coating process. On the other hand, crosslinking the external coating with pyridyldisulfide groups helped minimize passive release in the absence of the reductive agent, while this layer would be degraded in vivo allowing for the complete diffusion of the loaded protein.

3.3.2. Protein-Network Interactions: pH, Zeta Potential, and Isoelectric Point of the Protein

Proteins usually present a charged character at physiological pH due to charged amino acids in their structure. This effect is often exploited to increase the loading capacity and adsorption in NGs that contain opposite charges. In general, loading should be performed at a pH that guarantees opposite ionization of the corresponding protein and polymer network, leading to higher entrapment efficiencies. Some authors have also suggested that this charge difference has a stronger influence on the loading capacity when the protein has a low molecular weight.[125] For pH responsive NGs, variations in the environmental pH change

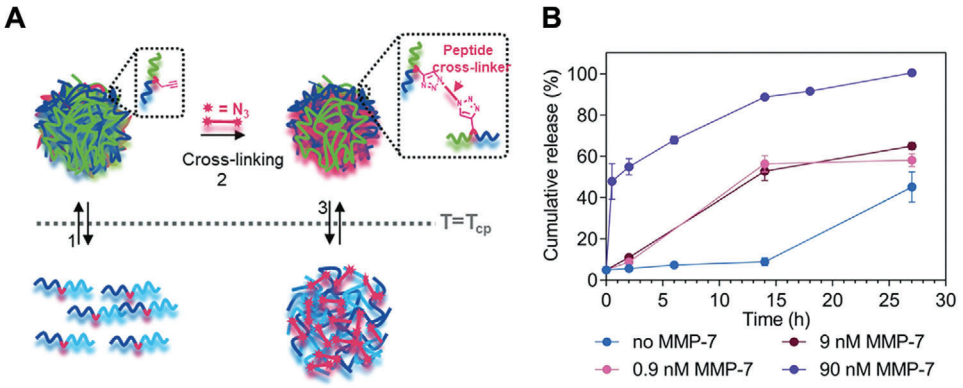

Figure 14. The thermo-responsive character of poly(NIPAM-*co*-NCPAM) was used in NG assembly and crosslinking with an enzyme-sensitive linker A). MMP-7 triggered the release of BSA at relevant enzyme concentrations: higher MMP-7 led to fast release, while in the absence of the enzyme only partial release of a small amount of protein due to diffusion was observed. Adapted under the terms of the Creative Commons CC BY 3.0 license.[121] Copyright 2020, The Authors, published by The Royal Society of Chemistry.

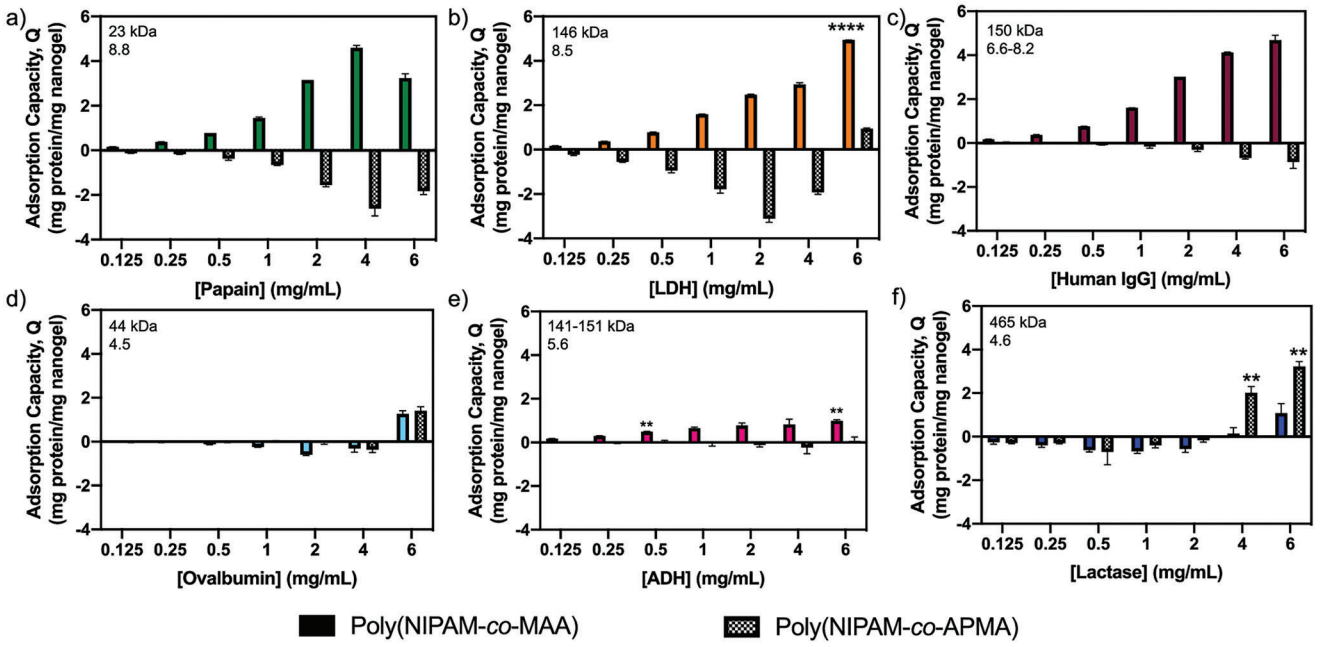

Figure 15. Adsorption capacity (Q) of proteins of varying molecular weight and high A) and low B) isoelectric points to anionic poly(NIPAM-*co*-MAA) or cationic poly(NIPAM-*co*-AMPA) NGs in PBS pH 7.4 (IgG and ADH in HBS pH 5.5). Reproduced with permission.[46] Copyright 2021, American Chemical Society.

this important ionization degree. As a result, electrostatic interactions with the protein are disrupted, which can induce cargo release.

Macdougall and co-workers systematically investigated the parameters that affect stabilization of high isoelectric point proteins.[46] They produced NGs of either anionic poly(NIPAM-*co*-MAA) or cationic poly[(NIPAM-*co*-*N*-(3-aminopropyl) methacrylamide hydrochloride] (AMPA) and loaded them with a set of proteins of different molecular weights and isoelectric points: OVA, lactase, BSA, alcohol dehydrogenase (ADH), immunoglobulin G (IgG), lactate dehydrogenase (LDH), papain and CytC. For this, the NGs were swollen and incubated with stock solutions of different concentrations of the proteins using different buffers. To release the proteins from the NGs and check their activity, the authors disrupted the electrostatic interactions between the polymer and the NG by the addition of high molar salt solutions. This process was dependent on the zeta potential of the NG, the isoelectric point of the protein, and the pH of the buffer used during the encapsulation procedure. In general, anionic nanogels loaded high amounts of proteins with high pI (**Figure 15**A), while cationic nanogels showed weak interaction with proteins of low pI due to low positive zeta potential of the NGs (Figure 15B). The protection capacity of the NGs toward different temperature treatments, time periods at room temperature, and freeze-drying were also evaluated. Anionic nanogels provided high stabilization of papain and IgG over time (LDH is already stable in solution), and protection of LDH and IgG to elevated temperatures (free papain is already stable at high temperatures). This study provides useful information on how to control the parameters during protein encapsulation, highlighting the need to use adequate conditions for the loading and release of the protein.

Another systematic study that highlights the importance of charge balance between the protein and the polymer is the one published by Culver and co-workers.[126] They produced PNIPAM-based NGs to study their potential as differential protein receptors in a turbidimetric sensor array. The ulterior objective of this study was to avoid the high cost and environmental instability of antibody-based tests. NGs were based on a NIPAM-methacrylic acid (MAA) copolymer (poly(NIPAM-*co*-MAA), where the MAA units act both as an ionizable unit able to establish electrostatic interactions with high pI proteins, and as reactive group to introduce other functionalities with sulfate, guanidinium, secondary and primary amine groups (**Figure 16**A). In this work, the authors examined how the ionizable groups affect swelling behavior, charge character, and protein binding of proteins with varied pI and molecular weight (Figure 16B). Adsorption capacity was determined indirectly by measurement of unbound protein in the supernatant using the MicroBCA assay. They also performed turbidimetric protein binding assays in buffers of different pH but comparable ionic strength. These tests revealed that turbidimetric examinations exhibit high accuracy, and could therefore be used for the proposed application when the polymer has high affinity for the protein.

3.3.3. Selective Factors: Presence of Affinity Binders

As mentioned above, protein loading via electrostatic interactions can lead to high entrapment efficiencies. However, the susceptibility of these forces to naturally occurring environmental changes may hinder selective protein delivery to the target site. Affinity binders in the polymer NGs are a promising alternative to overcome this effect, since they can provide strong

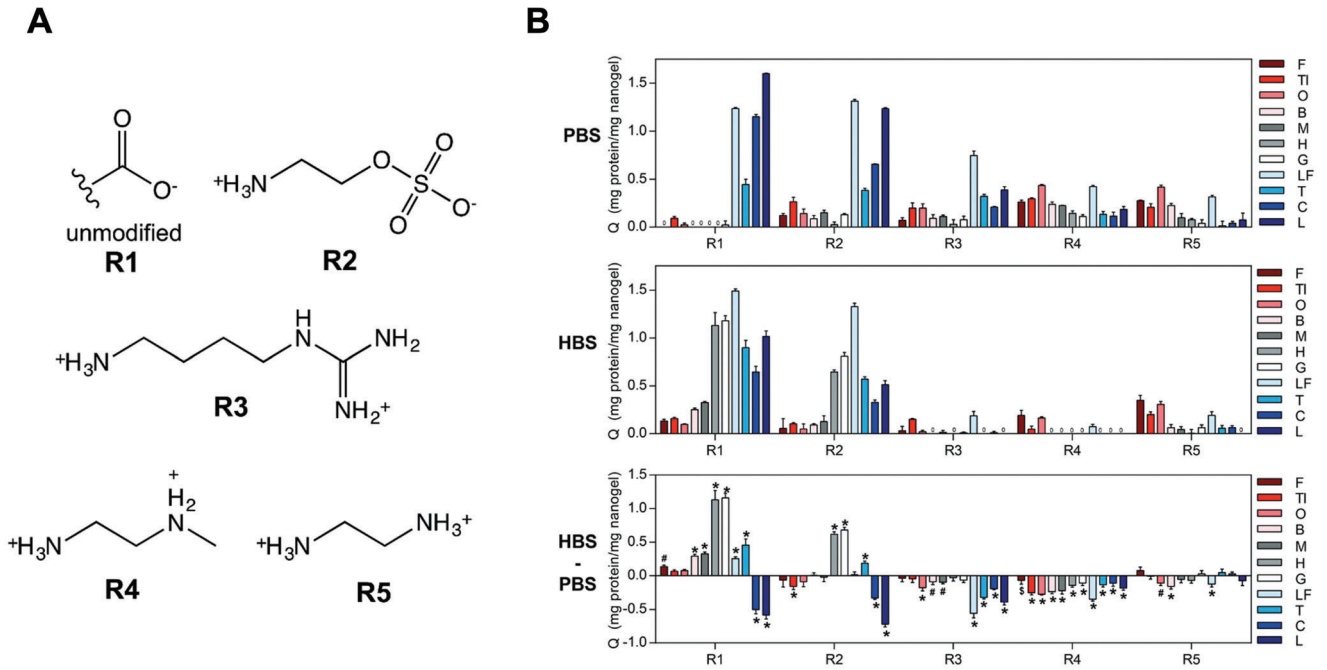

Figure 16. Ionizable groups used to functionalize poly(NIPAM-*co*-MAA) NGs A) provided NGs with a varied adsorption capacity (Q) for a library of proteins B). Reproduced with permission.[126] Copyright 2017, Royal Society of Chemistry.

but non-covalent interactions that are more stable in biological environments. The Haag group created NGs based on dendritic polyglycerol (dPG) that could be conjugated with avidin on their surface, thus offering the possibility to establish affinity interactions with biotin-modified biomacromolecules (**Figure 17**).[127] Loading of the NGs with biotinylated monoclonal antibodies (mAbs) provided higher activation of T cells with respect to conventional antibody carriers even at lower concentrations.

Besides incorporating specific affinity binders that show very strong non-responsive interactions with cargoes, NG structures can also incorporate groups that exhibit a stimuli-responsive affinity. This is the case in polymers containing phenyl boronic acid (PBA) groups. In aqueous media, these groups exist in a dynamic equilibrium between a hydrophobic trigonal-planar form and an ionized hydrophilic tetrahedral form. In presence of diols (e.g., glucose), the hydrophilic form is stabilized through dynamic covalent bonds. In NG networks, this hydrophilicity change can cause swelling of the NG and allowing for cargo release. This mechanism has also been used in competitive systems.[128] Here, PBA moieties in polymers containing multiple hydroxyl groups (such as PVA) cause the crosslinking. In the presence of high amounts of diols (sugars), competitive binding occurs and the crosslinks are destroyed. Both mechanisms are potential candidates for self-regulating insulin delivery systems. However, such systems still present limitations in terms of sugar affinity, and need to be carefully designed to provide a selective response to glucose with respect to other sugars (i.e., fructose, mannose).[129] In this regard, Li et al. developed a NG system composed of PEG and poly(cyclic phenylboronic ester) using a click chemistry approach.[130] Although the polymers were per se sensitive to glucose and H_2O_2, the release of insulin could be enhanced by incorporating glucose oxidase (GOx) that increased the amount of H_2O_2 in the presence of glucose. FITC-labelled insulin and rhodamine B-GOx were used to allow for direct loading and release determination. The "on-off" controlled release of insulin was emulated by alternating the release medium of the dialysis bag, from PBS with or without a glucose solution, and the system could be transferred in vivo for the treatment of diabetic mice.

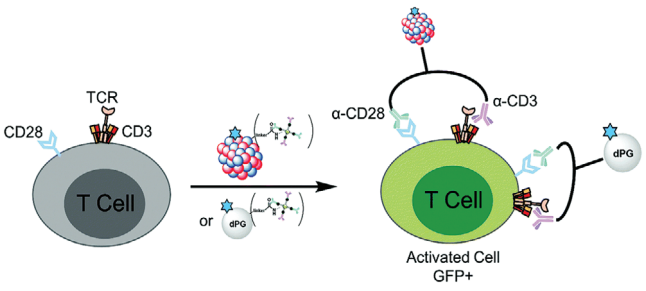

Figure 17. Biotinylated mAbs α-CD28 can be loaded by strong, non-covalent affinity-based interactions into dPG-based NGs conjugated with avidin, providing high specific targeting and activation of T cells. Reproduced under the terms of the Creative Commons CC BY 3.0 license.[127] Copyright 2022, The Authors, published by the Royal Society of Chemistry.

3.4. Strategies to Determine Protein Loading Capacity and Entrapment Efficiency

3.4.1. Indirect Determination

Encapsulated proteins can be detected either directly inside the NGs, or indirectly by measuring the remaining non-loaded

protein after the encapsulation procedure. The second approach is usually preferred since methods for free protein quantification are better established and avoid the interference of NGs. In this method, an intermediate step of centrifugation or ultrafiltration is usually needed to separate the loaded NGs from the supernatant. Encapsulation efficiency (EE) in percent is then calculated according to Equation (1):

$$\text{EE}\,(\%) = \frac{\text{initial mg enzyme} - \text{mg enzyme in supernatant}}{\text{initial mg enzyme}} \times 100 \quad (1)$$

3.4.2. Direct Determination

For direct determination, a method needs to be developed that does not suffer from interference by the NGs. In this case, equation (2) is used to calculate the encapsulation efficiency:

$$\text{EE}\,(\%) = \frac{\text{mg enzyme encapsulated}}{\text{mg enzyme initially fed}} \times 100 \quad (2)$$

The simplest approach is to use colorimetric methods for protein quantification such as the Bradford method, the Lowry method, or the bicinchoninic acid (BCA) assay.[131,132] However, absorption by functional groups in the polymer/crosslinkers or scattering interferences due to NG concentration may lead to inaccurate results.[133–136] A frequently used strategy is based on labeled proteins that can be quantified by colorimetric or fluorometric methods. For example, Duan et al. studied the loading of different FITC-labeled proteins into dual-responsive NGs to develop multifunctional carriers for combined cancer therapy.[137] These colloidal systems are based on β-CD-conjugated HA, polyethyleneimine (PEI), and cisplatin. In this case, cisplatin served two purposes: first, as small molecule drug and second, as coordinative crosslinker for electrostatic HA-PEI assemblies. The HA skeleton renders the NGs responsive to hyaluronidase, which is overexpressed in the tumor microenvironment. For loading, the NGs were incubated with FITC-labeled BSA, CytC, and GOx. Not encapsulated proteins were removed by dialysis. To quantify the encapsulation efficiency of the loaded proteins directly in the NGs, fluorescence spectroscopy was used. This method indicated high values of EE for glucose oxidase-FITC and BSA-FITC (> 55%) and moderate EE for cytochrome C-FITC. A wider range of proteins (including additional hemoglobin, hoseradish peroxidase, lysozyme, saporin, and melittin) was loaded to check the stability of the NGs. For this, the NGs hydrodynamic diameter was determined as a function of time. This study revealed that the complexes were colloidally stable for over 2 weeks. Further loading with other therapeutics in situ during crosslinking was also possible. In this case, the additional molecules can interact with the nanogels via electrostatic or host-guest interactions. An advantage of using fluorescent labeling in the proteins for quantification (FITC or rhodamine), is that they will also allow to monitor intracellular delivery by fluorescence microscopy.[80]

As an alternative to colorimetric methods, encapsulation efficiency can also be determined via electrophoresis. This was demonstrated by Yuki and colleagues, who used such methods to determine protein contents directly or indirectly.[138] They produced cationic CHP (cCHP) NGs by self-assembly and subsequently loaded them by incubation with pneumococcal surface protein A (PspA) fusion antigens. Native PAGE and densitometric analysis were useful to determine the amount of unencapsulated PspA. However, no free PspA was detected, thus suggesting complete encapsulation of the protein at the concentrations tested. Due to the cationic character of the cCHP NGs, native polyacrylamide gel electrophoresis (PAGE) analysis was not useful to determine the protein that was encapsulated directly inside the loaded NGs, but this issue was overcome by inversing the electrodes of the PAGE system. They also studied whether the proteins were simply adsorbed to the NG or encapsulated within. For this, the samples were treated with heparin, methyl-β-CD, or both. Heparin breaks the cCHP-PspA interactions without disturbing the integrity of the NG. Thus, heparin-treated NGs revealed a low quantity of protein in the PAGE analysis suggesting low amounts of surface-bound proteins. However, treatment with methyl-β-CD, which breaks the NG structure, revealed a much higher amount of protein, suggesting that most of the protein was efficiently encapsulated inside the NG. The amount of encapsulated protein could also be estimated by lithium dodecyl sulfate (LDS)-PAGE gel, since LDS is also able to break the physically crosslinked NG structure. The biologic activity of PspA could be measured by ELISA in the loaded NGs. These tests showed that the activity of the released PspA was similar to the native free PspA, thus suggesting the biologic activity was not irreversibly changed in the NGs and could be measured directly. The secondary structure of the PspA was studied by CD and changed upon encapsulation, being restored after release from the NG and suggesting a chaperone-like effect of the NGs in protein protection.

3.5. Protein Stability

As mentioned in the introduction, NGs can stabilize proteins through their immobilization inside the polymer network. The ability of NGs to protect the enzyme against different environmental factors can be evaluated from a functional or from a structural point of view.

To determine protein function, specific assays are available for each type of protein, e.g., immunoassays, cell studies, and reactions of enzymes with the substrate.[39,139] Depending on the application, the assays can be carried out directly inside the NGs or require a previous step for protein detachment.[46]

Protein structure can be determined by different techniques, depending on the level of complexity. Information on the primary structure can be obtained through HPLC and size-exclusion chromatography (SEC). These techniques allow to observe relevant (covalent) changes in the molecule, such as oxidation, deamidation, and formation of irreversible aggregates.[8] To check the secondary structure of the protein, Fourier-transformed infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), fluorescence spectroscopy, and circular dichroism (CD) are other common analytic tools.[140,141]

One of the main causes of protein instability is protein aggregation, which leads to decreased activity and in vivo immunogenicity. Encapsulation of proteins in the networks of NGs

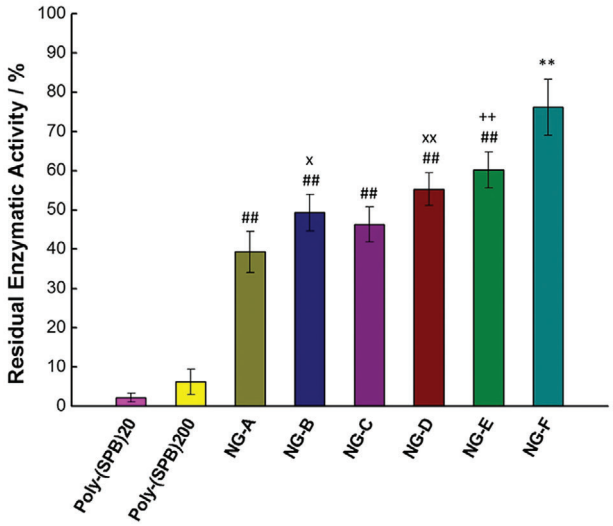

Figure 18. Enzymatic activity after thermal treatment following incubation of lysozyme with the linear polymers poly-(SPB)20 and poly-(SPB)200, and with all the NG formulations. NGs provided outstanding protection against protein aggregation and denaturation. Reproduced under the terms of the Creative Commons CC BY 4.0 license.[142] Copyright 2017, The Authors, published by Nature Research.

is proposed to inhibit such aggregation effects. This was examined by Rajan and co-workers, who used zwitterionic core-shell NGs (based on poly-sulfobetaine (SPB) and butyl methacrylate (BuMA)) to inhibit lysozyme aggregation upon thermal treatment.[142] Both the activity and structure of lysozyme were evaluated directly in the NGs. For this, lysozyme activity was determined by degradation of bacteria, and results showed very high degradation efficiency (**Figure 18**). Consequently, the NGs were able to protect lysozyme from intermolecular interactions between hydrophobic domains, which are the main cause for protein fouling and denaturation. The crosslinked network of NGs compared to the linear polymer analogs resulted in a much greater shielding from intermolecular interactions. This protection effect increased with the SPB content and BuMA content. The authors also evaluated the protein structure by circular dichroism (CD) and NMR. In CD, a remarkable decrease in the intensity of the bands was observed for the free enzyme after thermal treatment. While this indicates denaturation, the decrease was much lower in the encapsulated enzyme. Similarly, ^{1}H-NMR studies on the free enzyme showed a clear disappearance of signals corresponding to amino acids after thermal treatment, which is a clear indication of random-coil conformation of aggregated or denatured proteins. In the case of encapsulated enzymes, the original chemical shifts were better maintained.

4. Conclusions and Outlook

Polymeric nanogels are attractive candidates for overcoming the limitations associated with protein instability. Their ability to efficiently load and stabilize (therapeutic) protein cargoes enhances the bioavailability and enables targeted delivery. In this review, we have highlighted the key processes involved in the development of NGs for protein delivery. We focus on loading strategies, loading evaluation techniques, factors affecting loading efficiency and release kinetics, and strategies for optimizing protein-carrier interactions.

It is important to consider that the synthetic procedure used for NG preparation will influence protein loading capacity, protein distribution, and protein stability inside the carrier. NGs crosslinked by physical methods avoid harsh colloidal synthesis processes, thus allowing for in situ protein loading during NG synthesis. However, these colloidal systems tend to show more susceptibility to environmental conditions due to the weak and reversible nature of the physical crosslinks. Conversely, chemical crosslinking of NGs leads to more stable structures with enhanced stability in different environments. In this method, concurrent loading with the protein can only be performed under mild synthetic polymerization/crosslinking conditions (namely, the absence of organic solvents and low temperatures to avoid protein denaturation). Additionally, chemically crosslinked NGs must be provided with strategies that will ensure in vivo degradation– either via the incorporation of biodegradable polymers or inclusion of cleavable groups.

To finely adjust the encapsulation of the cargo within the polymeric framework, the loading process itself needs to be carefully chosen. Besides loading the cargo during the encapsulation process, diffusion-based strategies, such as incubation of a protein solution with swollen or dry NGs, and titration of a protein solution, can facilitate loading into preformed NGs. The efficiency of the loading process is highly dependent on the choice of polymer, crosslinking density, and swelling state of the NG network. Monitoring the loading process with techniques like DLS or electrophoresis can optimize loading contents and provide information on cargo location inside the NGs. Striking the right balance between protein diffusion into the NG and prevention of premature release is essential. To achieve this, functional groups in the network-forming polymer can be used to enhance affinity to the protein (via electrostatic interactions, hydrophobic interactions, or supramolecular/host-guest interactions, i.e., affinity binders). If the protein is entrapped tightly inside the NG (by a reduced mesh size, strong interactions, or covalent bonding), release can be prompted by changes in the swelling state or degradation of cleavable groups in the NGs.

Despite significant progress in the field of NG-based protein delivery, several opportunities remain: Future research should prioritize the improvement of cargo loading strategies, encompassing the optimization of encapsulation efficiency while maintaining controlled release. So far, this has been achieved and clinically translated in covalently-attached proteins for intracellular delivery, but purely physical entrapment approaches still need optimization to reach similar potential. Gaining insight into protein-compatible reactions is crucial for achieving this objective. Considering different types of proteins and their specific characteristics, and exploring advanced characterization techniques, including imaging, spectroscopy, and diffusion studies, can provide valuable insights into the mechanisms of protein encapsulation and release from NGs. This deeper understanding will contribute to the refinement of these systems. For in vivo applications, effort should be put into tailoring the polymer properties and polymer-protein interactions to overcome the physiological barriers such as mucus and skin. Furthermore, to facilitate clinical translation, it is crucial to address the scale-up of NG

synthesis and protein loading processes, ensuring reproducibility and batch-to-batch consistency.

In conclusion, the field of polymeric nanogels for protein delivery is rapidly advancing, thus offering exciting opportunities to improve the stability, bioavailability, and targeted delivery of protein therapeutics. By understanding the critical factors that influence NG performance, we should aim to continuously adapt our cargo entrapment strategies. This can pave the way for efficient and tailored protein delivery systems that address the challenges associated with protein-based therapies.

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Conflict of Interest

The authors declare no conflict of interest.

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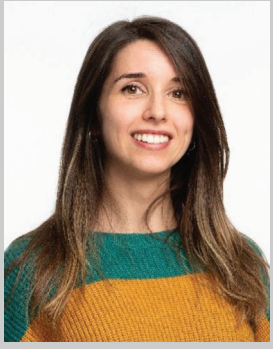

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