High-Resolution Analytical Characterization of Optibumin®: Structural Homogeneity and Functional Consistency Compared to Plasma-Derived HSA

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APPLICATION BENEFITS

- ***Optibumin® reduces aggregate burden in final formulations***
- ***Improves lot-to-lot consistency in conjugation and binding workflows***
- ***Preserves native Cys34 thiol for controlled maleimide chemistry***
- ***Maintains high drug- and ligand-binding capacity***
- ***Eliminates variability introduced by plasma-derived stabilizers***

INVITRIA SOLUTIONS

Optibumin 25 - Recombinant human serum albumin



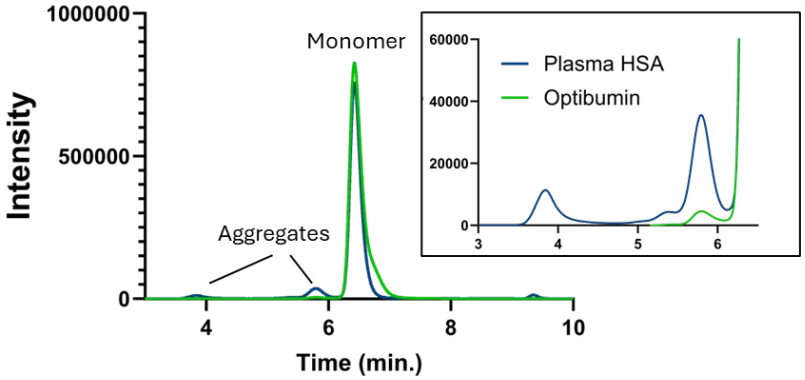
EXECUTIVE SUMMARY

Raw material consistency is critical to reproducible biopharmaceutical manufacturing and cell culture performance. Human serum albumin (HSA) is widely used as a functional excipient, yet plasma-derived HSA (pHSA) exhibits structural heterogeneity and lot-to-lot variability that can affect downstream applications. Structural homogeneity directly influences ligand binding and conjugation performance.

This application note presents a high-resolution analytical characterization of Optibumin, a chemically defined, animal-origin-free recombinant human serum albumin. Using SEC, IEX-HPLC, RP-HPLC, and functional binding assays, we primarily compare Optibumin to plasma-derived HSA, with additional comparisons to selected commercially available recombinant HSA sources. The data demonstrate Optibumins structural homogeneity, low aggregate burden, predominantly reduced Cys34, and preserved binding function, supporting consistent performance across production lots.

Key Highlights

- <0.5% total aggregates by high-resolution 3 µm SEC
- ~99% reduced Cys34 across 10 production lots
- Single-peak homogeneity by IEX-HPLC with no detectable charge variants
- ~3-fold higher maleimide conjugation efficiency vs pHSA
- ~3-fold higher binding affinity (K_d 2.58 µM vs 7.45 µM)



High-resolution SEC reveals distinct separation of monomer and aggregate species in Optibumin compared to plasma-derived HSA.

INTRODUCTION

The Evolution of Albumin Sourcing

The trajectory of albumin sourcing reflects the broader evolution of biotechnology: a progression from biological variability toward defined engineering precision. For decades, the industry has relied exclusively on plasma-derived HSA (pHSA), a byproduct of the fractionation of human blood. While abundant, pHSA is intrinsically heterogeneous. It is a biological record of the donor population, influenced by diet, disease state, pharmaceutical consumption, and age. Furthermore, the mandatory safety steps required to render blood products safe, specifically pasteurization at 60°C for 10 hours, impose severe thermal stress on the molecule, necessitating the addition of lipid stabilizers that alter its biophysical properties.

The advent of recombinant DNA technology promised a solution. First-generation recombinant albumins, expressed primarily in yeast hosts like *Saccharomyces cerevisiae* or *Pichia pastoris,* successfully addressed the risk of blood-borne pathogens. However, these systems introduced new complexities. Yeast-derived albumins often require extensive purification to remove immunogenic host cell proteins (HCPs) and are frequently formulated with surfactants like Polysorbate 80 to prevent aggregation during processing and storage.

Optibumin represents the next generation of recombinant technology. Expressed in a scalable non-mammalian system, it leverages the natural protein-storage machinery to produce albumin in a highly stable, protected environment. This system is inherently free of mammalian pathogens and prions. The expression system allows for the production of a "native-state" molecule that does not require harsh stabilizers or surfactants that compromise the functionality of yeast and plasma-derived alternatives.

The Functional Imperative

In modern bioprocessing, albumin is rarely used solely for its bulk osmotic properties. It is employed as a precise functional excipient. In the expansion of T-cells and Mesenchymal Stem Cells (MSCs), it serves as a lipid carrier and ROS scavenger. In diagnostic immunoassays, it acts as a blocking agent that must cover surfaces uniformly without interfering with specific binding events. In antibody-drug conjugates (ADCs), it functions as a scaffold where defined stoichiometry is paramount. Furthermore, albumin is increasingly utilized in medical device coatings to provide a biomimetic layer that enhances lubricity while imparting essential hemocompatibility and antithrombogenic properties to blood-contacting surfaces.

In these high-value applications, the "batch effects" associated with pHSA, where one lot performs perfectly and the next fails, are unacceptable. The industry requires a reagent that is chemically defined and strictly controlled. This report provides analytical evidence that Optibumin meets these rigorous demands, directly comparing its structural and functional characteristics to plasma- and yeast-derived HSA.

Accurate assessment of albumin structural integrity therefore requires analytical methods capable of resolving low-level aggregates and charge variants with sufficient sensitivity.

RESULTS AND DISCUSSION

Resolution Limitations in Standard SEC Methods

Protein aggregation is a critical quality attribute (CQA) that directly impacts the safety and efficacy of biologic products. Aggregates can serve as nucleation sites for further instability, reduce the effective concentration of the active protein, and, most critically, induce immunogenicity by breaking B-cell tolerance (Ratanji et al., 2014). Detecting these aggregates is the primary function of size exclusion chromatography (SEC). However, the ability to detect aggregates is entirely dependent on the resolution of the method employed.

The Limitations of the USP Standard

The United States Pharmacopeia (USP) monograph for "rAlbumin Human" and General Chapter "Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies" have long established the industry standard for SEC (United States Pharmacopeia [USP], 2017). These methods typically specify the use of an L59 packing material, a silica-based hydrophilic resin, with a particle size of 5 µm.

While robust and widely accessible, 5 µm SEC columns have inherent resolution limitations. Larger

particle sizes reduce column efficiency and increase band broadening, limiting separation of closely eluting species.

In albumin analysis, this limitation is critical. The hydrodynamic radius of an albumin dimer is ~5.2 nm (MW ~130 kDa) which is only modestly larger than that of the monomer at ~3.8 nm (MW ~66.5 kDa) (Ghuman et al., 2005). On a 5 µm column, the dimer peak frequently elutes as a shoulder on the leading edge of the monomer peak rather than as a fully resolved species.

Additionally, the USP rAlbumin Human monograph specifies an injection mass of 2 mg (50 µL injection volume of a 40 mg/mL solution). Because SEC is a non-binding technique, peak shape and resolution are particularly sensitive to injection parameters. High protein concentration increases local viscosity, and when the less viscous mobile phase starts to interact with the loaded sample, an effect occurs called “viscous fingering” (Rousseaux et al., 2007). This effect results in peak broadening, poor resolution and the potential for the monomer peak to mask the dimer peak.

Artificial Monomer Inflation

Under lower-resolution SEC conditions, incomplete separation of dimer species from the monomer peak can lead to what we describe as “artificial monomer inflation.” When dimers are not fully resolved, integration algorithms—particularly those using perpendicular drop methods—may incorporate the dimer shoulder signal into the monomer peak area. This results in overestimation of apparent monomer purity and underreporting of low-order aggregates.

High-resolution SEC using 3 µm particle size resins mitigates this limitation by physically separating dimer species from the monomer peak, creating a distinct valley between peaks and enabling accurate quantification of aggregate content.

Reported monomer purity values are therefore method-dependent and must be interpreted in the context of column resolution and injection conditions.

3 µm High-Resolution SEC

To rigorously validate the structural integrity of Optibumin, InVitria employs a more stringent analytical method utilizing a 3 µm particle size resin; moreover, the injection parameters, volume and concentration, are set in such a way to ensure resolution between the monomer and any aggregates. Reducing the particle size from 5 µm to 3 µm significantly increases the theoretical plate count, resulting in sharper, narrower peaks. This enhanced efficiency provides the resolution necessary to physically separate the dimer species from the monomer, creating a distinct "valley" between the peaks. Under these conditions, any aggregates present are clearly visible and accurately quantified.

When subjected to this high-resolution SEC analysis, the difference between Optibumin and clinical-grade pHSA is clearly observable. The chromatograms in **Figure 1A** reveal that Optibumin elutes as a single, symmetrical monomeric peak. The baseline in the region corresponding to high molecular weight species

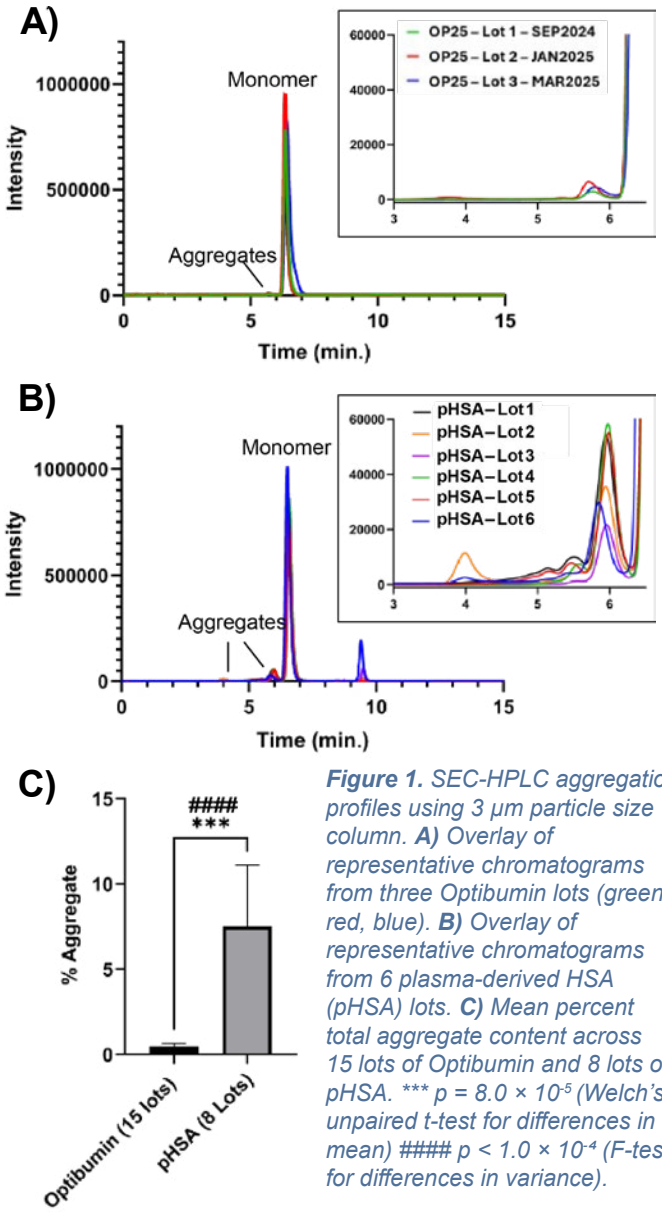


Figure 1. *SEC-HPLC aggregation profiles using 3 µm particle size column.* ***A)*** *Overlay of representative chromatograms from three Optibumin lots (green, red, blue).* ***B)*** *Overlay of representative chromatograms from 6 plasma-derived HSA (pHSA) lots.* ***C)*** *Mean percent total aggregate content across 15 lots of Optibumin and 8 lots of pHSA. *** $p = 8.0 \times 10^{-5}$ (Welch’s unpaired t-test for differences in mean) #### $p < 1.0 \times 10^{-4}$ (F-test for differences in variance).*

is flat. In contrast, **Figure 1B** shows the pHSA samples which display significant absorbance in the pre-monomer region indicating a heterogeneous mixture of oligomeric species. Optibumin contains less than 0.5% total aggregates (**Figure 1C**, **Table 1**), a value that approaches the limit of detection even for high-resolution methods. In contrast, clinical-grade pHSA contains an average of 7.5% aggregates (**Figure 1C**, **Table 1**). When analyzed with sufficient resolution to accurately quantitate aggregates, it becomes clear that using pHSA introduces a substantially higher aggregate fraction that is introduced into any downstream process.

Table 1: *Comparative Analysis of Aggregates via High-Resolution SEC-HPLC.*

Metric	Optibumin (Recombinant)	Plasma HSA (Clinical Grade)	Statistical Significance
Mean Aggregate Fraction (dimer + HMW)	0.469%	7.516%	Welch's t-test ($p = 8.0 \times 10^{-5}$)
Standard Deviation (Inter-lot)	0.20%	3.57%	F-test ($p < 1.0 \times 10^{-4}$)
Variance Factor	1x (Baseline)	~10x Higher Variability	

Furthermore, Optibumin demonstrates low inter-lot variability. The standard deviation of the aggregate fraction is just 0.20%, whereas pHSA varies by 3.57%, a more than 10-fold difference in variability (**Table 1**). This implies that a user of pHSA might receive one lot with 4% aggregates and the next with 11%, a fluctuation that may impact sensitive applications like cell culture expansion or conjugation efficiency.

Orthogonal Confirmation of Aggregates

To validate the SEC findings, two additional cornerstone techniques for protein separation were employed: Capillary electrophoresis (CE) and polyacrylamide gel electrophoresis (PAGE). When run in reduced and denatured conditions (i.e., CE-SDS and SDS-PAGE), both techniques separate proteins based on their hydrodynamic size in an electric field; however, when run in conditions that do not reduce or breakdown aggregates (i.e., Native-PAGE), these techniques separate proteins based on their native charge, size and hydrodynamic radius (shape). These techniques provide a separation mechanism distinct from the size-sieving effect of SEC and provide visual and quantifiable confirmation of the aggregation state of albumin.

Quantification of Aggregates via Capillary Electrophoresis

The CE electropherograms for Optibumin show a clean monomeric band running just below 66 kDa, with negligible signal in the high-molecular-weight region (**Figure 2A**). Conversely, analysis from four sources of pHSA exhibit characteristic "smearing" above the monomer band, along with distinct peaks corresponding to dimers and trimers (**Figure 2A**). Area per loaded mass quantification of the aggregate peak for Optibumin was 39,000 area/µg of loaded mass on average, whereas the pHSA samples averaged 403,000 area/ µg of loaded mass (**Figure 2B**). Nearly identical to the SEC findings, this represents a 10-fold reduction in aggregate burden ($p<0.05$) for Optibumin. The concordance between the high-resolution SEC and CE data confirms that the superior homogeneity of Optibumin is a physical reality, not an analytical artifact.

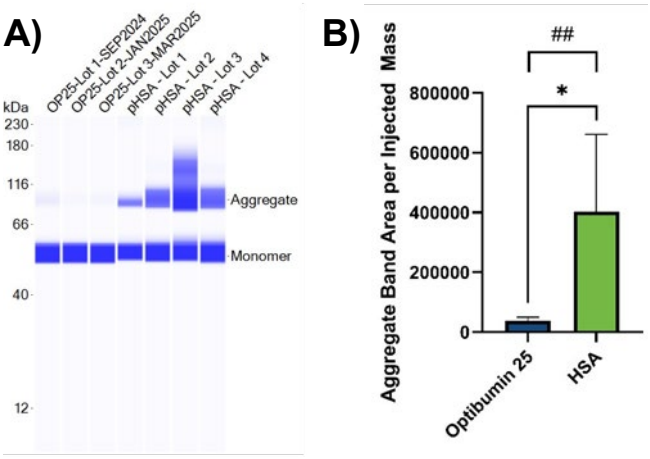


Figure 2. *Characterization of aggregation in Optibumin and plasma-derived HSA via capillary electrophoresis (CE).* ***A)*** *Native CE electropherogram of three Optibumin lots and four pHSA lots. Lane 1: molecular weight marker, Lanes 2-8: samples prepared at 0.5 mg/mL albumin concentration.* ***B)*** *Quantitative comparison of aggregate band area normalized to injected mass for Optibumin and pHSA * $p < 0.05$ (Welch's t-test for differences in mean); ## $p < 0.01$ (F-test for differences in variance).*

Native-PAGE and Understanding Product Versus Process Impurities

While harder to quantify, time-consuming, and considered outdated in many laboratory settings,

Native-PAGE is the gold standard for visualizing a product's purity. In fact, the USP rAlbumin Human monograph specifies testing "purity" by Native-PAGE. The guidance outlines loading and running 20 µg of rHSA product on the gel. This mass of protein undoubtedly saturates the main rHSA monomer band on the gel, but the intent is to show any low molecular weight bands which may be present: "If bands are present below the main albumin band in either or both lanes, quantify the relative amount, in ng, of protein present in each band against the calibration curve" (USP, 2017).

When running pHSA alongside Optibumin per the USP guidance (**Figure 3A**), both products would claim "100 %" per the USP; however, it is visually evident by the smearing and banding pattern above the pHSA main band that there are numerous aggregate impurities of pHSA. This is also the case when analyzing other rHSA manufactures alongside Optibumin (**Figure 3B**) (Oettl & Stauber, 2007; Olsen et al., 2004). Even when loading markedly less mass on the gel in the first four lanes, the rHSA USP reference standard material starts to show aggregates. This result begs the question: what does "purity" mean in the sense of a protein product?

"Purity" in the sense of a protein product can mean many things depending on how one chooses to classify the impurities. A process related protein impurity originates from the manufacturing process and is chemically distinct from the target protein: for example, a host cell protein or a protein-A fragment leached from resin. A product related protein impurity is derived from the product (i.e., target protein) and could be a fragment, aggregate or other variant caused by a post translation modification. High molecular weight HSA (i.e., aggregates) are never mentioned in the USP's Native-PAGE guidance for rHSA "purity", but that does not mean they are not present or that they should not be characterized as impurities.

Native Structural Homogeneity and Reduced PTM Burden

The manufacturing process of pHSA is inherently variable from the very beginning due to the starting biomaterial being plasma sourced from various healthy donors. Furthermore, the Cohn fractionation and viral heat inactivation steps used in pHSA manufacturing are generally considered a "harsh" environment for proteins. These steps are known to induce and isolate modified versions of human serum albumin (Oettl and Stauber, 2007).

Optibumin is manufactured from quality controlled, homogeneous starting material using low stress processing steps. The steps of the Optibumin process are specifically designed to isolate and produce a rHSA product that is structurally homogenous with reduced post translation modification (PTMs) burden. To show this, we have developed two analytical HPLC methods to show the modification state of the pHSA and Optibumin.

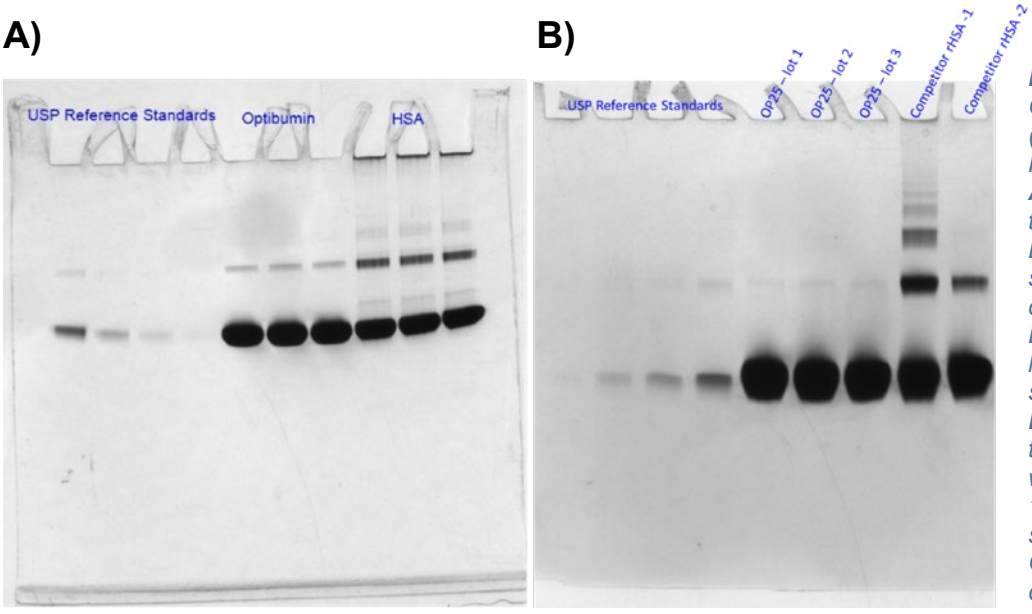


Figure 3. *Native-PAGE analysis of Optibumin, plasma-derived HSA (pHSA), and other recombinant HSA (rHSA) sources.*
A) *Native-PAGE gel run according to USP rAlbumin Human guidelines. Lanes 1–4: USP rHSA reference standards used for quantification of low molecular weight impurities. Lanes 5–7: single Optibumin lot loaded in triplicate. Lanes 8–10: single pHSA lot loaded in triplicate.*
B) *Native-PAGE gel run according to USP rAlbumin Human guidelines without replicate loading. Lanes 1–4: USP rHSA reference standards. Lanes 5–7: three Optibumin lots. Lanes 8–9: two commercially available rHSA sources.*

Analytical Characterization of Thiol Content via RP-HPLC

In the bloodstream, albumin acts as a sacrificial antioxidant. Over its several week half-life, the Cys34 thiol is progressively oxidized by circulating free radicals, converting mercapto-albumin, sometimes called HMA, into oxidized-albumin (Oettl and Stauber, 2007). This oxidized fraction includes mixed disulfides with cysteine or glutathione (reversible oxidation) and higher oxidation states like sulfinic or sulfonic acid (irreversible oxidation). The liver maintains albumin homeostasis by removing oxidized albumin from the bloodstream so that it can be replaced with unmodified, reduced albumin having a free Cys34 thiol group (i.e., mercapto-albumin) (Holte et al., 2023). As covered in numerous reviews (Oettl and Stauber, 2007; Wu et al., 2023) and with our own in vitro functional data later in this application note, the oxidation PTM is particularly detrimental to many of albumin's binding and carrier functions in the body.

To assess the redox state of Cys34 of Optibumin and pHSA, reverse-phase HPLC (RP-HPLC) was performed to separate the albumin species based on subtle differences in hydrophobicity induced by the oxidation of the cysteine residue. The RP-HPLC profile of Optibumin in **Figure 4A** displays a single, sharp, symmetrical peak at 13 minutes of retention time. This indicates that the population of albumin molecules is chemically homogeneous, with the Cys34 residue existing almost exclusively in the reduced state. Conversely, the chromatograms for pHSA are complex, showing split peaks and shoulders (**Figure 4A**). The main reduced-Cys34 peak at 13 minutes is accompanied by a significant secondary peak at 12 minutes representing the cysteinylated (oxidized) fraction.

To characterize the consistency of available reduced-Cys34, an analysis of 10 lots of Optibumin and 9 lots of clinical-grade pHSA were performed. **Figure 4B** and **Table 2** show that across the 10 lots of Optibumin there is 99% reduced Cys34 albumin on average, while the 9 lots of pHSA show only 60% reduced-Cys34 albumin on average. Moreover, Optibumin exhibits approximately ten-fold lower lot-to-lot variability in reduced-Cys34, with an inter-lot standard deviation of just 0.295% compared to 2.93% for pHSA's standard deviation.

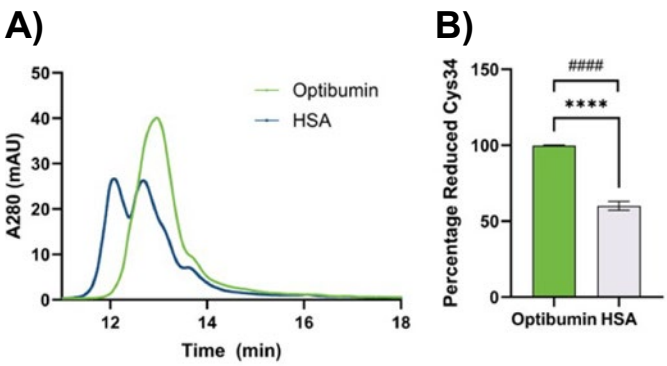


*Figure 4. Analytical characterization of Cys34 redox state in Optibumin and plasma-derived HSA by RP-HPLC. A) Representative chromatograms of Optibumin (green) and plasma-derived HSA (blue). B) Mean percentage of reduced Cys34 in across 10 lots of Optibumin 25 and 9 lots of plasma-derived HSA lots. **** $p = 1.0 \times 10^{-4}$ (Welch's unpaired t-test for differences in mean): #### $p < 1.0 \times 10^{-4}$ (F-test for differences in variance).*

Table 2: Comparative Cys34 Redox Consistency.

Metric	Optibumin (Recombinant)	Plasma HSA (Clinical Grade)	Statistical Significance
Mean Free Thiol (Cys34)	99%	~60%	Welch's t-test ($p < 1.0 \times 10^{-4}$)
Oxidized Fraction	~1%	~40%	
Inter-lot Standard Deviation	0.295%	2.93%	F-test ($p < 1.0 \times 10^{-4}$)
Variance Factor	1x	10x	

Charged Variant Analysis via IEX-HPLC

Parallel to the RP-HPLC, we wanted to further assess the level of PTMs in several lots of Optibumin and pHSA using Ion Exchange HPLC (IEX-HPLC). Whereas RP-HPLC uses differences in hydrophobicity for separation, IEX-HPLC separates HSA isoforms based on their net surface charge. With slight modifications, a previously detailed IEX-HPLC protocol was followed which uses an anion exchange resin and an increasing concentration of ammonium acetate at neutral pH to elute HSA (Leblanc et al., 2018). Modifications like deamidation and glycation typically add negative charge, causing species to elute later (acidic variants), while truncations can create basic variants that elute before the unmodified albumin.

(While beyond the scope of this application note, variants separated with this IEX-HPLC method have been previously characterized by mass spectrometry (Leblanc et al., 2018).

Analysis of three lots of Optibumin revealed a remarkably clean profile (**Figure 5A**). Optibumin elutes as a single main peak, accounting for essentially all detectable peak area under the conditions tested. No acidic or basic shoulders associated with deamidation or glycation were detected. This indicates that the Optibumin molecule is chemically homogeneous with respect to surface charge.

In contrast, all four lots of pHSA analyzed displayed a complex profile with several peaks representing several charged variants (**Figure 5B-E**). The chromatograms showed 3 to 4 distinct peaks, corresponding to a "Main" isoform surrounded by significant "Basic" and "Acidic" variants. The abundance of the "Main" isoform in pHSA was low and variable, ranging from 27% to 48% of the total protein. The "Acidic" fraction, likely representing glycated and deamidated species, constituted up to 53% of the total protein in some lots. Together IEX and RP-HPLC analysis indicate that Optibumin exhibits a single dominant species with minimal detectable PTMs.

The HSA Structure/Function Connection

The structure of a protein can shed light on its functional performance. **Table 3** summarizes the numerous incidents from literature showing how PTMs and aggregates of HSA can significantly impair its function. This structure/function framework provides the necessary context for our subsequent functional data, which demonstrates how the high structural integrity of InVitria's Optibumin recombinant albumin translates to improved functional performance compared to plasma-derived HSA.

Implications for Conjugation

As demonstrated in section "Analytical Characterization of Thiol Content via RP-HPLC", Optibumin contains 99% Cys34 thiol versus only 60% in pHSA. Under the conditions tested, this translated into improved conjugation efficiency. To evaluate this effect, we performed a comparative study using Alexa Fluor 488 C5 maleimide, directly comparing the conjugation efficiency of Optibumin and pHSA. Conjugation reactions were performed under

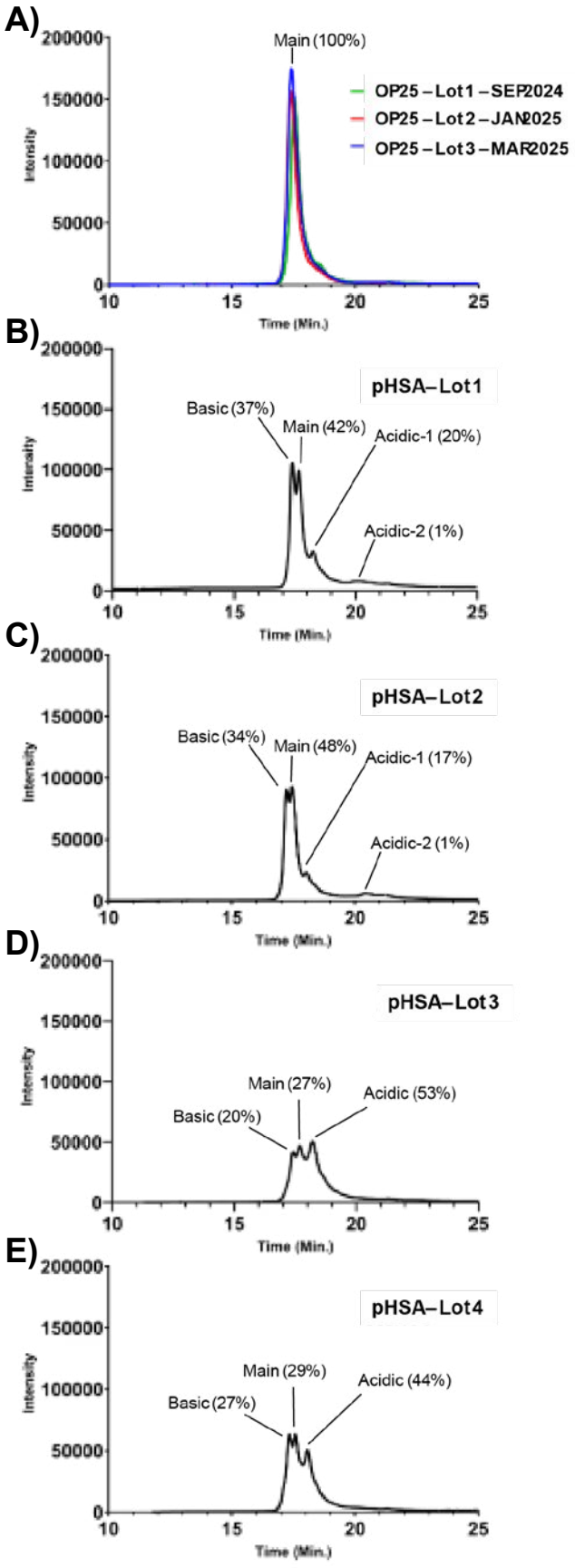
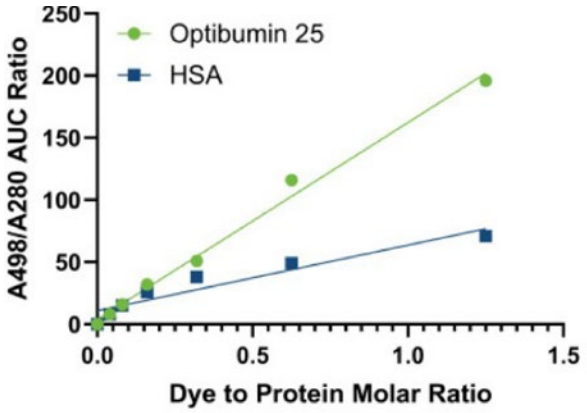


*Figure 5. Charged variant analysis of Optibumin and plasma-derived HSA by IEX-HPLC. **A)** Representative chromatograms of three Optibumin lots (green, red, blue). **B-E)** Representative chromatograms of four pHSA lots.*

controlled pH conditions using degassed buffer to minimize maleimide hydrolysis and reduce off-target amine reactivity (Kirkpatrick et al., 2024). Alexa Fluor™ 488 C5 maleimide contains a short carbon linker that facilitates access to the Cys34 thiol located within a surface pocket on albumin (Kratz et al., 2002). Proper reaction conditions are therefore essential to ensure selective thiol conjugation. Optibumin demonstrated a 3-fold higher conjugation efficiency compared to pHSA (**Figure 6**).



Albumin	*Slope*	*Intercept*	*R^2*
Optibumin 25	158.5	3.825	0.9920
HSA	52.74	10.92	0.9053

Figure 6. *Characterization of relative Cys34 conjugation efficiency in Optibumin and plasma-derived HSA (pHSA). Linear regression of the A498/A280 area-under-the-curve (AUC) ratio as a function of dye-to-protein molar ratio. Tabulated slope, intercept, and R^2 values are shown. AUC values were derived from RP-HPLC chromatograms.*

The extent of conjugation increased linearly (R^2=0.9920) across a broad range of dye-to-protein molar ratios, indicating that nearly every albumin molecule was available for reaction. pHSA, conversely, showed saturation at lower ratios and poor linear behavior (R^2=0.9053), reflecting its heterogeneous composition. Moreover, pHSA contains approximately 40% oxidized (non-reactive) albumin, which can act as a variable "sink" in the reaction mixture and may contribute to non-specific interactions, thereby reducing the fraction of reactive thiol available for controlled conjugation. In addition, pHSA exhibits approximately ten-fold greater lot-to-lot variability in reduced Cys34. This reduced reactive fraction complicates accurate dye-to-protein ratio calculations.

Many diagnostic reagents, drug linkers, and polymer conjugates rely on maleimide chemistry to covalently attach to the free thiol of albumin. For developers of Antibody-Drug Conjugates (ADCs) or albumin-binding prodrugs, consistency is critical. It ensures that the drug-to-protein ratio (DPR) can be tightly controlled, reducing batch failures and ensuring consistent pharmacokinetics in vivo.

Functional Implication for Molecule Binding

As demonstrated previously, when compared to pHSA, Optibumin has extremely low aggregates and PTMs. **Table 3** reviews the literature showing how these two factors affect HSA binding to ligands such as fatty

Table 3: *Albumin PTMs linked to functional performance.*

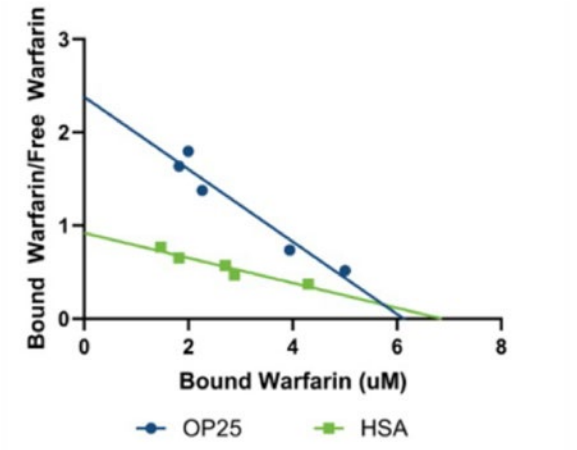
Albumin Form	Major Functional Change	Example	Reference(s)
Oxidized	Binding capacity	Metal ion	Ge et al., 2016
		Fatty acid	Ge et al., 2016
		Various Drugs	Anraku et al., 2001; Kawakami et al., 2006
		Fluorescent marker	Oettl et al., 2013
		Bilirubin	Oettl et al., 2008
	Receptor affinity	FcRn	Leblanc et al., 2019
	Transport	Fatty acid	Jalan et al., 2009
	Antioxidant	IMAR	Jalan et al., 2009
		Free radical scavenging	Kawakami et al., 2006
Glycated	Binding capacity	Fatty acid	Shaklai et al., 1984
		Drugs	Okabe et al., 1994; Baraka-Vidot et al., 2012; Joseph & Hage 2010
		Bilirubin	Shaklai et al., 1984
		Trp	Nakajou et al., 2003; Berzegar et al., 2007
	Receptor affinity	FcRn	Leblanc et al., 2019
Nitrated	Binding capacity	Fatty acids	Burczynski et al., 1995
Truncated	Binding/antioxidant	Metal ion/Free radical scavenging	Bar-Or et al., 2001; Naldi et al., 2013
	Receptor affinity	FcRn	Andersen et al., 2012; and Bar-Or et al., 2006
Aggregated	Binding capacity	Fatty acid	Chubarov et al., 2021

acids and drugs. This should translate directly into Optibumin having superior performance in drug binding assays.

To demonstrate this, we performed a comparative study using warfarin and compared the binding efficiency of Optibumin to that of pHSA. Albumin contains two primary high-affinity drug-binding sites: Sudlow Site I, also known as the "warfarin binding site," which binds bulky heterocyclic compounds, and Sudlow Site II, which binds aromatic carboxylates. To quantify the functional difference between pHSA and Optibumin, we performed equilibrium dialysis and UV-Vis spectroscopy to construct Scatchard plots, allowing for the precise determination of the dissociation constant (K_d) for warfarin binding.

Optibumin displayed a K_d of 2.58 µM for warfarin (**Figure 7**). This value is consistent with literature-reported values for native, unliganded albumin (~3 µM) (Sudlow et al., 1975; Diana et al., 1989; Loun and Hage, 1994). It confirms that the binding pockets in Optibumin are open, accessible, and functionally native. pHSA displayed a significantly higher K_d of 7.45 µM. This represents a nearly 3-fold reduction in binding affinity compared to Optibumin.

In addition to structural heterogeneity, there is another possible explanation for pHSA's poor performance in this instance. In order to survive the mandatory 60°C pasteurization step used during manufacturing, pHSA is formulated with high concentrations (typically 16-32 mM) of stabilizers, most notably sodium octanoate (caprylate) and N-acetyl-L-tryptophan. These are not passive additives; they are active ligands that bind with high affinity to specific pockets on the albumin molecule. Prior studies have demonstrated that sodium octanoate binds preferentially to Sudlow Site II with very high affinity (Faroongsarng and Kongprasertkit, 2014; Tramarin et al., 2019; Kragh-Hansen 1991; Harm et al., 2018; Olsen et al., 2004). Furthermore, the binding of fatty acids (like octanoate) induces conformational changes that can allosterically reduce the binding affinity of ligands at Sudlow Site I (Yamasaki et al., 1999; Ghuman et al., 2005). Consequently, plasma-derived HSA may contain stabilizers that occupy ligand-binding sites, effectively pre-occupying or sterically hindering these regions. This reduces the albumin's capacity to bind, transport, and solubilize drugs, hormones, and toxins.



Albumin	Slope (-1/KD)	Y-Intercept	X-Intercept (Bmax)	R^2	K_D
Optibumin 25	-0.39	2.38	6.13	0.94	2.58
HSA	-0.13	0.92	6.85	0.92	7.45

Figure 7. Scatchard analysis of warfarin binding to Optibumin and plasma-derived HSA (pHSA). Bound/free warfarin is plotted versus bound warfarin concentration. Linear regression was used to determine binding parameters (K_D and B_{max}). Slopes were significantly different between groups (F-test, $p < 0.01$).

The presence of aggregates, PTMs and stabilizers in pHSA may reduce ligand-binding capacity. For drug delivery applications, this means that pHSA will carry less drug, potentially altering the pharmacokinetic (PK) profile by increasing the free fraction of the drug in circulation. Optibumin, being structurally homogenous and free of caprylate and tryptophan stabilizers, offers a predictable, high-affinity carrier that mimics the native biology of unliganded albumin.

CONCLUSION

The data presented in this application note demonstrate that "albumin" is not a commoditized generic. The source of the protein and the processing it undergoes fundamentally influence structural quality and functional performance. While plasma-derived HSA (pHSA) remains a standard excipient, its utility in next-generation biotechnology is severely compromised by three factors:

- **Hidden aggregation**: Standard lower-resolution 5 µm SEC methods may under-resolve low-order aggregates (dimers and trimers) which averaged ~7.5% in pHSA and are clearly separated under 3 µm high-resolution analysis.
- **Structural heterogeneity**: The Cys34 thiol in pHSA is substantially oxidized (~40%), contributing to lot-to-lot variability that affects conjugation stoichiometry and antioxidant function.

- **Stabilizer interference**: The obligatory addition of sodium octanoate and related stabilizers occupies the protein's natural binding sites, reducing affinity for drugs and ligands by nearly 3-fold.

From a regulatory standpoint, the variance seen in pHSA, where the "native" protein may constitute less than a third of the total mass, poses a significant regulatory challenge. Under ICH Q6B guidelines, manufacturers must characterize and control impurities. Using pHSA as an excipient introduces a raw material that is essentially a mixture of distinct isoforms, each with potentially different stability and binding properties. This complicates the validation of analytical assays and the establishment of release criteria for the final drug product. Optibumin, with its single-peak homogeneity, provides a consistent, defined baseline that simplifies regulatory submissions and quality control.

Optibumin addresses these limitations. Produced in a non-mammalian expression system without blood sourcing, pasteurization, or added stabilizers, Optibumin demonstrates >99% monomeric content predominantly reduced Cys34 and preserved functional binding activity. For developers of cell therapies, gene therapies, vaccines, advanced diagnostics, and protein formulations, transitioning to Optibumin represents a move from biological variability to engineering precision.

MATERIALS & METHODS

High-Resolution Size Exclusion Chromatography (SEC-HPLC)

Samples were prepared at 1 mg/mL concentration in mobile phase buffer, 40 mM sodium phosphate, 200 mM sodium chloride, pH 7.0. Using a Shimadzu HPLC system capable of detecting 215 and 280 nm, 10 µL of samples were injected in triplicate onto a Phenomenex Yarra™ SEC-3000 column (300 Å pore size, 3 µm
particle size, 4.6 × 300 mm; 00H-4513-E0 Phenomenex, CA). The flow rate was 0.5 mL/min. Chromatograms were integrated to quantify the area of high molecular weight (HMW) species (dimers, trimers, oligomers) relative to the main monomer peak.

Reverse-Phase HPLC (RP-HPLC) for Cys34 Redox Analysis

Reverse-phase HPLC (RP-HPLC) was conducted on a quaternary pump (G7104A), 1290 Infinity II DAD FS Detector (G7117A), and an InfinityLab™ Bio-Inert Max-Light cartridge cell (10 mm; G5615-60018, Agilent Technologies, CA).

Chromatographic separation was achieved using an ACQUITY™ UPLC Protein BEH C4 column (300 Å pore size, 1.7 µm particle size, 2.1 × 150 mm; 186004497, Waters Corporation, MA). Mobile phase A consisted of LC-MS water with 0.1% trifluoroacetic acid (TFA), and mobile phase B consisted of 100% acetonitrile with 0.1% TFA. A shallow gradient of the mobile phase B (35% to 40%) was applied to resolve two major albumin species. Detection of albumin species was performed at 215 and 280 nm, while free Alexa Fluor™ 488 C5 maleimide dye (A10254, Thermo Fisher, MA) and dye-conjugated albumin were monitored at 498 nm. Data acquisition and analysis were conducted using MassHunter® Software (Agilent Technologies, CA).

IEX-HPLC for Charge Variant Analysis

Samples were prepared at 1 mg/mL in mobile phase buffer A, (50 mM ammonium acetate, pH 7.4). Using a Shimadzu HPLC system, 10 µL of sample was injected onto an Agilent Bio WAX NP5 column (non-porous, 5 µm, 2.1 × 250 mm, PEEK hardware; 5190-2491, Agilent Technologies, CA) with detection at 215 and 280 nm. The flow rate was 0.5 mL/min. Mobile phase A was held at 100% for 5 minutes, followed by a 22-minute linear gradient from 0 to 100% mobile phase B (50 mM ammonium acetate, pH 7.4). Valley-to-valley (drop-line) integration was used to quantify percent peak area.

Capillary Electrophoresis (CE)

Albumin samples were diluted to 1 mg/mL and 0.5 mg/mL in 20 mM phosphate buffer containing 50 mM NaCl (pH 7). Aggregation was assessed using chemiluminescence-based capillary electrophoresis on a Wes™ system (ProteinSimple®, a Bio-Techne brand, CA).

Samples were prepared by reconstituting the Fluorescent 5X Master Mix (PS-FL01-8, ProteinSimple, CA) in water and mixing with diluted albumin at a 1:5 ratio (1 part fluorescent master mix to 5 parts samples). Albumin aggregate content was analyzed using Compass for SW™ software (version 6.3.0; ProteinSimple, CA). Signal above the main

monomer band (~60 kDa) was quantified using drop-line peak integration. For each sample, the aggregate signal was normalized to injected protein concentration (1 mg/mL or 0.5 mg/mL) to calculate aggregate band area per injected mass.

Aggregate comparisons were evaluated using Welch's unpaired t-test for differences in mean and F-test for equality of variance.

Native-PAGE

Albumin samples were prepared in Native-PAGE sample buffer (62.5 mM Tris, pH 6.8, 0.1% bromophenol blue) to a final concentration of 2 mg/mL. Samples were loaded on to a 14% Tris-glycine gel submerged in native Tris-glycine running buffer. 10 µL of USP rHSA reference standards at various concentrations were loaded into lanes 1-4 for quantification of bands below the main monomer band. 10 µL (20 µg) of albumin samples were loaded in the remaining lanes.

Gels were run at 4ºC at a constant voltage of 140 V for 3 hours and 40 minutes. Gels were rinsed with purified water, fixed for 5 minutes, rinsed with water, stained with GelCode™ Blue Staining Reagent (24590, Thermo Fisher, MA), and destained until the background signal was minimal.

Images were acquired using a AlphaImager™ system and analyzed with AlphaView-FluorChem-Q™ software (version 3.4.0.0; ProteinSimple, CA).

Cys34- Maleimide Conjugation Reaction

Alexa Fluor 488 C5 maleimide was reconstituted in DMSO according to the manufacturer's instructions and diluted in degassed PBS adjusted to pH 6. Conjugation reactions were performed under controlled pH conditions to minimize hydrolysis and off-target amine reactivity. Reaction mixtures were prepared using defined dye-to-protein molar ratios as described in the results section.

Warfarin Binding Kinetics

Warfarin sodium salt was prepared at a stock concentration of 1 mg/mL in deionized water. The stock was serially diluted two-fold to generate concentrations spanning four orders of magnitude. UV-Vis absorbance spectra were recorded for each dilution to construct a standard curve and define the linear range of the absorbance range at 310 nm for quantitation of free warfarin.

Plasma-derived HSA was desalted by spin filtration to remove N-acetyl tryptophan, which inter-feres with absorbance measurements at 280 nm. This step was not required for Optibumin be-cause it contains no such additives. Stocks of Optibumin and pHSA were prepared at 0.2 mg/mL as determined by A280.
To evaluate the binding affinity, Optibumin or pHSA was first mixed 1:1 with serial dilutions of warfarin within the established linear detection range. Final albumin concentration was 0.1 mg/mL in a total reaction volume of 1 mL. Absorbance spectra were recorded to measure total warfarin (free and bound). Samples were subsequently buffer-exchanged using spin filtration with a 3 kDa molecular weight cutoff (MWCO) membrane to separate free and bound fractions. The flowthrough and retentate were each adjusted to total volumes of 1 mL each and quantified by UV-Vis.

The flowthrough contained free warfarin and the retentate contained albumin-bound warfarin. Albumin background absorbance was subtracted from both total and bound measurements prior to analysis.

Binding data were fitted to the Scatchard equation to determine the dissociation constant (K_d) from the slope and the number of binding sites (n) from the x-intercept.

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