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Review

Therapeutic Delivery

Protein-based nanomaterials: a new tool for targeted drug delivery

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Protein nanomaterials are well-defined, hollow protein nanoparticles comprised of virus capsids, virus-like particles, ferritin, heat shock proteins, chaperonins and many more. Protein-based nanomaterials are formed by the self-assembly of protein subunits and have numerous desired properties as drug-delivery vehicles, including being optimally sized for endocytosis, nontoxic, biocompatible, biodegradable and functionalized at three separate interfaces (external, internal and intersubunit). As a result, protein nanomaterials have been intensively investigated as functional entities in bionanotechnology, including drug delivery, nanoreactors and templates for organic and inorganic nanomaterials. Several variables influence efficient administration, particularly active targeting, cellular uptake, the kinetics of the release and systemic elimination. This review examines the wide range of medicines, loading/release processes, targeted therapies and treatment effectiveness.

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Nanotechnology has resulted in massive breakthroughs in diverse areas of research and technology and has assisted in the discovery of new therapies. However, the influence of nanoscience and significant research on biomedicine, particularly in the treatment and diagnosis of various diseases, is foreseeable. Drug-delivery systems manage the quantity and rate of drug administration and liberation and the solubility, bioavailability and biodistribution of the respective drug cargo. Recently, the great potential of nanoparticles (NPs) in the COVID-19 pandemic has been reported and shown significant impact [1–4]. Nanomaterials are specially engineered to target cells, organs or tissues of the human body and therefore only release the cargo, which can be a pharmaceutical, gene or diagnostic reporter molecule, once they reach their destination target [5,6]. When NPs are used as drug delivery systems, they may have several drawbacks. Once the drug is coupled to the NPs, it is conceivable that it will deactivate the therapeutics. Conjugating the drug with the NPs entails considerable consideration, as the coupling between the drug and the NPs would have to be able to prevent premature release [7,8]. However, bindings must degrade predictably according to the desired release rate once the cargo has reached the appropriate target without altering the drug activity under cellular and environmental conditions, such as in a cancer cell [9–12]. The proportion of drugs coupled to NPs can be very modest; however, excessive concentrations of NPs delivered *in vivo* might cause symptoms, including high blood pressure and renal failure. Hence, NPs must be highly effective as drug transporters. Since macrophages and other phagocytic cells engulf aggregated NPs, they are immediately removed from the circulation, preventing them from reaching target cells [13–15].

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Researchers from various disciplines are now investigating different dimensions of these essential biomolecule building blocks, including their assembly, cargo loading, interactions between the generated nanomaterials and the body and their potential applications in diagnostics and therapies involved in nanobiotechnology, pharmacy, toxicology and immunology. Nanocarriers resulting from proteins and peptides can control the release of their cargo to the targeted cells or tissues selectively [16–19]. Furthermore, the possibility of proteins transmitting the disease to humans is rare; only prions can behave like infectious agents to transmit disease [20], and for this reason, the use of protein nanomaterials to produce naturally occurring nanomaterials for therapeutic delivery has been considered by experts over the past two decades [21].

Naturally occurring NPs (proteins, aptamers & peptides)

Protein-based nanomaterials have various properties that make them potentially fascinating nanomaterials for controlling drug cargo release, and they are safe with no risk of transmitting the disease to humans [22–24]. Proteins are abundant, renewable and inexpensive resources. Furthermore, some protein-based macromolecules, such as viruses, can self-assemble to create hollow nanoarchitectures with well-defined geometries and highly organized capsids, polyvalency and amenability to genetic and chemical engineering [25]. Loading the internal cavities of protein viruses will lead to higher payload capacity than other nanomaterials. Therefore, a lower dose of therapeutic-loaded NPs is required to deliver an effective drug dose. As a result, they reduce the overall toxicity of the drug [26]. Viruses and protein nanomaterials have shown great advent as valuable naturally occurring nanomaterials in which the empty internal cavity has been used as a reservoir to carry and deliver pharmaceuticals, diagnostics and imaging agents. The term 'cage' implies that NPs can be unlocked in response to environmental stimuli in some cases but not always, taking advantage of distinct chemical and physical differences at the target site that alter their molecular structure, leading to the target release of therapeutics [27,28]. Proteins offer a smart drug carrier with precise engineering to selectively target diseased cells with high precision and the ability to release pharmaceutical cargos to their desired targeted destinations. Such smart nanocarriers naturally operate in a stimulus-responsive microenvironment as a response to an internal cellular or external stimulus that ultimately alters the therapeutic cargo release, enhancing the efficiency and safety of the administration of highly toxic therapeutic agents [23,29].

Proteins & nanomedicine

The organic composition and the nature of protein-based nanomaterials have the natural affinity to interact with living cells. Another significant advantage of such NPs is that they are often not recognized as foreign and are ultimately rapidly eliminated by macrophages. A distinguishing aspect of protein nanomaterial architectures is inherent size uniformity and the propensity to exhibit a very homogenous size distribution. Protein-based nanomaterials can be biologically or chemically altered or manipulated using various methods (genetic or chemical), making them particularly suitable for drug delivery [24,30–32]. To optimize the efficacy of nanomedicines, considerable efforts are being made to modulate the interaction of drug formulations with a range of proteins, notably immune system proteins. Numerous peptide sequences and proteins, including cell-penetrating peptides, phage peptides and antibodies, have also been considered to direct drug-containing NPs to the target tissue, tissues or cells, such as tumor sites [33]. Furthermore, proteins are crucial attributes in ailment diagnostics and are exploited in the development of biosensors to diagnose other diseases [34]. As pharmaceutical carriers, protein NPs are innovative drug-delivery technologies and systems.

Types of uncommon protein particles

The term 'uncommon' in this contest refers to the types of protein nanomaterials that are not frequently studied in the literature.

Collagen NPs

Protein polymers are naturally biodegradable and biocompatible macromolecules that are easily obtained from animals and plants. They are used as a renewable resource to prepare biocompatible NPs [35]. Collagen is an essential fibrous protein representing the most available biopolymer in the human body. Collagen is a flexible and robust molecule because of the long triple-helical parts in its structure. These helical parts are represented by the repeated sequence of amino acids: glycine-X-Y. X and Y could be lysine, leucine, proline or hydroxyproline. Collagens with this helical structure are known as tropocollagens; the binding of tropocollagens is responsible for

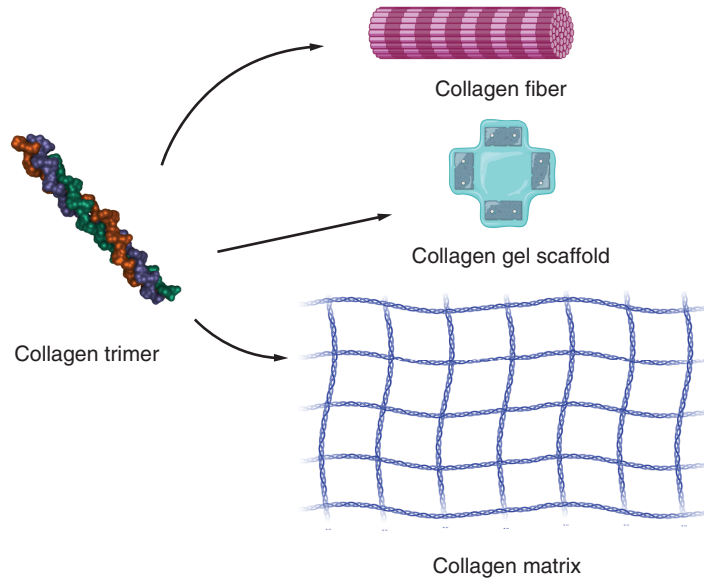

Figure 1. The collagen scaffold and the collagen fiber that hold great potential in nanotechnology.

the formation of a fibril structure. In tissue engineering, the crosslinking of these fibril structures is used to prepare appropriate cell scaffolds [35,36].

Collagen can induce the regeneration and remodeling of bone by inducing the differentiation of stromal cells located in the bone marrow. It is used primarily in the biomedical field because of its ability to act as osteoid in the mineralization process. In addition, it is used in skin grafting, cartilage and bone repair and wound healing, such as in the treatment of diabetic foot ulcers, as shown in Figure 1 [37–39]. Collagen is widely used clinically due to its abundance, low antigenicity, high biocompatibility and biodegradability. Furthermore, collagen has a high capacity to form fibrils with high tensile strength. Therefore, it is prepared in various forms such as coating material, sponges, sheets and membranes, hydrogels, beads, nanofibers and NPs [40–43]. Collagen can be prepared by different methods such as electrospinning, nanoemulsion, electrospray deposition and milling [44].

Collagen can resemble the microenvironment of tumor cells, and thus collagen-based NPs can infiltrate these spaces and release an antitumor agent. Furthermore, collagen-based NPs are considered good candidates to prepare controlled-release systems because their properties, such as size, surface are, and absorption capacity, can be configured easily [35,45,46]. In a recent study, collagen-poly (3-acrylamidophenylboronic acid) NPs encapsulating doxorubicin were prepared, and their effect on the treatment of ovarian cancer was studied. The transmission electron microscopy results showed that the NPs were spherical with uniform distribution of 75 nm in diameter. The encapsulation efficiency was high, and the *in vitro* drug release studies showed a sustained-release profile. *In vitro* cytotoxicity studies were performed on A2780 cells using the MTT test. In addition, the tumor model was conducted *in vivo* to evaluate the antitumor effect on BALB/c mice. The results showed that the blank collagen NPs did not have cytotoxicity in A2780 cells. It was found that tumor growth was low in doxorubicin-loaded collagen-based NPs than in free doxorubicin [47].

However, collagen is still suffering from a high rate of degradability and low mechanical strength. Therefore, in a recent study, bioactive glasses were added as a second phase to collagen. Bioglass nanofibers associated with collagen have reduced infection rates and induced skin renewal. Thus, the appropriate use of collagen with bioactive glasses forms a biomedical device that mimics bone composition [47].

Silk fibroin

Silk proteins are available in the glands of many members of the arthropod family, such as spiders, scorpions, mites, silkworms and bees. However, silk obtained from silkworms is the most used silk in textiles and biomaterials [48]. Silk fibroin is an abundant, cheap, natural protein-polymer mainly used to prepare biomaterials. It comprises 18 different amino acids; Gly is most available and accounts for 43% of these amino acids; then Ala accounts for 29%; and Ser accounts for 12%. Most of these proteins are generally obtained from allogeneic and xenogeneic tissues, thus requiring a high risk of infection. In addition, there is the high cost associated with their processing, purification and isolation. Silk fibroin is extracted from silk prepared by the *Bombyx mori* silkworm. This crystalline

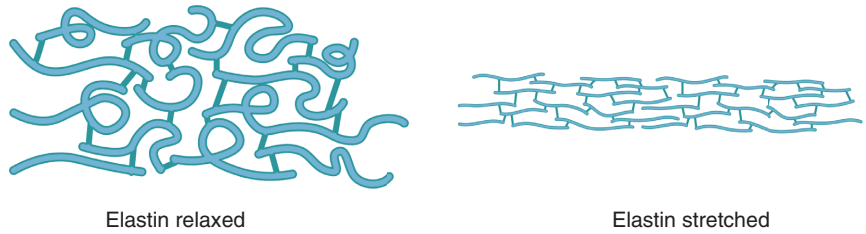

Figure 2. Elastin in both relaxed and stretched formats because of environmental stimuli.

structure could be modified to enhance the encapsulation capacity of many drugs while maintaining their activity. NPs prepared using silk fibroin were influential in delivering drug molecules with different molecular weights and degrees of hydrophilicity. Silk fibroin NPs have shown their potential to control the release rate of drugs in a sustained manner while preserving their stability. This protein was also successfully combined with various biopolymers such as albumin, insulin and synthetic polymers [35,48,49].

Three different methods are used to prepare silk fibroin-based NPs. The first one is direct mixing, in which NPs are mixed with silk fibroin solution; then a nanofiber, a film, a scaffold or a hydrogel is prepared from the mixture. Here, physical interaction is created between the silk fibroin macromolecules and the NPs. The second method is *in situ* synthesis, where silk fibroin is added to the NPs; they act as a template for the *in situ* growth and nucleation of the NPs. The third method is the silkworm feeding method, in which particles are obtained by directly feeding silkworms with diets containing metal NPs (silver, titanium dioxide, iron oxide, copper, graphene and carbon nanotubes) [50,51]. However, *in situ* synthesis is the simple, one-step fabrication of the nanopolymer that could utilize different reducing agents to generate the desired NPs. This technology enables the creation of nanocomposites *within situ*-produced NPs from appropriate precursors in a single step. The following requirements must be met: the solvent and antisolvent must be miscible under process circumstances and the solute should be insoluble in the solvent/antisolvent combination. As a result, when the polymer solution is mixed, the antisolvent will capture the molecules that solvate it, causing them to aggregate, whereas the feeding of fibroin cocoons with the desired functionalized doping moieties will avoid the need for external chemical processing and the further use of toxic chemical solvents [52].

Furthermore, because of its nonantigenic and nontoxic character, this biopolymer has delivered many antitumor drugs such as doxorubicin, paclitaxel, methotrexate, floxuridine and curcumin. The results are promising compared with traditional preparations. Furthermore, silk fibroin has recently been used in injectable and implantable drug-delivery systems [53–55]. Polymer-based natural dressings have been extensively used to treat skin injuries due to their biocompatibility, biodegradability, safety and nonallergic nature. Therefore, silk fibroin-based wound dressings deliver active ingredients, bioactive molecules and growth factors to the affected area. In addition, they provide the proper support for perfect healing [56–58].

Elastin

Elastin is a component of the extracellular matrix present in many connective tissues and offers particular physiological elasticity. Keratinocytes and fibroblasts are responsible for systemizing this protein, supporting the skin, lungs and blood vessels [59,60]. The construction of biomaterials containing elastin with its biological and mechanical merits was shown to enhance the hemocompatibility of the biomaterial [61]. Elastin defects were detected, and genetic cardiovascular disorders may be acquired. These defects are presented as changes in the mechanical properties of the arteries [62]. Elastin is a highly hydrophobic biomaterial with extensive crosslinking that assembles into elastic fibers, as shown in Figure 2, whereas its precursor, tropo-elastin, is water soluble [62,63]. Elastin is rich in residues of glycine, alanine, proline, valine and leucine. Its structure is presented as short, repeated sequences of three to nine amino acids, resulting in a highly dynamic structure [59,63]. Urry and coworkers have shown that repetition of the natural sequence present in natural elastin leads to the formation of self-assembled structures known as elastin-like polypeptides, which have properties similar to those of natural elastin [64].

Elastin-like polypeptides are biocompatible and have low critical solution temperatures, making them appropriate materials for stimulus-responsive applications. Their size and sequence can be precisely determined because of their recombinant synthesis and genetically encoded structure, whereas these properties are missing in synthetic polymers.

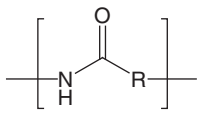

Figure 3. Molecular structure of zein with the molecular formula $C_7H_7FO_2S$, a molecular weight of 25–40 kDa and the IUPAC name phenylmethanesulfonyl fluoride.

Therefore, elastin polypeptides were primarily used in drug delivery on many platforms because of these features. Elastin-like polypeptides were used to deliver radionuclides, biological agents and small-drug molecules to treat many diseases such as cancer, osteoarthritis, neuroinflammatory disorders and Type 2 diabetes [65–67]. NPs based on elastin-like polypeptides were prepared to encapsulate hydrophobic drugs, and it has been shown that most of these NPs had a micellar structure and rarely self-assemble into vesicular structures. Therefore, elastin-like polypeptide-based NPs can be used as promising drug-delivery systems in nanomedicine [67]. These polypeptides are highly versatile, biocompatible and stimuli-responsive and they can self-assemble. Therefore, they have gained an increasing interest in biomaterials in tissue engineering, cell and tissue culture, protein purification and controlled drug-delivery systems [68–71].

Zein

Zein is a natural, water-insoluble, storage protein derived from maize with a molecular structure as shown in Figure 3. Zein was isolated for the first time from the entire white maize and was named by John Gorham in 1821, but zein was not commercially available until 1938 [72]. From a physicochemical point of view, this prolamine-rich protein is among the few water-insoluble natural plant proteins [73]. The distinctive solubility characteristic of zein is directly ascribed to its composition in amino acids. Polypeptides have gained an increasing interest in biomaterials in tissue engineering, cell and tissue culture, protein purification, and controlled drug-delivery systems [74]. Furthermore, zein differs from other proteins due to its lack of lysine and tryptophan, as well as its limited arginine and histidine content [75].

In the past, zein was considered a low-valued material, since zein is water insoluble and deficient in basic and acidic amino acids. Also, it mostly lacks tryptophan and lysine; thus, it is not considered good in nutritional quality [76]. Zein comprises a biocompatible polymer with good biodegradability and biocompatibility characteristics. Thus, it can be used to manufacture many materials such as textile clothing fibers, food packaging and coatings [77]. In 1985, the US FDA approved zein as a generally recognized as safe polymer for film coating of oral pharmaceuticals [72]. Therefore, zein today is used as a film and coating material in the food and pharmaceutical areas, particularly in drug-delivery studies [73,74].

Zein protein has many properties, including high hydrophobicity and thermal resistance properties. These distinctive characteristics make zein an attractive matrix for encapsulation/association with hydrophobic, temperature-sensitive and oxidative-sensitive compounds [78]. One of the advantages of high zein hydrophobicity is that it can be easily transformed into NPs (100 and 400 nm, with a higher payload of therapeutics of various charges) by changing the solubilizing capacity of the primary solvent by the addition of a nonsolvent, using the desolvation/coacervation technique of zein 'precipitate' forming stable NPs. However, zein freeze-dried NPs tend to aggregate/agglomerate because of their poor physical stability and dispersibility. For instance, specific hydrophilic or amphiphilic materials, such as sodium caseinate, are used to stabilize zein NPs [79]. Zein NPs have numerous advantages. For example, they can protect therapeutic drugs from digestive enzymes, which are relatively resistant. This agrees with the observation that zein NPs have a long residence time in the intestine after 24–48 h [80].

Based on the unique and important properties of zein NPs, many compounds have been loaded into them that have a huge potential in drug delivery [81]. In one work, Inchaurraga *et al.* developed zein NPs to enhance oral absorption of insulin. In this study, the results were interesting, as the *in vivo* pharmacological availability and relative oral bioavailability of diabetic rats (male Wistar rats) were significantly improved by 13.5 and 5.2%, respectively [82].

In a study by Zhang *et al.*, the research team designed a potential methodology for the topical treatment of skin fungal infections using ketoconazole-loaded lecithins–zein NPs. From this study, lecithin–zein NPs loaded with ketoconazole were developed to enhance the therapeutic effects of ketoconazole; an *in vitro* penetration test showed a higher drug concentration in the stratum corneum (2.98-fold) and deeper skin layers (1.51-fold) compared with free ketoconazole. The increased penetration capability of zein NPs is associated with improved drug retention in different layers of skin, which is the potential for sustained drug release. Furthermore, a reduction in systemic toxicity was also observed [83]. In addition, Lima *et al.* conducted a study to evaluate the antimicrobial effect of zein

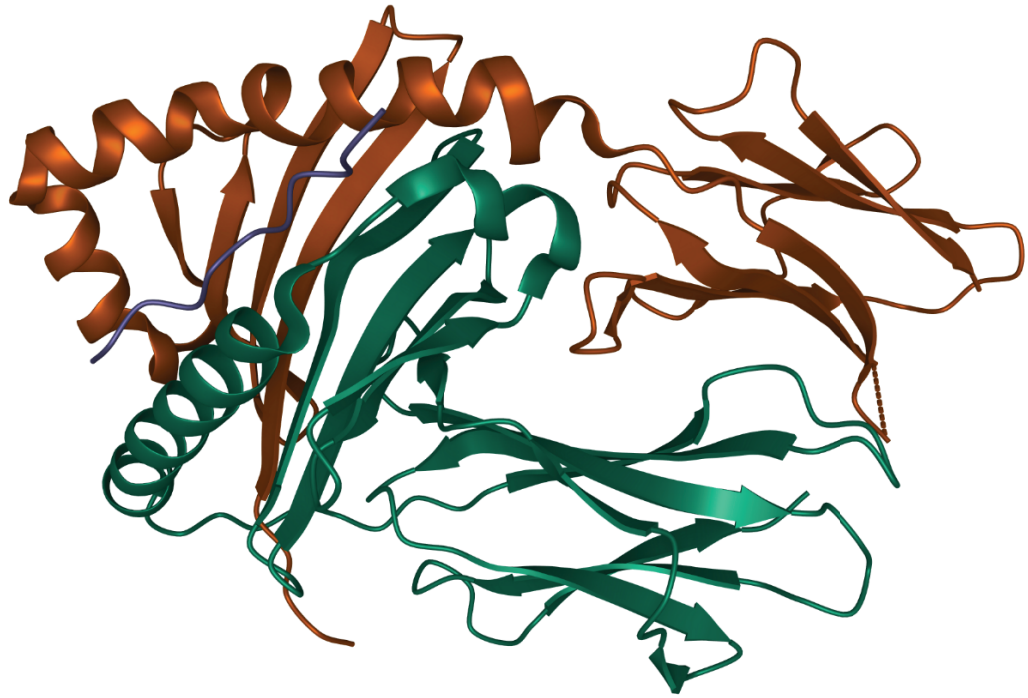

Figure 4. The crystal structure of gliadin with the Protein Data Bank entry 1S9V.

NPs against biofilm formation. This study loaded the zein NPs with anacardic acid and tested them against the *in vitro Streptococcus mutans* biofilm model [84]. In another study, Yu *et al.* evaluated the use of zein NPs loaded with maytansine in tumor cell targeting. The maytansine zein NPs showed a better tumor inhibition rate for the A549 (human lung cancer) cells, 97.3% compared with 92.7% when using maytansine alone [85].

Gliadin

The main storage proteins in bread wheat are the two proteins gliadin and glutenin, which make up gluten [86]. Jacopo Beccari, in 1728, isolated sticky paste from wheat grain flour dough and called it gluten. After 90 years, two gluten fractions were identified as gliadins (soluble in alcohol) and zymon (insoluble in alcohol). Subsequently, the insoluble alcohol fraction was given a new name, glutenin [87]. Therefore, based on the classification previously discussed gliadin is considered a typical prolamin.

Four classes of gliadins are known, based on electrophoretic mobility in SDS-PAGE at low pH: α-gliadin, β-gliadin, γ-gliadin and ω-gliadin. Specifically, α-gliadin is the fraction with the fastest mobility, ω-gliadin is the fraction with the lowest mobility and both β-gliadin and γ-gliadin have intermediate mobility. Meanwhile, α-gliadin and β-gliadin are gathered in the same fraction based on their structural homology, as shown in Figure 4 [88–90].

From the structural point of view, ω-gliadin is named sulfur-poor gliadin, while the other three types are called sulfur-rich gliadins. In sulfur-rich gliadin, the amino acid sequences are divided into two main domains: the N-terminal (consisting of repetitive amino acid sequences) and the C-terminal (consisting of nonrepetitive amino acid sequences). Sulfur-poor gliadins have a lon, repetitive domain with absent cysteines [91]. Gliadin is a versatile biomaterial with extraordinary properties; it is safe, nontoxic, biocompatible, metabolizable and biodegradable. Thus, gliadin is suitable for preparing NPs for the delivery of hydrophobic and amphiphilic drugs [32,33].

Gliadin NPs are prepared mainly by the liquid antisolvent precipitation method [92]. In an investigation of NPs prepared by this method, NPs in the size range of 450–475 nm were the best for drug delivery, and the loaded amount of drug increased with increasing drug hydrophobicity [93]. Gliadin has good interaction with the biological membrane, and it is rich in neutral and hydrophobic amino acids. Therefore, gliadin has mucoadhesive properties, making it suitable for oral delivery of lipophilic drugs [94].

Gliadin NPs ensure an efficient drug-delivery approach to targeting the upper gastrointestinal tract. One of the potential applications of gliadin NPs is their use in treating *Helicobacter pylori*, as it has a good affinity for the upper

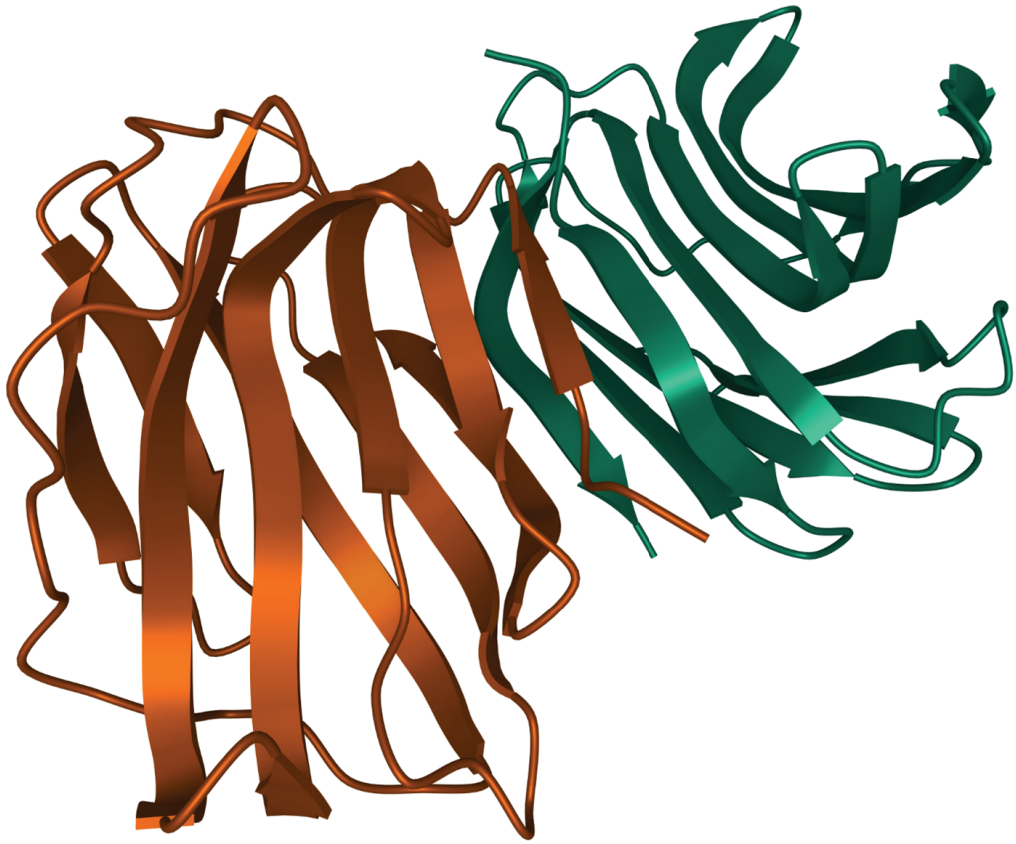

Figure 5. The x-ray crystal structure of lectins with the Protein Data Bank entry 1W6M.

gastrointestinal tract. In one work by Umamaheshwari *et al.*, gliadin NPs loaded with amoxicillin were developed. *In vivo* investigations showed that amoxicillin-loaded gliadin NPs were superior to free amoxicillin in the eradication of *Helicobacter pylori* [95]. The potential of gliadin NPs to deliver anticancer drugs, mainly cyclophosphamide, for breast cancer treatment was investigated by Gulfam *et al.* Gliadin NPs loaded with cyclophosphamide showed superior drug release by being gradually released for 48 h compared with gliadin–gelatin-loaded NPs, which showed rapid drug release [96].

Lectin

The history of lectin goes back to 133 years ago, when Stillmark described a very toxic protein isolated from the seeds of the castor bean (*Ricinus communis* L.), and he reported it as ricin. This ricin was the first lectin reported with hemagglutination activity. 10 years later, Elfstrand used the term 'agglutinin' to indicate all proteins with agglutination activity of red blood cells (hemagglutination) [97]. Lectins are carbohydrate-binding proteins present in all kingdoms of life, with the basic structure shown in Figure 5. This protein family has the exceptional ability to recognize and bind reversibly to specific carbohydrate structures in cells. Lectin's carbohydrate specificity is of great importance. However, plant lectins gain greater interest, as more than 500 lectins have been isolated and studied; many reflect potential activity against fungi, viruses, cancer and more [98–100].

Plant lectins are classified by molecular structure and the three-dimensional folds into merolectin, hololectin, chimerolectin and superlens. Furthermore, lectins are classified according to their various structural feature – for example, galectins of types S, C, M, L, P, I, R and F [97,101]. Significant work by Carneiro *et al.* was done to review patent publications (1988–2020) of lectins with antifungal, antibacterial and antiviral activity retrieved from Espacenet. A total of 46 patents were reviewed. The study indicated that mannose-binding lectins were the best antiviral agents proposed, since glycans with mannose residues are frequently associated with viral entry into the host cell. Also, they have a role in trapping viral particles and preventing their spread and replication. The

study showed various patented lectins based on viral inhibition *in vitro*. In addition, this review indicated lectin's antibacterial and antifungal activities. For example, *Portunus trituberculatus* mannose-binding lectin is active against *Vibrio alginolyticus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, with an MIC value of 0.8–1.6 μmol^{-1}, and *Bacillus subtilis* extracellular lectin is active against *Alternaria* sp., *Botrytis cinerea* and *Rhizoctonia solani* with an IC_{50} of ~0.1 $\mu g\ ml^{-1}$, 2.7 $\mu g\ ml^{-1}$, 4.0 μmol^{-1}, respectively [102].

Auth *et al.* evaluated the potential of lectin in the inhibition of SARS-CoV-2 infection. In this study, *Triticum vulgaris* lectin, named wheat germ agglutinin (WGA), was evaluated for its activity against SARS-CoV-2, where African green monkey kidney cells (Vero B4 cells) were inoculated with the patient isolate SARS-CoV-2PR-1 (Wuhan type). The inoculated cells were treated with different concentrations of WGA. The results indicated that WGA has activity against SARS-CoV-2 with IC_{50} < 10 ng/ml in Vero B4 cells [103]. In one work by Estrada-Martinez *et al.* [104] the role of lectins in cancers of the digestive system was evaluated against various digestive system cancers such as esophageal, small intestine, colorectal and pancreatic cancers. The results showed that plant lectins and digestive cancer cell lines seemed to cause apoptosis in a way that depended on both dose and time. Furthermore, the *in vivo* assays showed tumor growth inhibition and complete remission in a few cancer cases [104]. Yasin *et al.* isolated lectin from *Lepidium sativum* seeds, purified and synthesized lectin-loaded chitosan–sodium tripolyphosphate NPs and assessed their anticancer potential against hepatocellular carcinoma cell lines (HepG2). The findings indicated that synthesized lectin-loaded sodium tripolyphosphate chitosan NPs have better activity, with IC_{50} values of 105 μg/ml compared with 265 μg/ml for the lectin protein. Thus lectin-loaded chitosan–sodium tripolyphosphate NPs are potential anticancer agents [105].

In one work, the role of lectin-modified solid lipid NPs was investigated for a potential oral administration of insulin. Insulin-loaded, lectin-modified, solid lipid NPs (SLNs) and WGA-modified, lectin-modified SLNs were prepared. In the *in vitro* experiments, both insulin-loaded SLNs and WGA-modified SLNs could protect insulin from digestive enzymes. In contrast, *in vivo* experiments resulted in relative bioabilability of 4.46 and 6.08%, respectively, and 4.99 and 7.11%, respectively, after oral administration. Compared with subcutaneous administration of insulin, the lectin-modified SLNs are the proposed mode of insulin administration [106].

Casein

Casein is the primary protein of milk. Casein comprises approximately 94% protein with 6% low-molecular-weight molecules called colloidal phosphate of calcium. Phosphoryl peptides weigh between 19 and 25 kDa and have an isoelectric pH of 4.6–4.8. Casein and calcium phosphate proteins produce enormous colloidal particles called casein micelles, which have long been of interest. The hydrophobic inner surface of these spherical micelles is covered by a layer of hydrophilic casein kappa that stabilizes the micelles, producing electrostatic and spatial discharge between the molecules. Thus, dual proteins produce copolymers of blocks with strong tendencies to self-regulate micelles between 50 and 500 nm with an average hydrodynamic radius of 250 nm [4]. In reality, casein micelles in milk are natural nanocarriers, which carry amino acids and calcium phosphates from mother to child [107]. These micelles are highly stable and maintain their structural integrity when various dairy products are prepared through various procedures. Casein/copolymer micelles have recently been used to transport hydrophobic charges using other polymers to effectively block saturated fatty acids saturated with vitamin D, omega-3 and beta-carotene (a precursor to vitamin A) [108].

Gelatin

Gelatin is a naturally flexible protein biomaterial obtained from collagen hydrolysis exhibiting unique degradability, bioavailability, low immunogenicity and a multitude of different functional, modifiable chemical side chains that can be exploited as anchoring points for therapeutic attachments [109,110]. The gelatin generated (type A or type B) is determined by the collagen hydrolysis process (i.e., acidic and basic hydrolysis, respectively). Every gelatin has a different drug-release capability when used to manufacture different kinds of NPs. Each gelatin has a specific drug-delivery capability for different types of release profiles. The drug-delivery potential of type B gelatin NPs has been demonstrated to be superior to that of type A gelatin NPs [111]. Moreover, it has been reported that gelatin type B actively adheres to the DNA molecules, enhancing the carriers' transfection efficacy. Gelatin is a commercially heterogeneous mixture of polypeptide chains having different molecular weight range from thousands to millions of Daltons [112]. Different possible mechanisms for medication release from gelatin NPs are release induced by polymer degradation (hydrolysis); self-diffusion through available surface pores; drug release driven by polymer surface erosion; and pulse delivery triggered by the deployment of oscillating magnetic or ultrasonic

stimulation [113]. The most common type of drug release is biphasic release, which has two stages. The first stage is the rapid release of drugs that are adsorbed (have weak interactions) on the surface of gelatin nanoparticles (NPs). The second stage is the slow diffusion of drugs from the matrices that are covalently coupled, which means they have strong bonds with each other. This gives the drugs more time to get out of the matrices [114,115]. Both the size and loading efficiency of the gelatin NPs, as well as solubilities, determine the effectiveness of drug release. Tiny particles have a strong burst impact, but larger particles provide a comprehensive, continuous and sustained release. In addition to size, increasing the density of gelatin crosslinking was shown to influence swelling ratios and drug dissolution patterns of the matrices [17,109]. Another element that influences the release of gelatin NP drugs is pH. Proteolytic enzymes also help gelatin NPs release drugs faster.

Lactoferrin

Lactoferrin, with a molecular weight of 77–80 kDa, is a naturally occurring cationic glycoprotein that has iron-binding capabilities. The primary purpose of this protein is to monitor and control the number of free irons in biological fluids by solubilizing or sequestering ferric ions (Fe^{3+}) [116]. This unique feature emphasizes its distinct properties, which also include anticancer, antibacterial, antioxidant, anti-inflammatory and immune-stimulating attributes. Due to the targeted delivery potential of its expression on the surface of different cells, lactoferrin has a wide range of potential uses for different hydrophobic therapies [117]. Lactoferrin mostly in circulation could also be used as a prognostic marker in different inflammatory responses, including severe acute respiratory syndrome or septicemia. Lactoferrin has two lobes, according to structural studies. Each lobe combines multiple domains separated by a cleft which may bind Fe^{3+} and CO_3^{2-} simultaneously. Substrate association or release is intimately connected to conformational changes such as opening and closing. Lactoferrin was shown to retain its iron-binding capabilities despite heating at temperatures ranging between 65° and 90°C with ionic strength of 0.01 or less. When the temperature was elevated, considerable precipitation of lactoferrin was observed, as well as a considerable decrease in its iron-binding activity. Lactoferrin could tolerate heating at an ionic strength of up to 0.37 at pH 3.5, yet aggregation was reported at an ionic strength of more than 0.47, suggesting that the thermostability of lactoferrin was highly reliant on both ionic strength and pH [118,119]. Lactoferrin is one of the very few proteins possessing a positive overall net charge at physiological conidiation and an isoelectric point of 8.0–8.5 [120]. Moreover, documented studies have revealed that lactoferrin remains relatively stable in the gastrointestinal tract, with numerous receptors that improve the oral absorption and bioavailability of NPs. Furthermore, overexpression of the lactoferrin receptors improves nutritional absorption and demand for these highly proliferating cancerous cells [121]. Furthermore, the pH-dependent release profile of lactoferrin-based nanocarriers was reported. The accelerated release of drugs is noted at acidic pH, which might promote drug release in acidic environments such as the tumor tissue microenvironment and hence boost the therapeutic effectiveness of the entrapped hydrophobic active compounds [122]. Lactoferrin NPs loaded with doxorubicin are prepared by the sol–oil approach [123] to utilize the natural affinity of the lactoferrin nanocarrier to the targeted cancerous cells. It has been reported that doxorubicin-loaded lactoferrin is stable for 3 months with only 2.5–5% drug loss, which means it does not damage the membranes of erythrocytes [123]. Additionally, oral administration of doxorubicin-loaded lactoferrin NPs resulted in no toxicity reported in terms of physical weight loss and liver and kidney function, deeming the safety and biocompatibility of such carriers [121]. In another report, lactoferrin was used to encapsulate zidovudine (an antiviral drug). The 50–60 nm-sized particles that are made have a drug encapsulation efficiency of 67% and are very stable at room temperature and 4°C without changing much in size. Surprisingly, drug release was negligible in both simulated stomach and intestinal fluids, revealing that lactoferrin NPs are stable under harsh circumstances. The anti-HIV-1 impact of zidovudine-loaded lactoferrin NPs was equivalent to that of free medication when administered orally. Furthermore, drug-loaded NPs had a better pharmacokinetic profile than free drugs, which was linked with decreased organ toxicity, suggesting that this nanoformulation is a safe nanoplatform for improving drug delivery [124].

In vitro experiments revealed that both 5-FU-loaded and oxaliplatin-loaded lactoferrin NPs exhibited improved antiproliferative activity in human colon cancer (COLO-205) cell lines when compared with their free drugs [117]. Furthermore, azoxymethane carcinogen was used to produce aberrant crypt foci in the colon of a Wistar rat's animal model through intraperitoneal injection of two doses of azoxymethane at a dosage of 10 mg/kg body weight in the same week [117]. The findings suggested that the nanoformulation outperformed free drugs in terms of antitumor efficacy and systemic toxicity. This increased impact might be ascribed to drug-loaded lactoferrin NPs' superior

Table 1. Examples of drugs that were successfully loaded into different types of proteins for improved drug-delivery applications.

Types of protein NPs	Drug loaded	Application	Ref.
Albumin NPs	Paclitaxel	Cancer treatment	[128–131]
Zein NPs	Insulin	Diabetes	[82]
Collagen-based NPs	Doxorubicin	Cancer treatment	[47]
Lecithin–zein NPs	Ketoconazole	Fungal infection treatment	[83]
Gliadin NPs	Amoxicillin	*Helicobacter pylori*	[95]
Lectin-modified solid lipid NPs	Insulin	Diabetes	[106]
Ferritin NPs	Doxorubicin	Cancer treatment	[5]
Vault protein	New York esophageal squamous cell carcinoma tumor-specific antigens	Tumor vaccine	[132]

NP: Nanoparticle.

pharmacokinetic profiles and better tissue biodistribution when compared with free medicines, as well as their greater cellular absorption due to lactoferrin's active targeting characteristic.

Modification & targeting approaches

Drug-loading approaches

Several approaches can be used for the loading of drugs into protein nanomaterials. In a simple description, drugs can be loaded through a passive process that is efficient for loading metals and ions, while high-molecular-weight molecules and drugs can be loaded by the formulating approach, which is an approach based on a disassembly and reassembly process [125]. For example, the ferritin superfamily and APO are among the most investigated protein nanomaterials as drug-delivery systems; other systems are summarized in Table 1. The APO structures are composed of an octahedral scaffold that has both lipophilic and hydrophilic channels that connect the external surface of APO to the internal cavities [126]. These movements of therapeutic agents to the core of the protein nanomaterials through these channels represent the passive loading of therapeutic agents [127]. This passive-loading process is considered adequate for loading and stabilizing low-molecular-weight molecules. However, in terms of high-molecular-weight molecules, passive loading is not very efficient in loading and stabilizing these molecules, as they will be mainly adsorbed on the surface and will not diffuse to the inner cores [125].

In this regard, several approaches and hypotheses were developed to improve drug loading into protein nanomaterials actively. Some of these approaches were based on enhancing the permeation of prominent-molecular-weight drugs into the core of these nanomaterials. Improvement in penetration has been investigated, and studies have been performed using several techniques, such as NMR relaxation techniques and electron paramagnetic resonance spectroscopy. Several proteins, including APO, have high stability profiles under harsh conditions such as high temperature (up to 85°C), high concentrations of ionic strength and a wide pH range (3.40–10) [125,133,134]. For example, when APO is exposed to different pHs, it will undergo reversible disassembly, where the original structure will be almost completely restored when the physiological pH is restored.

Active drug loading will occur after protein disassembly; the drug present in the same solution will interact with the disassembled protein chains. When conditions are restored to physiological conditions, reassignment of protein chains will be associated with loading of the drug by entrapment in the spherical structure of the protein [135]. The level of encapsulation inactive drug loading is determined by Several methods, such as UV and HPLC, can be used to figure out the level of inactive drug loading for encapsulation. The encapsulation efficiency is then calculated as a percentage of the initial drug concentration.

Drug targeting

Protein nanomaterials have the same clearance challenges that are encountered with other types of NPs, mainly through the rapid liver and renal clearance. Several attempts have been made to enhance the targeting of drug-loaded protein nanomaterials, and many have been investigated *in vitro* and *in vivo* [5,136]. The accumulation of drug-loaded protein nanomaterials can be enhanced based on passive and active targeting. Passive targeting of protein nanomaterial formulations is based on specific pathophysiological changes in diseased and tumor tissues [137]. These tissues are characterized by a leaky vasculature, which will enhance the permeation of larger molecules

compared with normal tissues. In one study, these leaky vasculators improved the permeation of drug-loaded protein nanomaterials designed with an average size of 200 nm [138].

Moreover, among the pathophysiological changes in diseased tissues is a drop in the lymphatic drainage from these tissues. This means that after drug-loaded NPs penetrate these tissues, they remain longer [139]. Several efforts have been made to prepare engineered protein nanomaterials formulations modified with a targeting ligand on the surface of protein nanomaterials to achieve active targeting. The concept of active targeting of protein nanomaterials is based on determining the most upregulated receptor at the target site, and then the ligand that can specifically bind to this receptor is attached to the surface of the drug-loaded protein nanomaterials [140]. The binding of the ligand to its receptor enhances the uptake of this engineered delivery system, with subsequent improvement of the loaded active drug. The development of effective actively targeted protein nanomaterial formulations is based on the passive accumulation of these formulations in tumor tissues. This means that active targeting of protein nanomaterials cannot be achieved without designing these formulations based on passive targeting. Therefore, even for actively targeted formulations, these particles should be carefully designed to accumulate in target tissues based on the effect of EPR [141].

Several types of drug carriers have been generated, comprising water-soluble, high-molecular-weight polymer carriers, polymer-based NPs, micelles, liposomes, dendrimers, viral NPs, carbon-based system nanomaterials (e.g., carbon nanotubes, carbon dots, graphene and graphene oxide), magnetic NPs (e.g., iron oxide) and silica and gold NPs. Certain drug delivery platforms (e.g., polymer–drug conjugates or single magnetic nanomaterials) are more amenable to covalent drug conjugation than others (e.g., polymer NPs or magnetic NPs). Furthermore, they can include diverse functional groups such as amines, carboxyl and thiol groups and aldehydes derived from oxidizing saccharide moieties that provide several anchoring points for the conjugation to polymer carriers containing the suitable complementary reactive group [142,143]. For example, covalent conjugation of therapeutic moieties on the surface of magnetic nanomaterials has been reported with great potential for magnetic hyperthermia applications in both *in vivo* and *in vitro* models [144]. The development of a dynamically therapeutic target demands extensive knowledge of specific receptors that are much more prevalent on cancerous cells than on healthy cells. Identifying ligands that adhere strongly to such receptors, such as antibodies, peptides, folic acid and RGD-motif lectins, is also essential [145,146]. It is feasible to effectively direct the delivery system to the intended region of drug action and minimize nonspecific distribution to healthy cells and tissues by conjugating the delivery system with these kinds of ligands. Galactose, for example, has been utilized to increase adherence with parenchymal liver cells, oligosaccharides such as mannose and fucose to Kupfer cells and folic acid for cancer cells that express the folate receptor. Antibodies, especially IgG, with a molecular weight of approximately 150 kDa, and antibody single-chain variable fragments are the most selective ligands. By addition or substitution, a sulfhydryl group incorporated into the Ab framework via moderate reduction or reactivity with 2-iminothiolane can be used for conjugation with a polymer carrier containing vinyl, dithiopyr or maleimide groups [147]. An effective and chemoselective approach to covalent attachment drug carriers with polypeptide-targeting functionalities is 'click chemistry'. This approach relies on azide–alkyne cycloaddition chemistry in which an unprotected peptide with a terminal azide group is directly coupled to a polymeric or any drug carrier even without the requirement for other intermediates to complete the conjugation [148–150].

Conclusion

Protein nanomaterials are a flexible biomedical application framework. The most significant benefit of protein nanomaterials is the spatial distribution of functional groups displayed at well-defined sites that can be amenable to genetic or pharmacological manipulations. The conjugation of ligands, such as cell-penetrating peptides, to protein nanomaterials accelerates therapeutic cargo delivery inside cells through indiscriminate cellular absorption. Targeting moieties displayed on the external surface of protein constructions have been found to increase specificity and localized accumulation on target tissues. However, when presenting several ligands on the same protein nanomaterial, spatial control of ligand attachments on protein NPs is limited. The precise presentation of peptides, proteins or nucleotides with such precision makes it superior and has the potential to pave the way for the design and fabrication of novel, self-assembling, functional, hierarchical, supramolecular architectures. Even though most of the research has been focused on using protein nanomaterials to deliver anticancer drugs, using protein NPs to modulate the immune response is a relatively new field that has the potential to be the most interesting. Protein nanomaterials are fascinating candidates for providing immune modulator chemicals employed in cancer immunotherapies or autoimmune disease therapeutics. Viruses have an intrinsic capacity to encapsulate and transport nucleic acids via

cell receptor links. Innovative gene transfer platforms may be designed from scratch by examining the structure and activities of the protein subunits of viruses that are responsible for nucleic acid packing and release. The continued use of protein nanomaterials demands a greater understanding of the underlying concepts that are presently underexplored. Many protein nanomaterials, for example, do not have a precise mechanism for self-assembly. A comprehensive mechanistic understanding is required to improve drug-loading and drug-release capabilities. Biocompatibility, site-specific change, modulation of self-assembly in response to environmental stimuli, stability and drug/nucleic acid unloading may be substantial benefits of *de novo* creation of hybrid nanoscaffolds using advanced synthetic materials. These groundbreaking biomimetic nanomaterials, created by the self-assembly of specially tailored protein subunits for several purposes, have enormous potential in nanomedicine and other health sectors.

Future perspective

This paper provides an overview of the current global interest in the development of protein nanomaterials for clinical applications. Diagnostic and therapeutic moieties have been loaded into protein nanomaterials, and their exterior surfaces have been modified to improve biocompatibility and targeting abilities. Modifications to intersubunit interactions have affected the self-assembly profile, with implications for controlling the molecular release. With their distinct properties, including biodegradability, bioavailability, safety and amenability to chemical and genetic engineering, such naturally occurring particles would be pivotal for clinical development.

Executive summary

- Protein nanomaterials have been utilized for selective targeting of diseased cells with high selectivity through targeting moieties. Utilization of the natural binding affinities with some of the protein nanomaterials with overexpressed receptors on the targeted cells makes them ideal nanomaterials.
- Protein nanomaterials can self-assemble to a precise shape and the formed structure can be engineered chemically or genetically to impart new functionalities suited for various clinical applications.
- Protein nanomaterials are fascinating scaffolds that hold great potential as a novel class of nanoparticles (NPs), as they are biodegradable, biocompatible, inexpensive to produce and deemed safe for clinical applications.
- Protein NPs can be produced from various proteins such as fibroins, albumin, gliadins, gelatin, ferritin, lipoprotein and viral NPs. They can be prepared through various methods, isolated or expressed in a suitable expression system.
- When compared with other colloidal carriers, protein NPs have the benefits of being more stable and easier to produce. Furthermore, great potential *in vivo* usage is anticipated, since protein from multiple sources can be formed into NPs employing simple, cost effective and environmentally friendly production approaches.

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