Postprint of the Application of Human Serum Albumin in Long-acting Formulation of Protein and Peptide Drugs

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Abstract

Recombinant protein/peptide drugs exhibit a relatively short half-life in human serum, which substantially limits their clinical applications. Human serum albumin (HSA) offers advantages including a prolonged half-life, excellent biocompatibility, and low immunogenicity, rendering it an ideal drug carrier. Various HSA-based protein drug half-life extension technologies have been extensively applied and developed, currently encompassing primarily the construction of HSA fusion proteins, covalent conjugation to HSA via chemical bonds, and reversible non-covalent binding to HSA. This review summarizes the recent advances in albumin-based drug half-life extension technologies, the merits and drawbacks of each approach, and the current status of drug development.

Full Text

Application of Human Serum Albumin in Half-Life Extension of Protein and Peptide Drugs

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Abstract

Recombinant protein and peptide drugs exhibit short half-lives in human serum, which substantially limits their clinical applications. Human serum albumin (HSA) offers an ideal drug carrier due to its long half-life, excellent biocompatibility, and low immunogenicity. Various albumin-based strategies have been widely developed and applied to create long-lasting protein therapeutics, primarily including the construction of HSA fusion proteins, covalent chemical

conjugation to HSA, and reversible non-covalent binding to HSA. This review summarizes recent advances in albumin-based half-life extension technologies, discusses the advantages and disadvantages of each approach, and examines the current status of drug development employing these methods.

Keywords: Albumin fusion; linker; chemical coupling; modification with fatty acids; albumin-binding peptide

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Recombinant protein and peptide drugs offer numerous advantages over traditional small molecules, including high activity, low immunogenicity, and reduced toxicity, playing an increasingly important role in clinical disease treatment. However, conventional recombinant proteins and peptides exhibit short half-lives in human serum, necessitating frequent dosing that increases both economic burden and inconvenience for patients [1]. Extending the half-life of such drugs can improve patient compliance and facilitate self-management of chronic conditions, representing a key trend in next-generation protein and peptide therapeutics [2]. Current strategies for half-life extension include glycoengineering [3], PEGylation [4], human serum albumin fusion [5], transferrin fusion [6], and Fc fragment fusion [7].

Human serum albumin (HSA) is the most abundant soluble protein in human plasma, comprising 585 amino acids with a molecular weight of approximately 66.5 kDa. With a half-life of about 19 days in humans, HSA exhibits safety, nontoxicity, excellent biocompatibility, and low immunogenicity, making it an ideal drug carrier [8]. Various HSA-based half-life extension technologies have been widely applied and developed [9], currently encompassing three main approaches: construction of HSA fusion proteins, covalent conjugation via chemical bonds, and reversible non-covalent binding, which are detailed below.

1 HSA Fusion with Protein/Peptide Drugs

HSA fusion technology involves the recombinant expression of a chimeric protein combining HSA with a therapeutic protein or peptide. Leading companies in albumin fusion technology include GSK, Teva, Cogenesis, and CSL Behring. To date, two albumin fusion drugs have been approved for market: GSK' s GLP-1-HSA product Tanzeum (albiglutide) and CSL Behring' s Factor IX-HSA product Idelvion (Table 1). Native human GLP-1 peptide has an extremely short half-life (1.5–2.1 min) [10], requiring extension to become a viable therapeutic. Albiglutide achieves this by mutating the 8th amino acid of GLP-1

to glycine (8Gly), tandemly linking two copies, and fusing them directly to the N-terminus of mature HSA, extending the half-life to 5 days and enabling once-weekly dosing [11]. Idelvion is an albumin-fused coagulation factor IX that prolongs the half-life of recombinant FIX from 17.2 h to 92 h (a 5-fold increase), allowing weekly administration and, after titration, dosing every two weeks [12]. Additionally, multiple albumin fusion drugs have entered clinical development (Table 2).

The primary advantage of HSA fusion is the genetic linkage of albumin to the target protein/peptide, eliminating the need for additional chemical modification and simplifying purification, preparation, and quality control. However, a major bottleneck is the potential interference with proper folding of the therapeutic protein [22] and steric hindrance from HSA that can impede the biological activity of the effector molecule [23], necessitating optimized linker design between HSA and the therapeutic moiety. Optimization strategies primarily involve selecting the fusion orientation and appropriate linker peptides.

1.1 Direct Fusion of Proteins/Peptides to HSA

Early fusion protein designs employed direct fusion without linkers. The marketed drug albiglutide, for instance, consists of two GLP-1 molecules with an alanine-to-glycine mutation at position 8, fused directly to the N-terminus of mature HSA [24]. Direct fusion avoids potential immunogenicity from additional peptide sequences but presents significant drawbacks. First, the N- or C-terminal fusion of HSA with effector domains may compromise proper folding and native conformation, impairing function [25]. Second, direct fusion can sterically shield the effector molecule from receptor binding, reducing drug activity. For example, the interferon- /HSA fusion protein Albuferon exhibits only 1/8th the biological activity (EC50 ratio) of the native protein, while the granulocyte colony-stimulating factor/HSA fusion protein Albugranin shows approximately 1/10th the activity. Consequently, researchers have introduced linker peptides between HSA and effector molecules to mitigate these structural and functional interferences.

1.2 Fusion via Linker Peptides

Linker peptides are sequences interposed between fused proteins or domains, ranging from a few to over a hundred amino acid residues. Various peptide sequences have been successfully employed as linkers to prevent interference between domains during folding. Three major types have been extensively studied: flexible linkers that adopt random coil conformations, rigid linkers that form stable helical structures, and cleavable linkers that can be processed in vivo [26]. The characteristics of these linkers are summarized in Table 3 .

1.2.1 Flexible Linkers Flexible linkers are used when the fused domains require a degree of freedom. Typically composed of small, flexible non-polar amino acids (e.g., glycine) or polar residues (e.g., serine, threonine) [27], these

linkers provide the necessary flexibility for drug action while preventing domain interference. The most commonly used flexible linker is the "GS" linker (GGGGS)n proposed by Huston et al., whose length can be optimized by adjusting the number of repeats [28]. Multiple proteins fused to HSA via this linker have achieved stable expression and activity. Other flexible linkers, such as KESGSVSSEQLAQFRSLD and EGKSSGSGSESKST, have been applied in constructing biologically active single-chain antibodies (scFv) [29].

1.2.2 Rigid Linkers Rigid linkers form stable secondary structures and are less flexible than their counterparts, more effectively separating functional domains. The most widely used rigid linker is the -helical linker (EAAAK)n, whose structure is stabilized by salt bridges between Glu and Lys residues [30]. Another rigid linker is proline-rich (XP)n, where X can be any amino acid (preferably alanine, lysine, or glutamic acid). The proline residues increase rigidity and more effectively separate protein domains [31].

1.2.3 Cleavable Linkers With conventional rigid or flexible linkers, the albumin and effector molecule remain permanently fused. While this extends half-life, potential drawbacks include steric hindrance, reduced bioactivity, and altered biodistribution. Cleavable linkers address these issues by dissociating the functional domains at an appropriate rate in vivo, thereby restoring therapeutic activity and biodistribution. This approach combines the benefits of half-life extension and controlled release, balancing pharmacokinetics and pharmacodynamics—a significant challenge. Cleavable linkers exploit unique metabolic processes and are cleaved under specific conditions (e.g., in the presence of reducing agents or proteases).

One approach utilizes the reduction of disulfide bonds in vivo. Introducing a disulfide bond into the linker allows it to be cleaved upon reduction, releasing the effector molecule [32]. Zhao et al. incorporated a disulfide cyclic peptide (CRRRRRREAEAC) between interferon- 2b and albumin. The two cysteines form an intramolecular disulfide bond, while the intervening sequence is sensitive to signal peptide processing proteases in the yeast secretory pathway. During expression, the linker is sequentially cleaved by Kex2 and Kex1/Ste13, removing all amino acids between the cysteines and leaving IFN- 2b and HSA connected via a disulfide bond (IFN-SS-HSA) [33]. Compared to IFN-HSA, IFN-SS-HSA showed an 82% decrease in AUC but a 47% increase in antiviral activity, demonstrating that cleavable linkers can eliminate steric hindrance and improve fusion protein bioactivity.

Another strategy involves incorporating protease recognition sequences (e.g., for thrombin, furin, or factor Xa) that slowly release the native therapeutic protein in vivo. For instance, fusing factor IX (FIX) to albumin via a "GS" flexible linker reduced its activity by 88%, likely because albumin hindered FIX interaction with other coagulation factors. The marketed Factor IX-HSA product Idelvion (rIX-FP) overcomes this spatial effect using a cleavable linker.

Schulte et al. introduced a sequence from the FIX N-terminal activation region (VSQTSKLT↓RAETVFPDV) as a linker between FIX and albumin (Figure 1 [Figure 1: see original paper]). During FIX activation, both the activation region and linker are cleaved by FVIIa or FXIa, releasing the FIXa molecule [34]. This increased clotting activity 10- to 30-fold compared to non-cleavable linker fusions while preserving albumin's half-life extension, prolonging FIX half-life 2.8- to 3.9-fold in rabbits and achieving similar extensions in cynomolgus monkeys and hemophilia B dogs [35-36].

2 Covalent Conjugation of HSA with Protein/Peptide Drugs

Beyond genetic fusion, albumin can be covalently linked to protein/peptide molecules through chemical conjugation, achieved by introducing chemical linkers that react with albumin. Compared to fusion expression, chemical conjugation offers the advantage of not being limited to N- or C-terminal attachment sites, though it suffers from non-specific modification and complex processes. ConjuChem developed the Drug Affinity Complex (DAC™) and Pre-Coupled Drug Affinity Complex (PC-DAC™) platforms for half-life extension [37, 38]. DAC™ is an in vivo HSA coupling technology where a protein/peptide is conjugated to a linker bearing reactive groups (N-hydroxysuccinimide esters, isocyanates, or maleimides) that rapidly and specifically react with the free thiol at Cys-34 of circulating HSA, forming a therapeutic molecule-albumin complex. PC-DAC™ is an ex vivo pre-coupling technology where the protein/peptide-linker conjugate is reacted with HSA in vitro to directly form the therapeutic complex. ConjuChem applied these technologies to GLP-1 and exendin-4. The DAC™ derivative CJC-1131 significantly extended GLP-1 half-life to 15–20 h in rats and 9–15 days in humans after coupling with endogenous albumin. The PC-DAC™ derivative of exendin-4, CJC-1134-PC, demonstrated a one-week half-life in Phase II trials, enabling weekly glucose control [39].

A major challenge of chemical conjugation is the difficulty of site-specific modification, generating heterogeneous conjugates that complicate quality control and downstream processing. Lim et al. achieved site-specific albumin conjugation of urate oxidase (Uox) using genetic code expansion [40]. They incorporated p-azido-L-phenylalanine at two predetermined positions in Uox and conjugated HSA' s C34 thiol to DBCO-PEG4-MAL via thiol-maleimide reaction to yield HSA-PEG4-DBCO. The azido groups on Uox then reacted with the dibenzocyclooctyne moiety via strain-promoted azide-alkyne cycloaddition (SPAAC) to produce a homogeneous, site-specific HSA-Uox conjugate (Figure 2 [Figure 2: see original paper]). While native Uox has a 1.3 h half-life in mice, the HSA-Uox conjugate extended this to 8.8 h.

3 Non-Covalent Binding of Protein/Peptide Drugs to HSA

Non-covalent association of peptide/protein drugs with albumin represents another effective half-life extension strategy [41]. This approach involves introducing an HSA-binding ligand (such as a small peptide or fatty acid) onto the therapeutic molecule. The reversible nature of non-covalent binding avoids the impact of albumin on drug bioactivity and biodistribution, truly achieving a balance between pharmacokinetics and pharmacodynamics.

3.1 Fatty Acid Modification

HSA possesses five fatty acid binding sites, enabling proteins/peptides modified with appropriate fatty acid chains to reversibly bind circulating albumin for half-life extension [42]. Novo Nordisk' s long-acting technology platform employs fatty acid chain modification, with marketed products including insulin detemir, liraglutide, insulin degludec, and semaglutide (Table 4), while fatty acid-modified long-acting growth hormone (somapacitan) is in Phase III trials [43-44]. Three key parameters define this technology: fatty acid chain length, terminal group, and linker. Native insulin has a 5–15 min half-life; insulin detemir, created by removing Thr at B30 and adding a myristic acid (C14) side chain to Lys at B29, extends half-life to 5–7 h [45]. Insulin degludec, featuring a 16-carbon diacid at B29 after B30 Thr removal, achieves a 25 h half-life [46]. Human GLP-1 has a 1.5–2.1 min half-life; liraglutide replaces Lys34 with Arg and adds a palmitic acid (C16) side chain to Lys26, extending half-life to 13 h [47]. Semaglutide builds upon liraglutide with systematic optimization, including an Arg34 mutation and Aib8 substitution, plus an 18-carbon diacid fatty chain at Lys26, achieving a 40 h half-life [48].

Compared to albumin fusion, fatty acid modification involves more complex chemical reactions, purification, and quality control, requiring careful consideration of several factors. First, site-specificity of modification remains a bottleneck for broader application. Lysine residues are crucial for linking proteins to chemical modifiers [49], but while insulin and GLP-1 have few lysines (1–2), most proteins contain multiple lysines with similar reactivity, yielding heterogeneous mixtures. Achieving single-site modification while minimizing non-specific products is a critical challenge. Second, detection and control of process-related impurities is essential. The complex fatty acid modification process introduces organic impurities; for example, insulin acylation requires solid-phase synthesis of the acylation reagent, dissolution in DMF, dropwise addition to insulin in Na CO solution at pH 11.2, and subsequent RP-HPLC purification [50]. Comprehensive tracking and analysis of these impurities is necessary to ensure product purity and quality.

3.2 Albumin-Binding Peptides

Beyond small molecules like fatty acids, specific peptide sequences (e.g., the albumin-binding domain ABD from streptococcal protein G) can bind albumin

[51] and can be identified through phage display screening. Dennis et al. used phage display to isolate albumin-binding peptides with the core sequence DICLPRWGCLW; fusing this peptide to an antigen-binding fragment (Fab) increased its half-life 37-fold in rabbits [52]. Ablynx applied albumin-binding peptides to extend nanobody half-life, developing the trispecific antibody ATN-103 (ozoralizumab) comprising two TNF -targeting nanobodies and one HSA-binding nanobody, which has completed Phase II trials for rheumatoid arthritis [53]. Genentech' s albumin-binding fragment technology employs similar principles; fusing an albumin-binding peptide to trastuzumab' s Fab fragment increased albumin affinity 10-fold, significantly reducing plasma clearance and extending half-life 6-fold in mice and rabbits while preserving antigen (HER2) binding activity [54].

Human serum albumin can be utilized for half-life extension of protein and peptide drugs through either chemical modification or genetic fusion. Compared to chemical modification, albumin fusion offers several advantages: it eliminates additional chemical steps, yields more homogeneous products, and simplifies manufacturing and scale-up. However, conventional albumin fusion often sacrifices pharmacodynamic properties to improve pharmacokinetics due to HSA' s steric effects. Fusion proteins not only exhibit reduced in vitro biological activity but also show fundamentally altered biodistribution, with significantly impaired penetration into peripheral target organs and tissues, limiting therapeutic efficacy. This represents the major bottleneck for HSA fusion in half-life extension. Cleavable albumin fusion technology offers a solution to this pharmacokinetic-pharmacodynamic 矛盾矛盾. The 2016 approval of rIX-FP, featuring a carefully designed cleavable linker between HSA and FIX that preserves both albumin' s long half-life and the coagulation factor' s native function, brings new promise to albumin fusion technology. Chemical modifications like fatty acid acylation enable reversible albumin binding that balances half-life extension and biological activity but face challenges with complex processes and non-specific modification. Future research must advance site-specific modification technologies to achieve homogeneous products with minimal off-target effects.

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