Protein based nanomedicine: Promising therapeutic modalities against inflammatory disorders

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Abstract
The safe and targeted delivery of pharmaceutical formulations has relied extensively on synthetic chemistry and other physicochemical approaches. The research paradigm is now especially changing towards green (or environmentally friendly) approaches, and several biopolymer-based nanocarriers are marked as emerging nanomedicine tools. These have been developed to overcome problems with conventional drug carriers, which induce severe side effects, especially due to non-specificity with detrimental effects on non-targeted normal healthy cells. In addition, the phagocytosis of classic nanoparticles (NPs) and their degradation associated with the formation of oxygen radicals by the immune system are significant barriers for drug delivery. In this regard, protein nanocarriers appear as a promising approach to escape unwanted immune reactions. Moreover, these protein-based NPs are generally non-toxic, biodegradable, and even cost-effective. As a less appreciated advantage, the surface properties of proteins are easily modified. There are, however, several challenges and limiting choices to make when choosing the type of modification to enable effective drug delivery. Here, we shed light on the role of protein nanocarriers for enhancing the bioavailability of different anti-inflammatory drugs, including better macrophage targeting and overcoming biological barriers. This insight helps one to understand their broad utility in the treatment of inflammatory diseases and bridges the gap between naturaltherapeutic products and nanotechnology-based delivery approaches, creating perhaps an optimal blend to meet some of our most persistent healthcare problems.

Keywords
biological barriers, biopolymer, drug delivery, inflammation, macrophage targeting, nanomedicine, protein nanoparticles, therapeutics

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1 | INTRODUCTION

Inflammation is the initiating phase of both several homeostatic functions (such as microbial elimination as well as autoimmune diseases) and other inflammation-derived, chronic injuries that leads to tissue degeneration and dysfunction.\[^1\] The incidence of inflammatory diseases are increasing world-wide, which appears to have some connection with increased exposure to long-term irritants from air pollution and industrial chemicals.\[^2\] This is added to other life-style related risk factors such as obesity, smoking, and alcohol consumption. Additionally, the long-term use of non-steroid anti-inflammatory drugs (NSAIDs) results in severe cardiovascular and gastrointestinal complications.\[^3\] All of these risk factors trigger a biochemical cascade mechanism resulting in chronic inflammation. This may probably explain why the clinical treatment of, for instance, asthma, inflammatory bowel disease, hepatitis, and glomerulonephritis still involves several unmet challenges.\[^4\] Different epidemiological studies have revealed that NSAIDs cause liver toxicity if taken at high doses with low-to-moderate incidence.\[^5\] In general, NSAIDs are believed to inhibit the biosynthesis of prostaglandins by inhibiting enzymes COX-1 (Cyclooxygenase) and COX-2.\[^6\] Most NSAIDs exhibit slow blood distribution and low targeting potential. Therefore, in order to obtain the desired response at the inflammation site, a high dose is required. Unfortunately, this ultimately also induces deleterious effects in normal tissues.\[^7\] For instance, drugs with a long plasma half-life and/or slow-release have been associated with an increased risk of upper gastrointestinal bleeding, likely due to persistent exposure of the gastrointestinal tract to circulating NSAIDs.\[^8\]

In this scenario, to minimize the off target affects and to further augment these risk-associated therapies, drug carrier system with controlled and targeted release features appear as a promising aid. Progress can currently be seen in the design of versatile NP based platforms with programmed cell- and tissue-specific delivery.\[^9\] Moreover, established surface engineering protocols to append the targeting ligands on the NP surface enable efficient delivery and thereby enhance a drug’s therapeutic index.\[^10\] There are ample NP choices available. However, to explain the superiority of protein NPs in anti-inflammatory therapies, which is part of the intention of this review, we have divided this review in different sections.

Section 1 includes a brief description of how inflammation is triggered and regulated by the immune system relevant to the influence of therapeutic protein NPs. This is followed by addressing current therapeutic strategies and the potency of protein NPs in preclinical models along with prerequisite attributes of protein NPs to target and down regulate inflammation. Section 2 sheds light on the multifaceted aspect of protein NPs in targeting different cells associated with inflammatory pathways to cure chronic inflammation diseases. Section 3 focuses on the role of protein NPs in controlling tumor-associated inflammation (TAI) by modulating or re-educating macrophages, macrophage escape and suppression of tumor associated cells/proteins. Finally, Section 4 describes commercialized protein NPs, their limitations in current healthcare challenges and required improvements.

1.1 | How the inflammation phenomena influences the immune system

The activation of an inflammatory response is initiated by receiving different molecular signals that directly or indirectly report tissue damage or the intrusion of a microbial foreign genome, or, as happens quite often, both. The signals, which report infection and injury, activate various cells, such as epithelial or endothelial cells, as well as a range of leukocytes mainly belonging to the innate arm of immunity, namely macrophages or monocytes, mast cells and neutrophils. Constituents of the coagulation system, such as platelets, may also be involved. These cells undergo a cascade of recruitment, activation, and programming of APCs that generate binary signals in association with microbial products and relevant cytokines. Then, the signals from the APCs together with the antigen-receptor ligand activate and program T cells to respond. In this way, inflammation marks the immune response with a unique pattern of signals.\[^11\]

One of the key soldiers of the immune system is the neutrophil, which recruits, activates and instructs APCs. For instance, chemo-tactic signals are generated by neutrophils, which further attract dendritic cells (DCs) and monocytes. Neutrophils also govern the transformation of macrophages into a predominantly pro-(M1) or anti-inflammatory (M2) state.\[^12\] M1 macrophages are essential characters of inflammation and exert proinflammatory activities by producing high levels of reactive oxygen species, proinflammatory cytokines, inducible nitric oxide synthase (iNOS), reactive nitrogen species and COX-2. A decrease in the level of proinflammatory cytokines at the place of origin can effectively alleviate chronic inflammatory diseases (a detailed discussion can be found in Section 2). M2 macrophages present in tumors are called tumor-associated macrophages (TAMs) and constitute as much as 50% of the TME (tumor microenvironment) population and play a major role in tumor development and poor prognosis.\[^13,14\] In addition to this, the TAMs are inversely proportional to the patient’s survival period.\[^15\] Furthermore, M2 macrophages produce various types of...
cytokines that take part in the anti-inflammatory reaction. Based on the dual action of macrophages, nano-drugs are designed that can mostly target negative macrophages in different disease conditions or re-educate the TAMs in order to converse the ailment back to normal as discussed in Section 3.

1.2 Current therapeutic approaches in preclinical models and the need of protein based NPs

Currently, one of the focuses of nanomedicine-based, preclinical research is to distribute anti-inflammatory therapeutics across different biological barriers, including the endothelium and blood brain barrier. In this perspective, different nanocarrier systems are being investigated for their in vitro and in vivo potential against degenerative, inflammatory, and infectious nervous system diseases. However, despite some promising results, NP-based drug delivery has still many impediments from the formulation stage to efficacy in preclinical animal models and eventual clinical use. While there could be reasons from a chemical standpoint to consider metal-based NPs as relatively inert to immune reactions, by contrast, evidence now suggest that their metallic nature per se may be a source of inflammatory responses. Similar issues of immunogenicity exist for synthetic polymer based NPs, which may also confer undesirable side effects. Furthermore, once distributed in vivo, the accumulation of blood proteins on NPs (in a process called the protein corona effect), especially opsonization by complement proteins, have a very influential role that might expand the hydrodynamic size of NPs and, in turn, affect their stability and surface recognition by other cells. This event assists in NP recognition and clearance from the blood by disseminating immune cells like phagocytes and tissue macrophages. The composition of the coated plasma proteins is expected to be varied on the basis of actual physicochemical features of the NPs, heterogeneity and circulation time of the NPs, the dosing course, and the pathological condition being treated. It has been found that most abundant plasma proteins, such as albumin are deposited on the surface of almost all NPs.

Even NPs that are functionalized with polyethylene glycol (PEG) may not be entirely “inert” in the body, as recent studies have shown that the immune system can develop antibodies to PEG, thus, recognizing it as foreign.

With the perspective of clinical aspects of nanoformulations, proteins are endogenous materials, which provide many benefits over synthetic polymers, especially with regard to immunogenicity and biocompatibility. For instance, most of the proteins used in therapeutic formulations are of high molecular weight, which help to maintain formulation stability for a longer period of time and prolonged circulation time in serum. Some of the commercialized protein-based NPs are discussed in Section 4. Additionally, to address inflammation, one of the promising approaches is to target macrophages using tailored protein nanocarriers, which can transform the therapeutic paradigm of inflammatory diseases like prostate cancer, brain cancer, colon cancer and leishmaniasis. In Section 3.3, this approach is used to address the tumor associated macrophages using PEGylated silk fibroin NPs.

1.3 Required attributes of nanocarrier system to target inflammation

The design of nanomaterials (NMs) has a profound link with the expected nano-bio interface, which is further responsible for promoting biodegradability, targeting capacity, and metabolism of the resulting carrier. To make an effective bio-interface, nanocarriers are required to exhibit the following characteristics:

- Must be non-toxic and safe for in vivo usage with negligible immunogenicity
- Be sufficiently stable for at least a moderate shelf life
- The rate of in vivo degradation must be slow to inhibit abrupt drug release
- The degradation by-products (if any) must be nontoxic, easily metabolized, and cleared from the body
- Be cost effective and have high reproducibility in production

All of these above mentioned traits are mostly manifested in biopolymer-based NPs. Biopolymers are polymers that are synthesized under natural conditions by living organisms. These biopolymers mostly consist of proteins and polysaccharide-based materials. The widely used polysaccharide-based materials seem to fulfill the above conditions, but a few of them may have cytotoxicity issues. More recently, however, another type of biopolymer-based systems has gained interest, namely the protein-based NPs. These have proved quite effective in nutraceuticals and pharmaceutical delivery due to their biodegradability and low toxicity. Different protein nanocarriers like albumin, gelatin, fibroin and protamine NPs are being investigated. It can even be argued that protein-based NPs have already been part of human medicine for more than 20 years when looking through the inventory of immunomodulatory therapeutics (Table 1). The formulation glatiramer acetate (GA) was used for ameliorating symptoms in relapsing-remitting multiple sclerosis and was among the first treatments showing...
### TABLE 1  Pros and cons of Protein NPs

<table>
<thead>
<tr>
<th><strong>Protein NPs</strong></th>
<th><strong>Source</strong></th>
<th><strong>Advantages</strong></th>
<th><strong>Disadvantages</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>Serum of humans, cattle and eggs</td>
<td>Thermally stable, soluble in physiological fluids, biocompatible and non-toxic</td>
<td>Could cause allergic reactions</td>
<td>[40,41]</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Derived from collagen</td>
<td>Water soluble, easy to crosslink and sterilize, inexpensive, FDA approved and can be used as a food supplement</td>
<td>Fast degradation and low mechanical strength</td>
<td>Chances of prions transmission from animal source</td>
</tr>
<tr>
<td>Fibroin</td>
<td>Silk worm, spiders and other arthropods</td>
<td>Non-toxic, biocompatible, approved by FDA, easy to modify and synthesize, high encapsulation efficiency and yield, can withstand heat sterilization process</td>
<td>Slow rate of degradation</td>
<td>[42-45]</td>
</tr>
<tr>
<td>Protamine</td>
<td>Salmon fish</td>
<td>High cell penetrating ability, controllable size, can efficiently deliver gene and can be used against heparin activity</td>
<td>Complex synthesis process and low yield</td>
<td>[46]</td>
</tr>
</tbody>
</table>

Abbreviation: NP, nanoparticles.

any therapeutically useful impact on the inflammatory response in this autoimmune disease. The pharmaceutical active ingredient is a random co-polymer of four amino acids, each polypeptide with a length of approximately 50 residues. The pharmacological mode-of-action is complex and involves mechanisms of both the innate and adaptive immune response.\(^{[36]}\) It is increasingly clear, however, that the formulation itself also presents complex traits. Indeed, the co-polymer interacts to form a nanoparticle material,\(^{[37]}\) which seems to prolong the half-life in the lymphoid system.\(^{[38]}\) As a surprising consequence of the positive charge of the co-polymers, it was recently demonstrated that GA may work as a delivery system for nucleic acids.\(^{[39]}\) As a starting point for understanding protein NPs in pharmacology, GA may consequently be helpful.

Like all other nanocarriers, the ultimate parameters for designing protein NPs are size, internal attributes and morphology, which decide their suitability in different in vitro and in vivo models. It has been revealed that NPs with 100 nm sizes have 2.5 times more cellular uptake in contrast to 1 µm size particles and likewise six times greater uptake as compared to microparticles of 10 µm in size.\(^{[47]}\) Furthermore, it has been reported that rod-shaped NPs have five times less cellular uptake than spherical NPs.\(^{[48]}\) The size of NPs also plays a critical role in stability, drug loading, and release.\(^{[49]}\)

The size can be manipulated by modifying the physico-chemical properties, such as temperature, ionic strength, and pH. Some protein NPs need to be modified chemically (as discussed in Section 1.4)\(^{[50]}\) that are less toxic than most synthetic options. Surface-coatings can also be accomplished by modifying anionic surfaces of protein NPs. For instance, to attach and deliver negatively charged therapeutic molecules, the surface of protein NPs can be amended to be cationic (discussed in Section 2.3)\(^{[51]}\)

### 1.4  Remodeling tools for proteins

In addition to their biodegradability and reduced immunogenicity, protein NPs exhibit versatile functionalities for therapeutics or imaging payloads. Quite established genetic and biochemical tools available for protein modification enable targeted and controlled release of therapeutic payloads to match the need of the type and severity of the disease.\(^{[52]}\)

The peptide backbone and the side chains of the amino acid residues of proteins, both facilitate ligand conjugation, cross-linking, and several other need-based modifications.\(^{[53]}\) The presence of amino, carboxyl and hydroxyl groups makes them receptive for chemical remodeling. Both natural (antibodies and protein/peptide receptors) and synthetic (aptamers and smaller peptides) ligands are used to enhance cell/tissue uptake of NPs as well as delaying their clearance.

For example, PEGylated albumin NPs encapsulating AZT (azidothymidine), have been surface modified with a ligand “transferrin” used to target the BBB.\(^{[54]}\) As a result,
FIGURE 1 Depiction of different interfaces offered by protein NPs to add/tag various functionalities. Internal interface is often hydrophobic (oil phase) and is used to encapsulate hydrophobic drugs. External and inter-subunit interfaces are used to carry hydrophilic compounds. Modification of external and inter-subunit interfaces can be used to enhance the colloidal stability and biocompatibility of the nanocarriers, which, in turn, also enhances the bioavailability of encapsulated drug

the enhanced localization of the drug in the brain was confirmed. Therefore, proper surface engineering of NMs is needed to influence the interactions between NPs and a biological system.

1.5 Surface modification of protein NPs

In the material design at nanoscale level, besides size, shape and material composition, assembling interfaces in nanostructures also have a profound impact on the material properties. Like several other biopolymers, protein NPs are formed by the self-assembly of different protein subunits. In this perspective, for specific modifications and loading the therapeutic cargoes, three interfaces (as shown in Figure 1) are offered by protein NPs for drug delivery purposes, that is, external, inter-subunit and internal surfaces. These interfaces can be redesigned chemically or genetically with natural or synthetic ligands as discussed in Section 1.4 in order to insert multiple functionalities.

The NP internal surface is modified to enclose therapeuti- c or diagnostic cargoes including nucleotides and imaging agents. The internal surface can also be used to design controlled release attributes. For this direct conjugation approach is investigated extensively in order to encapsulate drug and enable its controlled release from nanoparticles (NPs). Different functional groups and amino acid residues from protein NPs present active sites for drug conjugation like amines,[55] cysteines,[8,52] carboxyl,[53] phenol, tyrosines,[56] imidazole,[9,27] and guanidine. These active sites can either be native or introduced by mutagenesis. Additionally acid labile chemical bonds have been used to initiate drug release because of their pH-dependent hydrolysis. At neutral pH, these chemical bonds remain intact, whereas at acidic pH these were hydrolyzed, and encapsulated drugs could be released. At the cellular level, delivered NPs are supposed to go through a physiological pH change from extracellular pH 7.4 to intracellular pH, that is, < 6, during endocytosis.[19] This pH transition could effectively be used for drug/payload release.

As one example, researchers modified internal surface of nanoscaffolds based on virus-like proteins by introducing sixty cysteine molecules.[8,10] The external surface of protein NPs provides the potential engineering sites to append the targeting moieties. The delivery aims to target drug carriers directly at tumor or inflammatory sites with the help of receptor ligands. These ligands are involved in recognition and specific binding to overly expressed receptors on the cancerous cells.[57,58] Numerous targeting ligands have been identified by researchers such as small molecules (folate/folic acid),[59,60] antibodies, peptides (62), antibody fragments,[62] and nucleic acid aptamers.[63]

To engineer the subunit interfaces, one must understand the self-assembly and protein-protein interaction in the biological system. The interaction between inter-subunits can be regulated to control the disassembling and assembling of NPs. Therefore, cargo release can be more targeted and robust by modifications in individual subunits and interactions of NPs.[64]
VERSATILITY OF PROTEIN NPS TO TARGET IMMUNE SYSTEM-ASSOCIATED INFLAMMATION

2.1 Targeting neutrophil apoptosis

Neutrophils are widely circulating leukocytes in human blood and have a fundamental role in innate immunity. Their defensive mechanism involves the digestion and disruption of pathogenic microorganisms and the repair of tissues.\[^{11}\] They have a short life span of about 8 to 20 hours in circulation, and their life span is specifically regulated by apoptosis. Apoptosis is characterized by programmed cell death in order to maintain a constant number of neutrophils in circulation.\[^{65}\] Evidence revealed that the inflammation usually promotes the life span of these cells.\[^{66}\] Prolonged activation and unrestrained neutrophil infiltration cause inflammatory diseases, such as ischemia/reperfusion (I/R),\[^{67}\] acute lung inflammation/injury,\[^{68}\] sepsis,\[^{69}\] and rheumatoid arthritis.\[^{56}\] Currently, anti-cytokine therapies and non-steroidal anti-inflammatory drugs are used to treat these diseases. However, systemic immune suppression can cause off-targeting effects leading to severe side effects such as gingival hyperplasia caused by cyclosporine A,\[^{70}\] hyperlipidemia due to rapamycin consumption,\[^{71}\] cancer, and proneness to infections.\[^{72}\]

Considering the role that NPs play in treating such conditions, it has been reported that AgNPs can delay neutrophil apoptosis and also hinder the de novo synthesis of proteins.\[^{73}\] The toxicity of these NPs is generally linked with the formation of a “protein corona” as discussed in Section 1.2. This protein corona imparts human neutrophil (PMN) toxicity by interacting with scavenger receptors.\[^{74}\] Similarly, ZnO NPs have the ability to prompt cell spreading as well as cell shape changes. These NPs prompt cytoskeleton re-organization by inducing actin polymerization and might delay neutrophil apoptosis.\[^{75}\] Therefore, in comparison to these NPs, protein NPs can be a better solution to target autoimmune and inflammatory diseases.

In a recent study, DOX conjugated with bovine serum albumin (BSA) via a hydrazone bond to the NPs were synthesized and employed to resolve the anti-apoptotic state of neutrophils hindering acute lung inflammation as shown in Figure 2.\[^{76}\] After administration, NPs were able to target specifically the anti-apoptotic state of neutrophils circulating in the blood and taken up by the neutrophils (responding to inflammation).\[^{77}\] DOX molecules were selectively released when hydrazone bonds were cleaved by the acid produced in the neutrophil environments, resulting in the induction of neutrophil apoptosis and reducing neutrophil transmigration.\[^{78}\] Moreover, this protein based NPs did not damage neutrophil generation.
in the bone marrow. Intercalation of DOX molecules into DNA double helices hindered the growth of topoisomerase II, generating DNA damage to cause cell death. Furthermore, BSA NPs alone did not impose any cytotoxic effects at various concentrations.

2.2 Inhibition of T cell progression and reversion of activated macrophages

In the pathogenesis of collagen-induced arthritis and RA, T-cells play an important role because of their activation and interaction with other immune cells that continuously produce inflammatory cytokines. So, it is needed to target T-cells in order to reduce inflammation at an arthritic site.

Interestingly, it has been revealed that albumin has a special anti-arthritic potential as it accumulates well in inflamed tissues. Moreover, due to the up-regulation of synovial cell metabolism, arthritic joints utilize higher levels of albumin as an energy and nitrogen source than normal tissues. This increased demand for albumin paved the way to exploit the potential of albumin based NPs to arthritic sites. It seems probable that tacrolimus (TAC)-encapsulated albumin NPs repressed inflammation because TAC selectively obstructs the IL-2 transcription that is essential for T cell activation. A scheme of this phenomenon is shown in Figure 3A.

Osteonectin also known as SPARC (secreted protein acidic and rich in cysteine), is a glycoprotein, which is known to be highly expressed in arthritic joints, tissue remodeling (modulates the interaction between cells and the extracellular matrix) and wound repair. However, at the site of various aggressive tumors such as ovarian, colorectal, head and neck, and breast cancer, SPARC is overexpressed by increasing tumor aggression and metastasis affecting cell adhesion and matrix composition. Methotrexate (MTX) is a suggested drug for RA. It is a folic acid analogue and widely used as a first-line disease-modifying antirheumatic drug (DMARD) at low doses in the clinical treatment of arthritis. It is important to target this therapeutic drug with high efficiency and specificity to inflamed joints, otherwise its long term administration results in suboptimal responses and systemic toxicities, including opportunistic infections and an impaired immune system.

By exploiting the increased demand of albumin in arthritic joints, MTX-encapsulated albumin NPs were designed to target arthritic inflammation. These MTX-loaded albumin NPs enhance the therapeutic index of a drug because they are retained longer in joints and accumulate in greater amounts at arthritic joints. Importantly, the impressive clinical efficacy of these NPs is linked with the overexpression of SPARC in various types of cancers. Followed by systemic administration, MTX encapsulated albumin NPs accumulate in the synovium of arthritic joints via leaky blood vessels because of an inherent high affinity between overly expressed SPARC and albumin. After that, these drug loaded albumin NPs were phagocytosed by synovial macrophages.
Due to the acidic microenvironment of lysosomes in macrophages, these NPs were prompted to release MTX (Figure 3B), blocking the expression of inflammatory cytokines by inhibiting the multiplication of activated macrophages.\(^{63}\)

While treating arthritis, sudden contraction of synovial hyperplasia is a quite common complication that leads to early erosion of bone and cartilage.\(^{99}\) This is due to the presence of a large number of M1-activated macrophages, which continuously secrete a variety of pro-inflammatory cytokines and drive the RA process. In this way, targeting synovial macrophages can be a potential therapeutic strategy.\(^{100}\) Lipid-based NPs could be a competitive approach due to their drug accommodating potential. However, different lipid NP-based formulations have been proved inefficient due to destabilization of the lipid bilayer, which results in rapid unwanted drug release.\(^{101}\) The use of protein NPs can be an alternative approach. Many anti-inflammatory drugs depending on their chemical structure can form a tight complex with proteins (such as HSA) and so can be tuned for sustained drug release.\(^{102}\)

HSA NPs encapsulating prednisolone (PD) and curcumin (CU) complexes were used to target synovial macrophages and hinder the inflammatory cytokine secretion. Both CU (a hydrophobic polyphenol) and PD (a glucocorticoid) are hydrophobic and have remarkable anti-inflammatory properties.\(^{103}\) These drugs, however, when administered without a proper delivery system, were unable to reach the inflammatory site.\(^{104}\) The co-delivery system of HSA NPs-PD/CU hindered pro-inflammatory cytokines, such as IL-6,\(^{105}\) and promote the secretion of IL-10 more effectively.

This type of combination therapy for RA revealed a remarkable reduction in the level of pro-inflammatory cytokines and enhanced secretion of anti-inflammatory cytokines due to a synergistic effect produced by the drug complex.\(^{106}\) Additionally, HSA NPs-PD/CU have been found to target inflamed joints more than free CU/PD revealing the fact that their encapsulation by HSA NPs improve their bioavailability and efficacy.\(^{107}\)

**2.3 | Suppression of overexpressed inflammatory cytokines**

Dry-eye syndrome (DES) involves inflammation of the eye surface and hyperosmolality of tears. It has been recognized as major mediator of an inflammatory response.\(^{108}\) The disease is associated with visual disruption, unstable tear film, and discomfort. Overexpression of many pro-inflammatory cytokines (including IL6, IL1β, CCL2, IL17, TNF-α, IFN-γ, etc.) has been reported in DES-bearing patients.\(^{109}\)

Different antibiotics and steroid-based ophthalmic solutions have been used for treatment.\(^{110}\) Usually these ophthalmic solutions (eye drops) are quite cheap and have been widely used for the treatment of this disease. However, only \(<5\%\) (w/v) of the drug in solution can penetrate the cornea because of physiological and anatomical barriers.\(^{111}\) The macrolides and tetracycline (hydrophilic drugs) antibiotics have proven to possess insignificant anti-inflammatory effect on patients with DES\(^{112}\) In addition, the prolonged usage of corticosteroids can result in other eye infections such as cataracts and glaucoma.\(^{113}\) All these antibiotics, if not delivered selectively, have their own side effects and sometimes depend on the patient’s sensitivity, and can make symptom seven worse.\(^{114}\)

In comparison to synthetic antibiotics used for DES, natural compounds including polyphenols have been used as an efficient alternative\(^{115}\) as they possess anti-inflammatory and antioxidant properties.\(^{116}\) Many studies have focused on elevating the drug concentration retained on the cornea using various ocular drug delivery systems such as nanospheres,\(^{117}\) liposomes,\(^{118}\) and other NPs.\(^{119}\) However, typically, for the treatment of DES, the lipophilic corneal epithelium is the major barrier for the distribution of hydrophilic polyphenols (e.g., EGCG).\(^{120}\) In order to cross this lipophilic corneal epithelium barrier, other protein based NPs (like gelatin) have been employed for topical delivery and as an ocular drug carrier\(^{121}\) due to their lack of toxicity, biocompatibility and biodegradability.\(^{122}\) Moreover, EGCG contains multiple hydroxyl groups, which can be used for cross linking to drugs and to develop electrostatic interactions between the negatively charged EGCG and positively charged protein-peptide chains of gelatin.\(^{123}\)

These cationic gelatin based mucoadhesive (GEH) NPs are capable of being retained in the cornea for longer durations compared to than any other ophthalmic solutions.\(^{124}\) Additionally, due to the slow-release rate of GEH NPs, a significant decline was observed in the gene expression of inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) compared to an EGCG solution alone (Figure 4) and other ophthalmic solutions.\(^{125}\)

**3 | PERSPECTIVE OF PROTEIN NPS TO TARGET TUMOR-ASSOCIATED INFLAMMATION**

**3.1 | Escape of NP’s interaction with macrophage receptors to reach tumor cells**

During the last few decades, various chemotherapeutic strategies have been developed to treat cancers, which are among the most malignant diseases.\(^{127}\) However,
different chemotherapeutic agents, once administered, often pose several side effects due to their physicochemical properties (solubility and hydrophobicity) and size.\[128\]

An efficient carrier system is needed that can selectively deliver chemotherapeutic drugs to tumor cells.\[129\] Indeed, a large number of carrier systems have been investigated in this regard. However, considering how quickly cancer cells grow and also exploiting other patho-physiological needs of tumor tissues, albumin seems quite suitable to intercept tumor cell requirements. Therefore, has-based NP carrier systems have been designed due to the intrinsic biocompatibility and solubility behavior of HSA with human blood cells as discussed in Sections 1.3 and 1.4.\[72\]

One of the large hurdles to deliver anticancer drugs is macrophages, which engulf the carrier system and restrict it from reaching the target site. In the case of HSA NPs, the uptake ratio was strongly linked with alcohol (desolvating agent) concentration. For example, in a method optimized by Langer et al.,\[130\] HSA NPs synthesized at 80% (v/v)ethanol were found to be rapidly denatured and eagerly engulfed by macrophages due to scavenger receptors\[131\] (gp 30 and gp 18)\[132\] as shown in Figure 5. However, PEGylation of the same type of nanocarriers can be used to prevent their uptake by the reticuloendothelial system (RES) and opsonization.\[133\] The in vivo denaturation of HSA NPs was found to be minimal at low concentrations of ethanol and at a neutral physiological pH resulting in less susceptibility of cellular uptake by macrophages.\[134\] Interestingly, by reducing alcohol concentration, circulation time of these NPs was enhanced without adding any additional chemical modification steps, like PEGylation, to prevent opsonization.\[135\]

### 3.2 Down-regulation of tumor cell proteins

Several flavonoids, such as CU, possess a great deal of antioxidant properties, but their inherent hydrophobicity limits their physiological absorption and therapeutic efficiency.\[136\] Several types of nanocarriers have been being designed to enhance the intracellular delivery of curcumin, such as inorganic NPs\[137\] and solid lipid NPs.\[138\] Due to their benefits (as discussed in Section 1.3), different proteins are also being explored for this purpose.

For example, silk fibroin (SF) NPs are considered as a natural polymeric biomaterial. As bioactive carriers, SF NPs exhibit a wide variety of features such as tunable amphiphilic chemistry, excellent mechanical properties and availability of established chemical/biochemical tools to incorporate modifications.\[139\] In the perspective of biomedical applications, SF possesses intrinsic anti-inflammatory properties because of their ample disulfide bonds, imidazole, amino and carboxyl groups.\[140\] It offers an ability to tune sizes from 60 to 1000 nm range as well as high encapsulation and drug loading capacities which are ideal attributes required to penetrate and accumulate into cancerous and inflamed tissues.\[141\] Recent studies have...
clearly highlighted multi-responsive and on demand drug release features of SF NPs against tumor biomarkers like reactive oxygen species (ROS)/pH/glutathione (GSH) \[142\] and lysosomal enzymes.\[143\] SF based nanocarriers have also been used to deliver and enhance the efficiency of some active anti-inflammatory ingredients (like CU) for the medicament of tumors.\[136\] The in vivo application of these CU-F NPs did not affect the viability of healthy tissues. The CU-SF NPs also enabled the down-regulation of the bcl-2 protein that is expressed in the tumor microenvironment, which ultimately led to the apoptotic death of cancerous cells.\[144\]

3.3 | Re-educated TAMs

The mechanism of phagocytosis, although vital for many physiological processes and key for biological events, is considered to be an undesirable event for nanomedicine due to the risk of hypersensitivity reactions.\[153\] The approaches to avoid this unwanted clearance is active targeting by conjugating NPs with polymeric (poly-ethylene glycol) or targeting moieties (aptamers, peptides or monoclonal antibodies).\[154\] Another approach that can be used is to passively target NPs using the EPR effect.\[155\] This allows NPs to evade macrophage targeting and maximizing therapeutic delivery to tumor sites.

Presently, research interest is focused on exploring the immunomodulatory aspects of novel NMs to serve as immunological adjuvants that can specifically influence TAI.\[156\] TAMs are abundantly present in the tumor microenvironment.\[157\] During tumor development, TAMs are highly plastic.\[158\] They are polarized into two major phenotypes: TAM 1 (the anti-tumor M1) and TAM 2 (the pro-tumor M2).\[159\] TAM 1 is a highly expressed pro-inflammatory cytokine, such as TNF-\(\alpha\), whereas TAM 2 shows the expression of anti-inflammatory cytokines, such as TGF-\(\beta\).\[160\] TAM 2, acting as a driving factor in suppressing TME, for example secreted TGF-\(\beta\) inhibits CD8\(^+\) T-cells and induces Treg cell response to promote apoptosis of cancer cells. In cancer immunotherapy, both the populations of TAM 1 and TAM 2 can be utilized as a prognostic factor,\[161\] which can be another focus of nanomedicine design. Different protein based NMs have been tested for TAM reprogramming which ultimately modulates their inflammatory profiles and maximizes their intratumoral potential.\[162,163\]

From a clinical translation perspective, promising results have been obtained while using SF based anticancer NPs, like SF coated with PEG.\[164\] The interaction of either PEGylated or unmodified silk fibroin NPs caused
SF is revealed to have anti-cancer potential that is explored using PEG. Modification with PEG is because it improves the colloidal stability of SF and helps to efficiently target TAM no damage to the macrophage plasma membrane. Due to the inherent antioxidant potential, when a high dose of unmodified SF NPs was administered in vivo, significant increases in the levels of antioxidant activity and reduction in pro-inflammatory mediators were observed such as nitric oxide, TNF-α and ROS. Increased cellular glycolytic activity is indicated by a significant increase in glucose consumption.[166] upregulation of pyruvate to lactate conversion and marked by increased lactate and alanine excretion from cells.[167] However, at a low dose, unmodified SF nanocarriers were still able to mediate a pro-inflammatory shift in the metabolic profile in a concentration dependent manner.[168] On the other hand, PEG modification has been used to enhance the colloidal stability of SF NPs, modulate drug release profiles and degrade NPs.[164] These modified NPs do not impose a negative impact on the delivery potential of NPs to lysosomes.[169] However, when administered at a high concentration of PEGylated SF NPs, it produced moderate levels of the proinflammatory mediators TNF-α, NO and ROS as shown in Figure 6.[170]

3.4 Backtracking of macrophages from the M2 phenotype to anti-tumor M1 phenotype

Macrophages play a crucial role in the aggression and regression of tumor cells.[171] Under different physiological conditions, they have potency to transform into distinct functional phenotypes. The abundancy of M2 macrophages in the tumor microenvironment has a key role in tumor progression.[172] Studies are being focused to reverse the immunosuppression induced by M2 macrophages into anti-tumor M1 macrophages using different agents such as cytokines (IL-12[173] and IL-21,[174] cationic polymers,[175] glycopolymers[176] and MicroRNA.[177] Due to instability issues and difficulty penetrating across the cell membrane, advancements in gene delivery vectors are still needed to design efficient delivery carriers using biocompatible vectors.[178] IL-12 (a heterodimeric cytokine) is particularly considered as an ideal candidate to promote macrophage reversal from a tumor supportive M2 phenotype to an anti-tumor M1 phenotype because it activates cytotoxic T lymphocytes and natural killer cells as well as promotes the Th1-type immune responses to represses tumor growth.[179] Recently, the use of protein-based delivery systems has been a quite popular approach. Table 2 shows protein-based NPs that can target tumor cells using the EPR effect and receptor mediated endocytosis which was used to reverse the TAMs phenotype into a tumor suppressed M1 phenotype. In addition to different synthetic drugs, certain peptides and nucleic acid constructs play a promising role to re-program tumor associated macrophages.[180,181]

A multifunctional protamine based nanocarrier system comprised of fusion peptides and IL-12 gene was recently designed. This positively charged nano-sized hybrid core was decorated with HA to form negatively charged
### Table 2

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Anti-inflammatory drugs</th>
<th>Type of modification</th>
<th>Application</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methotrexate</td>
<td>Conjugation of Ce6</td>
<td>Targeting SPARC for the treatment of Rheumatoid Arthritis</td>
<td>[62]</td>
</tr>
<tr>
<td>2</td>
<td>Mesalamine</td>
<td>Glutaraldehyde as a crosslinker</td>
<td>Targeting Myeloperoxidase (MPO) for treating inflammatory bowel disease</td>
<td>[145]</td>
</tr>
<tr>
<td>3</td>
<td>Piceatannol</td>
<td>Glutaraldehyde as a crosslinker</td>
<td>Inactivate neutrophil transmigration and alleviation of vascular inflammation</td>
<td>[146]</td>
</tr>
<tr>
<td>4</td>
<td>Tanshinone IIA</td>
<td>PEGylation</td>
<td>Modulate signaling pathways of neurons for the medication of cerebral ischemia</td>
<td>[147]</td>
</tr>
</tbody>
</table>

**Gelatin NPs**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Drug</th>
<th>Modification Used</th>
<th>Application</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Ibuprofen</td>
<td>PEGylation and crosslinking with CaCl₂</td>
<td>Enhanced the bioavailability of Ibuprofen and its pharmacokinetics for the treatment of chronic arthropathies and rheumatoid arthritis.</td>
<td>[148]</td>
</tr>
<tr>
<td>6</td>
<td>Epigallo- EGCG</td>
<td>Surface coupled with mucoadhesive hyaluronic acid</td>
<td>Enhanced retention time in the cornea ensures sustained release of drug promoting the treatment of DES.</td>
<td>[126]</td>
</tr>
<tr>
<td>7</td>
<td>Kaempferol</td>
<td>Crosslinking with glutaraldehyde</td>
<td>Reduction in the growth of blood vessel on cornea leading to the treatment of DES.</td>
<td>[149]</td>
</tr>
</tbody>
</table>

**Protamine NPs**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Drug</th>
<th>Modification Used</th>
<th>Application</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Heparin derivatives</td>
<td>PEGylation</td>
<td>Promote sustained release of heparin derivatives (LHsura) resulted in successful accumulation at tumor site.</td>
<td>[64]</td>
</tr>
<tr>
<td>9</td>
<td>VIP</td>
<td>Conjugated with Cy3 dye</td>
<td>Protection of VIP cargo by proticles from enzymatic degradation resulted in specific targeting of breast cancer</td>
<td>[150]</td>
</tr>
<tr>
<td>10</td>
<td>shRNA-expressed in plasmid DNA</td>
<td>Crosslinking with glutaraldehyde</td>
<td>Significant inhibition of Bcl-2 proteins resulted in inducing cell apoptosis and improved targeted gene apoptosis in cancer therapy</td>
<td>[151]</td>
</tr>
<tr>
<td>11</td>
<td>CAP</td>
<td>Crosslinking with glutaraldehyde and surface coated with Ca-alginate hydrogel</td>
<td>Enhanced absorption of wound exudates facilitated rapid treatment of diabetic skin wound</td>
<td>[152]</td>
</tr>
</tbody>
</table>

Abbreviation: Ce6, Chlorin e6; CAP, cationic antimicrobial peptide; EGCG, catechingallate; NP, nanoparticles; shRNA, small hairpin RNA; SPARC, secreted protein acidic and rich in cysteine; VIP, vasoactive intestinal peptide.

(HA/PS/CaCO₃/DNA) PHNPs. The multi-functional assembly delivered pDNA IL-12 to M2 macrophages that could shift its polarity into anti-tumor M1 phenotype is shown in Figure 7. The reminiscence of the ability of positively charged GA co-polymers to bind DNA and work as a gene delivery vector in anti-tumor phenomenon is striking.

Protamine sulphate (PS) is a nuclear protein rich in arginine which is able to condense DNA. It contains certain sequences which mimic the nuclear localization sequences (NLS), thus, NPs of this protein are very useful for nuclear delivery. This protein helps to overcome the intracellular delivery barrier by increasing the cellular uptake and nuclear transport because it has regions like the
FIGURE 7  Schematic illustration of backtracking TAM into anti-tumor M1 macrophages using protamine based NPs; conceived from[185] This multifunctional vehicle enters into the macrophage encapsulating plasmid DNA and coated with protamine protein. This remarkable protein helps to cross the intracellular barrier because it has sites similar to nuclear localization signals (NLS) and then pDNA-IL-12 released within the nucleus, effectively convert the TAM into anti-tumor MI stage

nuclear localization signal (NLS).[184] The PHNPs mediated transfection at the tumor site led to the up-regulation of HLA-1 and CD80, down-regulation of CD47 and production of IL-12, successfully indicating the reversal of tumor induced immunosuppression.[173] As immunotherapy is clinically approved for many cancers, this advanced multifunctional based immunotherapy therefore holds much clinical potential.[185]

4 | CLINICAL TRANSLATION OF PROTEIN NPS

From the perspective of the advancement of clinical medicine, protein based NP engineering therapy is now able to provide a better alternative to those formulations which have associated problems of solvent based toxicities. Protein NPs based products have been proven to increase intratumor drug concentrations by exploiting the endogenous albumin pathways, and they do not require premedication but do enhance the ease of administration and reduce the burden on the health care system.[186] As mentioned with the example of GA, protein NPs are likely to have excised previously unappreciated parts of medicines. With our increasing ability to analyze NPs, a more guided use of the promising aspects of protein NPs is now greatly encouraged.

Abraxane (nab-paclitaxel) is one of the first commercially available products of protein based NPs approved by FDA for breast cancer patients who suffer from repeated trials of combination or adjuvant chemotherapy. This product is cremophor polyethoxylated castor oil (Creomophor EL) free and only consists of human albumin and unmodified paclitaxel.[187] Upon excluding CrEL from nab-paclitaxel, the chances of hypersensitivity reactions are reduced, enabling its short time distribution i.e., (30 min) and without needing any special intravenous tubing[188]

Certain protein based products that are approved by FDA include ABI-008 (Paclitaxel albumin loaded NPs) for treating metastatic breast cancer,[189] Gelafusal,[190] which results in expanding plasma for intravenous inoculation, and Gelafundin,[191] which has marked a milestone in history as it utilized the advancement of protein NPs to serve as a drug delivery vehicle. Similarly, commercially available protein based drug delivery system for diabetes is Levemir and Victoza.[192,193] The ozoralizumab, a protein based product is used for the treatment of rheumatoid arthritis and albuferon, is prescribed in liver inflammation (hepatitis C) therapy.[194] The product albuferon is derived from binding gene sequence of human albumin to interferon alpha.[195] From a technological insight, it can be beneficial to have improved knowledge of different key elements involved in controlling release rates as well as
developing methods to prolong drug release and broaden pharmacokinetic studies. These investigations will prove to be significant in changing the purpose of protein engineered NPs into those materials practically applied as the next generation of drug delivery systems.

Despite having the benefits offered by these commercially translated products, some main concerns and unmet challenges regarding the safety usage of protein NPs include: (i) There may be an unexpected structural changes in physiological conditions which can alter the actual impact of endogenous proteins, (ii) development of nonspecific interactions in vivo, (iii) already available methods for proteins NPs, provide low yield, (iv) transmission of prions from especially naturally derived proteins, (v) rapid degradation of some proteins such as ELPs (Elastin-like polypeptides) (56) and protamines (46), (vi) biphasic drug release from protein NPs along with initial-burst liberation, (vii) unpredictable and sometimes complicated biodegradation in physiological environment, (viii) On utilizing available protein NP based drugs in the market such as Abraxane, some alarming side effects have been reported such as sensory neuropathy, myalgia, asthenia and fatigue was observed frequently. Finally, while protein NPs have excellent tolerability and low immunogenicity compared to other carriers, at least for long-term administration immunological hypersensitivity reactions cannot be excluded.

In overcoming the issues of having low yield regarding naturally derived protein NPs, a recombinant protein strategy can be employed along with advance studies on improving its downstream pathway. Likewise, endotoxins present in recombinant proteins can be averted by utilizing plants or yeasts as an expression system. Thirdly, in order to address issues regarding rapid degradation, protein precursors can be blended with other biocompatible polymers.

5 | CONCLUSIONS

In summary, we reviewed here the current status of protein nanocarriers in the context of targeting inflammatory disorders. We account for the ability of protein nanocarriers to cross different biological barriers and for the improvement of the biodistribution of drugs. Many proteins are extracted from plants and other natural resources, which lessen their toxicity concern and can effectively be utilized for novel drug formulations. In nature, each protein has an inherent mechanism for metabolism, which leads to the easy breakdown of NPs into non-toxic products within the host system. The present review highlights that protein NPs, as a carrier, holds a great deal of advantages in delivering exogenous substances such as peptide hormones, anti-cancer drugs, growth factors, DNA and siRNA. Several approaches have been applied for the surface functionalization of protein NPs with ligands of interest. These include the supramolecular interactions, modified biomaterials and post fabrications coatings of NPs with synthetic polymers. Taken together, studies based on protein NPs hold much potential for clinical translation against current pandemic diseases as the approaches are innovative, novel and rational to treat various types of inflammatory disorders.

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