ISSN 2706-6819 Vol.5, Issue 11: 39-45, DOI: 10.25236/IJFM.2023.051106

The Applications of Lactoferrin in Various Therapies and Nanotechnologies

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Abstract: *Lactoferrin (LF) is a natural glycoprotein that possesses iron-binding properties and multiple biological applications, including antiviral, anti-inflammatory, antioxidant, anticancer, and immunomodulatory effects. Due to the overexpression of LF receptors on many cells such as cancer cells and its ability to cross the blood-brain barrier (BBB), LF has demonstrated its potential for active targeting, making it an ideal nanocarrier for hydrophobic therapeutic agents. Therefore, LF, as a multifunctional protein, has shown broad prospects for application in cancer therapy and nanomedicine.*

Keywords: *Lactoferrin; nanocarrier; brain targeting; cancer cell targeting*

1. Introduction

Lactoferrin (LF) is a cationic iron-binding glycoprotein with a molecular weight of approximately 77-80 kDa, first identified in human milk in the early 1960s and later reported to be also found in other exocrine fluids such as blood, tears and saliva. LF is folded into two spherical lobes, connected by an α-helix and stabilized by disulfide bonds [1]. The main function of this protein is to control and regulate the concentration of free iron in biological fluids by binding or releasing iron ions(Fe3+), and this property is a key reason for its ability to perform a variety of functions, including antibacterial and antiviral, anticancer, anti-inflammatory, antioxidant, and immune stimulation [2]. LF extracted from milk or produced by microbial recombinant technology is widely used in a variety of products, including baby food, cosmetics, and food additives [3]. The level of LF in blood can also be used as a diagnostic basis for some inflammatory diseases, such as sepsis or acute respiratory distress syndrome, and LF in stool is a non-invasive and sensitive biomarker for the diagnosis and severity of intestinal inflammatory diseases such as Crohn's disease and chronic inflammatory bowel disease [4]. The LF consists of two blades, each of which can synergistically bind two Fe3+ ions via HCO3- or CO32- . The Fe3+ binding ability of LF is enhanced by lowering pH or using chelating agents. LF undergoes conformational changes through inter-domain cleavage opening and closing, and has a compact structure when binding Fe3+ ions and a loose structure after releasing Fe3+ ions. There is a glycosyl attachment site on each of the two blades of LF, which can participate in receptor recognition. The thermal stability of LF is strongly dependent on the degree of Fe3+ ion binding and pH, and LF in the iron-saturated state is less likely to form agglomerates and has better water solubility [5]. LF has a net positive charge under physiological conditions and is relatively stable in gastrointestinal tract, and can be actively targeted to tumor cells and brain endothelial cells via LF receptors [6], so it is commonly used to make non-immunogenic nanocarriers with good biocompatibility.

2. LF's own characteristics

2.1. Antimicrobial and antiviral

LF is an important component of the mammalian innate immune system and it has antibacterial, antiviral as well as antifungal functions. This action derives mainly from the protein's ability to bind

ISSN 2706-6819 Vol.5, Issue 11: 39-45, DOI: 10.25236/IJFM.2023.051106

Fe3+ and its ability to interact between cells or molecules of hosts and pathogens [7]. LF can affect a wide range of Gram pathogens. For Gram-negative bacteria, LF affects the release of bacterial lipopolysaccharides by binding Ca2+, leading to pathogenic cell wall instability [7]. LF competes for binding Fe3+ to reduce pathogenic bacteria's iron acquisition, leading to restriction of their growth. LF can bind directly to pathogenic bacteria, leading to destabilization of broad-spectrum bacterial cell membranes [8], e.g., the glycan fraction of LFcan attach to bacterial adhesins, leading to a decrease in the ability of bacteria to form biofilms [9]. LF can also inhibit certain bacterial strains, such as inhibition of Streptococcus periodontalis and Pseudomonas aeruginosa. LF also has a protein hydrolytic effect, and although its activity is lower than that of LF is also protein hydrolytic, and although its activity is lower than that of trypsin, this protein hydrolytic activity can still be used to degrade some virulence factors of viruses or secreted proteins of some pathogenic bacteria [10]. More importantly, the adsorption of LF on host cells is closely related to the activation of the immune system. LF binds to integrins and glycosaminoglycan receptors on the surface of host cells, which are required for viral entry [11], and LF binds to these receptors binding interferes with viral attachment, thereby reducing host cell infection.

2.2. Anti-inflammatory

LF has anti-inflammatory protective properties and can be used to treat both infectious and non-infectious inflammatory conditions. In infectious inflammation, the anti- inflammatory effect of LF is mainly attributed to the ability to neutralize microbial molecules by binding to the lipid A region to prevent further interaction of lipopolysaccharide-binding proteins with endotoxins, thus preventing lipopolysaccharide binding to CD14 on the membrane [12]. LF also stimulates the secretion of anti-inflammatory cytokines such as IL- 10, while inhibiting the synthesis of many pro-inflammatory cytokines, including IL-6 IL-8, IL- 1b, and TNF- α [13]. Another important function of LF is to inhibit the production of reactive oxygen species (ROS), which are produced by granulocytes during inflammation, and LF inhibits ROS production and further lipid peroxidation reactions mainly by chelating Fe3+, which is necessary forROS production [14].

2.3. Anticancer

LF exerts antitumor effects in multiple ways [15]. It inhibits tumor growth by activating cysteine aspartate proteases, upregulating pro-apoptotic protein levels to induce apoptosis in tumor tissues [16], and promoting cell cycle protein-dependent kinase and tumor suppressor protein production [17]. It also specifically inhibits the action of the plasma membrane V-ATPase proton pump, which is overexpressed in highly metastatic cancer cells, and decreases the acidity of the tumor microenvironment, by reduce the growth and invasion ofcancer cells [18]. In in vivo and in vitro experiments, LF in combination with temozolomide enhanced the inhibitory effect of temozolomide on human glioblastoma cells. LF has shown promising applications in combination therapy to enhance the antitumor effect of conventional chemotherapeutic agents.

2.4. Immune stimulation

Complex LF with monophosphatidylated lipid A acts as an effective adjuvant to induce cellular and humoral immune responses. The main mechanisms of this LF- induced immune response are stimulation of T lymphocyte differentiation, activation of phagocytes and maintenance of Th1/Th2 cytokine balance [19]. Oral bovine LFcan promote the transcription of some essential genes such as intestinal p40, IL- 12 and NOD2 by activating systemic host immune response. Under non-pathogenic conditions, LF can enhance the differentiation of immature precursor cells to T cells by inducing CD4 antigen. LF can also activate macrophages by stimulating TLR4-dependent and non-dependent signaling pathways to induce IL-6 and CD40 production [20].

Lf and Transferrin (Tf) belong to the transferrin family of proteins. Tf is a single-chain glycoprotein with approximately 700 amino acids, while Lf contains about 690 amino acids. Although Tf and Lf are structurally and sequentially similar, both coordinating iron in a similar manner, they exhibit distinctions in their lobe interfaces, salt bridges between helical lobes, disulfide bond binding patterns, and their receptor binding characteristics [21]. It has been established that Lf receptors (LfR) and Tf receptors (TfR) are present on the (BBB) in various species and participate in the transport of Lf and Tf across the BBB, both in vitro and in vivo. While Tf and TfR demonstrate a high affinity and exhibit multiple strong interactions that hinder their dissociation, leading to a significant proportion of nanoparticles being captured in lysosomes, Lf and its carrier can undergo unidirectional transport into the brain after binding

ISSN 2706-6819 Vol.5, Issue 11: 39-45, DOI: 10.25236/IJFM.2023.051106

to LfR. During this process, they effectively escape from lysosomes within the BBB endothelium, and their transport efficiency is much higher than that of Tf. Additionally, lactoferrin possesses the capability to reverse neuronal iron death, making it more promising in the treatment of certain diseases like diabetic encephalopathy [22]. These characteristics collectively highlight the advantages of Lf in the field of nanomedicine when compared to similar proteins.

3. Application of LF in Nanotechnology

Nanocarriers are nano-sized structures that can encapsulate a drug and precisely control its release when attached to a diseased site, and they should have the ability to enhance the bioavailability of the encapsulated drug and improve its pharmacokinetic profile. LF has many advantages that have prompted its widespread use in nanocarrier preparation. It is one of the few proteins with a net positive charge (pI: 8.0-8.5) under physiological conditions, and this high pI value makes LF positively charged over a wide pH range [23]. Moreover, LF is relatively stable in the intestine and has many intestinal receptors, which can improve the absorption and bioavailability in circulation of orally administered LF-based nanocarriers and enhance the encapsulated active within these nanoparticles molecules [24]. The presence of amino groups at the end of LF makes it easier to connect with other carriers (e.g. , starch, lipids) thus expanding the choice of carriers while they perform their targeting functions.

Adriamycin (DOX) is a potent anticancer drug with cytotoxic properties, but according to reports, DOX not only has limited oral absorption but also has extensive toxicity to the heart and spleen. LF receptors are highly expressed on the surface of cancer cells, so sol-gel-oil chemistry was used to prepareDOX-loaded LF nanoparticles to reduce the toxic effects of DOX. DOX-loaded LF nanoparticles have a particle size of about 68 nm and have good physical stability, with only 2.5%~5% drug loss over 3 months and very low hemolysis rate (<2%), which indicates that they have negligible damaging effect on erythrocyte membranes [25]. After oral administration to rats with hepatocellular carcinoma, the nanoparticle group showed enhanced anti-tumor efficacy due to its better bioavailability and liver tumor targeting ability. And it did not show any side effects of DOX, such as weight loss, cardiosplenic toxicity, and hepatorenal toxicity. This further confirms the safety and biocompatibility of nano formulations.

In another study, LF nanoparticles were used to encapsulate the antiviral drug zidovudine, which is an effective antiviral drug with good bioavailability (50~75%), but it has side effects such as myelosuppression, neutropenia and organ toxicity. To overcome these problems, LF nanoparticles loaded with zidovudine were prepared by sol-gel-oil chemistry, and the prepared nanoparticles had a particle size of about 50~60 nm with good physical stability and high drug loading rate at both room temperature and 4℃, and the particle size and loading rate did not change significantly with time. In vitro release studies showed little drug release from the nanoparticles in both simulated gastric and intestinal fluids, i.e., LF nanoparticles had good stability under extreme conditions and exhibited the same therapeutic effects and lower organ toxicity after oral administration, indicating that the nanopreparations are safe nanocarriers for enhanced drug delivery [17].

Efavirenz, a non-nucleoside reverse transcriptase inhibitor, is widely used in the treatment of HIV, but has the disadvantages of low bioavailability and high toxicity, which limit the use of efavirenz, so LF nanoparticles loaded with efavirenz were prepared by a sol-gel-oil method to reduce toxicity and increase efficacy. In vitro drug release studies showed that maximum drug release was observed at pH 5, while minimum drug release was observed in simulated gastric and intestinal fluids, which confirmed its stability in the gastrointestinal environment and hence its suitability for oral administration. In vitro studies showed that the anti-HIV- 1 activity of drug-loaded nanoparticles was increased twofold compared to the free drug and LF nanoparticles loaded with efavirenz showed a stronger pharmacokinetic profile and reduced the toxic side effects of the drug [26]. In another study, efavirenz and curcumin co-loaded LF nanoparticles were prepared by sol- gel-oil chemistry for use as multiprophylactic agents. In vitro drug release studies showed that the maximum drug release was observed to be about 80% at pH 5 and about 10% at pH values below 4 and above 6. In a vaginal environment with a pH range of 4 to 4.5, this release property of LF nanoparticles results in a slow release of both drugs in a normal vaginal environment, and when a viral infection occurs in the vagina, where the pH value exceeds 4.5, the drugs will be released more rapidly to kill the virus quickly. In addition, these dual-loaded nanoparticles exhibited good physical stability when incubated in PBS (pH = 7.4) at 4℃ and 25℃. When the formulation was applied to rats as a vaginal irrigation, not only did it have no effect on the viability of normal vaginal flora compared to the free drug, but the amount of delivered drug was significantly increased and showed a better pharmacokinetic profile with significantly lower toxicity levels in vaginal tissues. These results suggest that this dual-action topical formulation can act simultaneously as an anti-

ISSN 2706-6819 Vol.5, Issue 11: 39-45, DOI: 10.25236/IJFM.2023.051106

HIV- 1 and antibacterial agent [27]. Another LF nanoparticles loaded with zidovudine/ efavirenz/lamivudine, prepared by sol-gel-oil method, also showed stronger in vitro anti-HIV activity and better in vivo drug bioavailability and pharmacokinetic profile, and reduced tissue inflammation. This shows the multiple advantages of triple-loaded LF nanoparticles as anti-HIV nanoformulations [28].

Another nanoparticulate albumin paclitaxel prepared by the nanoalbumin binding technique was approved by the FDA in 2006 as the first nanotechnology-based chemotherapeutic drug, a method that allows the encapsulation of highly lipophilic drugs into protein nanoparticles to increase their hydrophilicity, showing better efficacy in patients with metastatic breast cancer in whom conventional combination therapies have failed [29]. LF-loaded garcinia cambogia and LF- loaded oleanolic acid [30] nanoparticles were prepared using this technique, respectively, which not only improved the water solubility of the otherwise hydrophobic drugs but also showed good slow release in simulated gastric and intestinal fluids of the drugs, suggesting that these nanocarriers are suitable for oral drug delivery. Cellular experiments showed that the therapeutic effects of LF-loaded drugs were almost identical to those of free drugs, while LF nanoparticles significantly improved the bioavailability and pharmacokinetic characteristics after oral administration. The use of nanocarrier technology can increase the oral absorption of hydrophobic drugs and improve the therapeutic efficacy of the drugs, thus reducing the possible hazards caused by intravenous administration.

In another study, a highly stable micelle formed by anamphiphilic copolymer synthesized from LF and zeatin was prepared. The micelles can self-assemble in aqueous media, have low critical micelle concentration and prolong drug retention time in the circulatory system, and are widely used in drug delivery [17]. Compared to unstable and toxic surfactant polymeric micelles, LF can be used as an alternative and have low immunogenicity, high biocompatibility and active targeting capabilities. Encapsulation of rapamycin and baicalein in these micelles formed dual drug cross- linked micelles through cross-linking reactions, which showed superior anti-cancer effects in vivo by improving cytotoxicity and enhancing cellular uptake against MCF-7 human breast cancer cells compared to single drugs [6]. It has also been reported that these micelles were used to prepare dasatinib-enriched magnetic micelles that enhanced the serum stability of the drug, increased toxicity to cancer cells, and reduced migration and p-c-Src protein expression levels of cancer cells in the presence of an external magnetic field [31]. Further encapsulation of these micelles in sodium alginate microspheres possessed sustained release and enhanced their cytotoxicity against MDA- MB-231 human breast cancer cells [32]. These results suggest that LF nanoparticle-loaded structures are a promising drug delivery system for applications.

4. Application of LF as ananocarrier target

4.1. Targeting the Brain

The tight junctions between brain endothelial cells and low cytosolic drinking activity, as well as the presence of efflux pump systems and inactivating enzymes, which hinder the transport of drugs and gene fragments across the blood-brain barrier (BBB) to the brain. A large number of LF receptors are present on the surface of the BBB, while LF receptors such as LDL receptor- associated protein 1 and LDL receptor-associated protein 2 are also expressed in microvessels and neurons. LF has a positive charge in the physiological pH environment, which facilitates its uptake by negatively charged brain capillary endothelial cells, making LF a suitable target for brain targeting and even neuronal targeting. In contrast to classical transferrin (TF) targets, endogenous LF has a lower plasma concentration and LF is transported in a unidirectional manner through a single layer of brain capillary endothelial cells. These characteristics lead to a greater accumulation of LF in the brain compared to TF, which is its advantage over conventional targets. In a recent study reporting the vascular system of a human astrocytoma xenograft model [33], the investigators found that 97% of nanoparticles could cross the endothelium rather than through a non-passive extravasation transport mechanism through the interendothelial gap as previously thought, a phenomenon that suggests that LF-modified nanocarriers enter the brain through brain capillary endothelial cells, where nanoparticles bind to endothelial cells and are then transported through vesicles vesicles and cross-cellular channels into the brain. Compared to untargeted nanoparticles, LF-targeted stigmasterol nanoparticles are more concentrated in brain cells through cytokinesis [34]. In another study for brain delivery drug carriers using LF-modified cationic liposomes (LF-PCLs), the addition of LF around PCL particles increased their brain uptake in vitro and in vivo, and LF greatly increased the uptake of LF-PCLs by the brain through receptor-mediated endocytosis [35], which shows the promise of LF as a brain-targeted drug carrier target.

ISSN 2706-6819 Vol.5, Issue 11: 39-45, DOI: 10.25236/IJFM.2023.051106

Another feature of LF is that LF can deliver carriers to most neuronal cells in the brain, and the corresponding LF receptors are also detected on the surface of mature neurons and astrocytes. Meanwhile, LF plays a key role in various neural pathways in both the CNS and brain physiology, and LF deficiency or abnormal function may lead to the development of typical CNS diseases such as Parkinson's disease, Alzheimer's disease, and brain tumors. LF has neuroprotective functions, including immunomodulatory effects and neuroredox regulation. A study successfully linked polyamide-polyethylene glycol (PAMAM-PEG) gene vectors with LF to generate nanocarriers with brain-targeting ability, showing that LF can be used as an effective target head for delivery of non-viral genes to the brain, and the results showed that PAMAM-PEG gene vectors were localized faster in the brain after linking LF, which has important implications for the treatment of neurological diseases. This has important implications for the treatment of neurological diseases. Another experiment synthesized LF-linked metal nanoparticles with antioxidant enzyme activity, which improved behavioral performance and neuronal damage in a mouse model of Parkinson's disease, effectively scavenged ROS and protected cells from oxidative stress, suggesting its application in the treatment of neurodegenerative diseases. An LF-modified PEG-PCL nanoparticle administered via the nasal cavity could delay the progression of Alzheimer's disease, and its cellular experiments showed that LF-modified nanoparticles accumulated in the brain and neuronal cells much more than before LF modification, which provided a non-invasive way for nanoparticles to cross the BBB and facilitate drug entry into the central nervous system [36]. LF-bound nanoparticles enhances the uptake of nanoparticles by the brain and improves nanoparticle transport. Compared to non-targeted nanoparticles, LF-bound nanoparticles showed more efficient uptake in cerebrovascular endothelial cells versus neuronal cells and increased distribution in brain parenchyma, especially in cortical, substantia nigra and striatal regions. LF becomes the best choice of target head in nanodrug carriers for the treatment of central nervous system diseases such as Parkinson's and Alzheimer's disease [37].

4.2. Targeting cancer cells

LF is able to bind to Toll-like receptor 4 (TLR4) expressed in tumor cells to internalize and initiate various signaling processes in macrophages and tumor-associated macrophages (TAM) associated with solid tumor progression [38], LF can interact with cancer-associated cell surface receptors, a feature that makes it a preferred target for targeted drug delivery. Various combinations of drug carriers have been developed, including mannosylated LF nanoparticles, complexes of LF coupled to the phthalocyanine-based photosensitizing dye IRDye700DX, and LF nanoparticles coupled to doxorubicin. Metal-based delivery systems such as LF-coupled FePt alloy nanoparticles and cisplatin-loaded LF have also been developed for targeted drug delivery into cancer cells. LF-based features allow effective targeting to different cancers: the LF receptor is overexpressed in gliomas and LF also has an affinity for capillary endothelial cells in the BBB, allowing it to enter the brain and target to gliomas via transcytosis. LF has been shown to be overexpressed in hormonally downregulated breast cancer cells, and for breast cancer patients who are not responding to hormone therapy LF-modified nanoparticles offer a new therapeutic strategy, and LF can also be used as a target for nanoparticles targeting triple-negative breast cancer cells. LF also exhibits protective properties against hepatocytes and has the ability to inhibit liver fibrosis [39], which allows LF to be used as a target for therapeutic agents for liver cancer. Due to the inhibition of VEGF-A165-mediated lung cancer as well as inflammation shown by LF ability, it has also been used as a target for lung cancer delivery systems.

5. Summary

In summary, LF is a cationic iron-binding glycoprotein with multiple bioactivities. LF can achieve its various functions such as antibacterial, antiviral, anticancer, anti-inflammatory, antioxidant and immune stimulation by binding or releasing iron ions. Due to its multiple bioactivities and good biocompatibility, LF is widely used in baby food, cosmetics and food additives. The unique ability of LF to interact with receptors expressed on brain microcapillary epithelial cells, neuronal cells, cancer cells and tumor-associated macrophages makes it a good choice as a ligand for many targeted drugs. These unique abilities make it an excellent choice for the development of vectors for targeted drug delivery across the BBB. Vectors can target neurons after crossing the blood-brain barrier to treat central nervous system diseases. Vectors can also target a variety of cancer cells, including glioblastoma, hormone-downregulated breast cancer cells, liver cancer cells, and lung cancer cells for cancer treatment. In this paper, several drug delivery systems developed based on LF are presented, such as glycosylated LF nanoparticles, LF-conjugated FePt alloy nanoparticles, and cisplatin-loaded LF nanoparticles. Although the stability of LF-related nanoparticles and the optical penetration ability in tissues still need further

ISSN 2706-6819 Vol.5, Issue 11: 39-45, DOI: 10.25236/IJFM.2023.051106

optimization, the stability, hydrophilicity and advantages of LF as a carrier for targeting various types of cancers as well as the central nervous system are still unparalleled and should be the focus of future research and development.

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