

# Thermodynamic Stability of Recombinant Human Serum Albumin-Peg-Water Systems

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There are much research addressing the behavior of mixtures of proteins and polymers. Until this moment, however, there are no papers that specifically address to the aqueous mixtures of recombinant human albumin. This work aims to evaluate the thermodynamic stability of the ternary mixture composed of water, polyethylene glycol and recombinant human albumin (rHSA) for use as a coating solution for disposable products from the extracorporeal membrane oxygenation (ECMO) systems. The experiments were carried out in an equilibrium cell and to quantify the rHSA concentration spectrophotometric assays were performed using the Brandford method. UNIQUAC thermodynamic model was used to calculate Gibbs energy of mixture as well as Gibbs distance function to identify whether the system is stable. The formation of a second phase was not observed, indicating that the mixture remains stable under the applied conditions, confirming what was seen in the experimental work. Diluted protein-PEG solutions do not form a two-phase system especially with low molecular weight PEG. However, it is important that future works can evaluate other experimental conditions to settle a range of stability, filling the lack of experimental data in literature.

## 1. Introduction

The Human Serum Albumin (HSA), the major protein contained in plasma, shows an extraordinary bonding capacity, being a versatile carrier for many compounds. In fact, HSA is responsible for carrying most of the fatty acids, which affects the pharmacokinetic behavior of many drugs, enhance the metabolic modification of some binders, and makes potential toxins ineffective, being also responsible for the antioxidant function in human plasma and contains pseudo enzymatic properties. The secondary structure of HSA contains many cysteine residues that are shaped in helical form on the protein chain (Perez, 2013).

HSA has an attaching capacity which allows to link many primary physiological binders at different bonding points, having a long half-life period, compared to other proteins. The term PEGylation describes a biological modification in the molecules through a covalent bonding with polyethylene glycol (PEG), a nontoxic and non-immunogenic polymer. Studies observed that PEGylated products showed resistance to antibodies and proteolytical enzymes and they were eliminated slower from the human organism. Many factors must be considered during the PEGylation such as: the number of PEG molecules reacted with the polypeptide, molecular weight and PEG structure, the PEG location on the peptide and the chemical conditions adopted during the reaction (Fanali, 2012).

It was observed that PEG hydrophobicity increases as in PEG molecular weight increases due to the two nonpolar groups contained in the PEG monomer. Longer PEG chains are more capable of interacting with proteins in aqueous solution, than short ones showing low interaction capacity with protein, whilst the optimal chain size to form the protein-polymer complex is unknown (Bekale *et al.* 2015). In order to understand how these two characteristics influence the protein-polymer interaction, the study reported by Bekale *et al.* (2015)

showed that HSA and bovine serum albumin (BSA) presented slightly difference in their hydrophobicity's due to the structural similarities resulting in a higher stability in the protein structure. Besides, an important observation was that the formation of the protein-polymer complex is spontaneous under environmental conditions.

In 1997, the behavior of HSA in a low polyethylene glycol concentration environment was analyzed by Farruggia *et al.* (1997) with different PEG molecular weights. PEG is a solute commonly used to precipitate or crystallize proteins in aqueous solutions at high concentrations. However, the mechanism where the partitioned proteins favor the PEG phase is unknown. The study concluded that PEGs with the molecular weight of 8,000 Da and 10,000 Da stabilized the native HSA compact showing a negative preferential interaction with the protein. This observation is consistent with the fact that PEG is, by nature, a hydrophobic compound interacts well with the lateral hydrophobic exposed chain when unfolded.

PEG with lower molecular weight favors the ionization of albumin tyrosine residues. The HSA thermal stability drastically decrease with PEG 1,000, with a slightly increase in PEG 10,000. Furthermore, it also takes to a higher hydration of HSA and have a preferential positive interaction and an influence in phenolic groups present in HSA. The medium becomes more hydrophobic with the increase of the PEG concentration, favoring the ionization of HSA, probably because of the dielectric constant variations (Farruggia *et al.* 1997)

Although the existence of some studies to understand the thermodynamic behavior of water, PEG and protein, few works present experimental data for the phase equilibrium of these three components. Systematic research was performed in the Web of Science (WOS) database from Clarivate Analytics main collection using the keywords from the technological field of interest as shown in Figure 1.

Search results from lines #5, #6, #7 and #15 indicate that there is no record in the WOS Main Collection database for a combination of thermodynamic modeling of LLE with HSA. Line #8 was added to retrieve information from BSA, which is most used as a protein model. Only 6 records were retrieved with the combination of thermodynamic modeling of LLE with BSA, in line #10. To enhance record retrieval the keyword "recombinant" associated with HSA in line #11 was omitted and this was combined with thermodynamic modeling of LLE with PEG in line #12, resulting in the retrieval of only 1 record.

To have a broader search, the term HSA was replaced with "albumin" and "protein" in line #16 and the polymer-related keyword was included in line #13. The result of the combination of the 4 categories of keywords is presented in search line #17, representing the technological field of LLE thermodynamic modeling with Protein-PEG system, containing only 12 records.

Therefore, no experimental data was found so far addressing the thermodynamic stability of the PEG-HSA-Water system, nor specific data related to the partition of the system into two liquid phases or liquid and solid phases.

	Results	
# 17	12	#16 AND #14 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 16	4 111 080	TÓPICO: (albumin OR protein) Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 15	0	#14 AND #1 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 14	51	#13 AND #3 AND #2 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 13	103 284	TÓPICO: ("polyethylene glycol" OR PEG) Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 12	1	#11 AND #4 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 11	21 091	TÓPICO: ("human serum albumin") Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 10	6	#9 AND #3 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 9	42	#8 AND #2 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 8	39 418	TÓPICO: ("bovine serum albumin") Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 7	0	#3 AND #1 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 6	0	#2 AND #1 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 5	0	#4 AND #1 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 4	723	#3 AND #2 Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 3	31 898	TS=(thermodynam* NEAR/2 model*) Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 2	8 973	TÓPICO: (liquid-liquid equilibri*) Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos
# 1	394	TÓPICO: ("recombinant" NEAR "human serum albumin") Índices-SCI EXPANDED, SSCI, ABHCI, CPCI-S, CPCI-SSH, ESCI Tempo estipulado=>Todos os anos

Figure 1. Search Results related to the Human Serum Albumin + PEG + Water System at Web of Science Database.

Addressing all this previous knowledge and aiming to fill the lack of experimental data about the thermodynamic behavior of water, PEG, rHSA, and also to understand the viability of using this solution as coating solution, the objective of this paper was to evaluate the thermodynamic stability of the ternary mixture composed of water, polyethylene glycol and recombinant human albumin (rHSA) for use as a coating solution for disposable products from the extracorporeal membrane oxygenation (ECMO) systems.

## 2. Methodology

This work was divided in two parts, (1) the experimental part, where assays were performed to observe the phase behavior of the mixture composed by water, PEG 300 and rHSA, and (2) the phase equilibrium modeling, which consisted in thermodynamic description the phase behavior of the mixture studied. The procedure for both parts will be detailed as follows.

### 2.1 Experimental Procedures

Nine equilibrium experiments with the aqueous solution containing water, PEG 300 and rHSA were performed by varying the amount of PEG300 and water, and keeping the rHSA 20% aqueous aliquot constant, as shown in Table 1.

Table 1. Experimental planning to study the ternary equilibrium of the water, PEG 300 and rHSA system

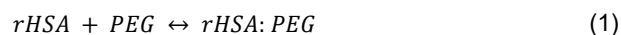
SAMPLE	rHSA ( $\mu\text{g}/\mu\text{L}$ )	PEG300 ( $\mu\text{g}/\mu\text{L}$ )	WATER ( $\mu\text{g}/\mu\text{L}$ )
1	3	17	988
2	3	17	988
3	3	17	988
4	3	17	967
5	3	37	967
6	3	37	967
7	3	47	957
8	3	47	957
9	3	47	957

The tests consisted of adding the mixture to an equilibrium cell and let it under stirring for two hours. Posteriorly, the mixture was let to rest for 12 hours to observe the possible phases formation. After the rest period, the absorbance of the solution was measured following the Bradford spectrophotometric method to be implemented in the Thermodynamic model (Walker, 2002).

### 2.2 Thermodynamic model

To identify if the system was stable, it was used the UNIQUAC thermodynamic model to calculate Gibbs energy of mixture and the Gibbs distance function from the Gibbs Tangent Plan Criterion. The values of the structural parameter "r an q" in this model for water, PEG 300 and rHSA, were, respectively: 0.920; 11.0160; 2497.8000 for "r parameter" and the values adopted for "q parameter" were: 1.4000; 9.2340; 1883.7000 (Agena, Bogle, and Pessoa, 1997)

According to the study made by Ragi *et al.* (2005) who performed an assay to analyze the HSA interaction with an aqueous solution of PEG at physiological conditions and stated that the protein physicochemical properties can be altered by the polymer interaction once the presence of many bond locations with high HSA affinity became a possible target for various organic and polymeric molecules. Also, they concluded that PEG does not induce protein conformal alterations in low polymer concentrations, indicating that it stabilizes the protein conformation while protein unfolding occurs in high PEG concentration. It was considered that PEG and rHSA bond through the following interaction.



The bonding constant would be:

$$K = \frac{[rHSA:PEG]}{[rHSA][PEG]} \quad (2)$$

Considering  $C_b = [AR:PEG]$ , the following equation is obtained:

$$K = \frac{C_b}{(C_b - C_{rHSA})(C_b - C_{peg})} \quad (3)$$

where  $C_{rHSA}$  and  $C_{peg}$  are the *rHSA* and PEG analytical concentrations in solution. According to the Lambert-Beer Law (Equations (4) and (5)):

$$C_{rHSA} = \frac{A_0}{\epsilon_{hsa} \cdot \mathcal{E}} \quad (4)$$

$$C_{rHSA} = \frac{A - A_0}{\epsilon_b \cdot \mathcal{E}} \quad (5)$$

where,  $A_0$  and  $A$  are the absorbances of *rHSA*, at 595 nm, in the absence and presence of PEG, respectively;  $\epsilon_{rHSA}$  and  $\epsilon_b$  are the extinction coefficients of *rHSA* and the complex, respectively;  $\mathcal{E}$  is the path of light in the cuvette (1cm). By manipulating the equations, we obtain Equation (6):

$$\frac{A_0}{A - A_0} = \frac{\epsilon_{rHSA}}{\epsilon_b} + \frac{\epsilon_{rHSA}}{\epsilon_b \cdot K} \times \frac{1}{C_{peg}} \quad (6)$$

### 3. Results and Discussion

#### 3.1 Experimental Procedure Results

During the execution of all tests, the formation of a second phase was not observed, indicating that the mixture remains stable under the applied conditions. For the *rHSA* quantification, the Bradford spectrophotometric method was used. Initially, it was necessary to do an analytical curve with solutions of known concentration of *rHSA* as described in the aforementioned methodology. Table 2 presents the absorbance values of the each *rHSA* concentration point of the calibration curve. The data obtained were plotted on a scatter plot (x,y) and fitted to a linear regression curve as shown in Figure 2. The Coefficient of Determination ( $R^2$ ) value was 0.996, showing that the absorbance and concentration of *rHSA* exhibits linear correlation as expected.

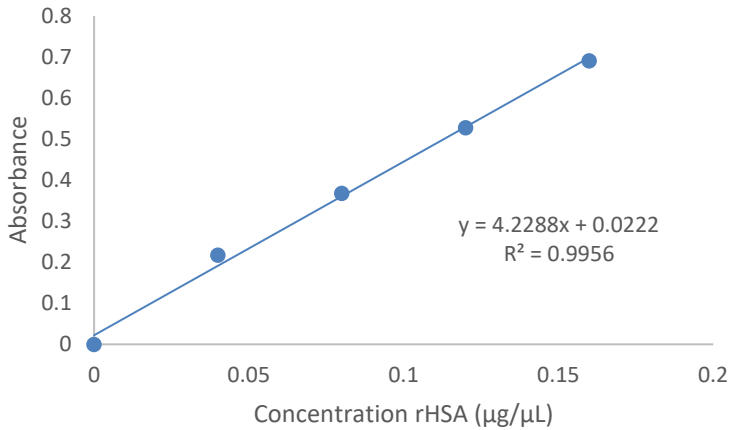


Figure 2. Analytical Curve for Recombinant Human Albumin

The absorbance values of the samples taken from the equilibrium cell were taken. The values obtained and the concentration calculated for each sample are presented in the Table 2.

Table 2: Absorbance measured in the experiments.

Sample	Absorbance	rHSA Concentration ( $\mu\text{g}/\mu\text{L}$ )
1	0.4170	0.093
2	0.3145	0.069
3	0.2975	0.065
4	0.3045	0.067
5	0.3128	0.069
6	0.2800	0.061
7	0.3170	0.070
8	0.2645	0.057
9	0.2705	0.059

It was observed only one phase in all planning conditions of mixture presented in Table 1. With the data of  $A$  and  $A_0$  in the equation (6) and  $C_{\text{peg}}$ , linear and angular coefficients and  $K$  can be obtained. The value found by the authors was  $K = 1.56 \times 10^8 \text{ M}^{-1}$ , which indicates a high stability in the conformation of the rHSA: PEG complex formed. Both the Gibbs energy graph and the Distance Function graph shown in the Figures 3 and 4, respectively, confirm this observation. One can see that Gibbs energy of mixture behavior indicates a stable homogeneous mixture (Figure 3) e the distance function is always positive as expected for stable mixtures.

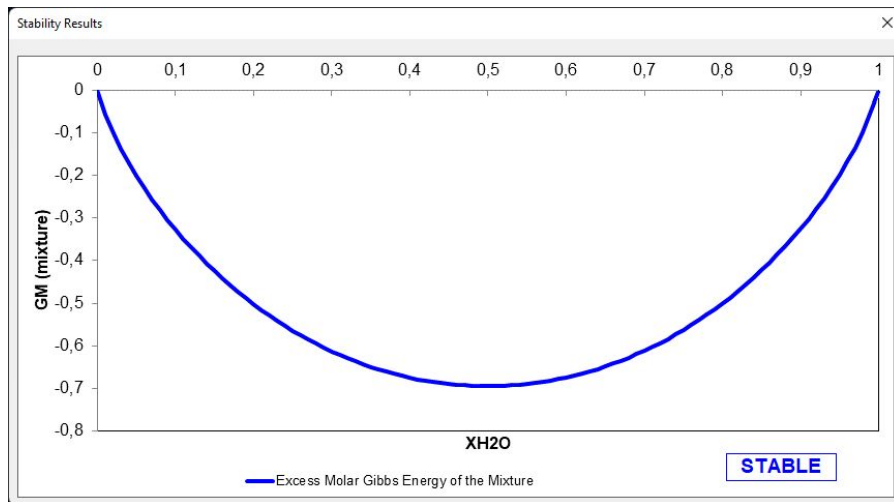


Figure 3: Graph of Gibbs Free Energy with the molar fraction of water.

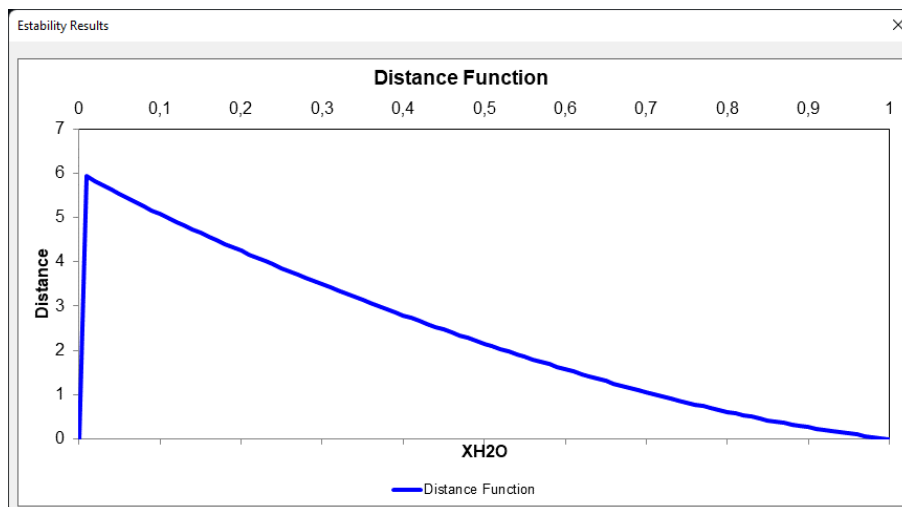


Figure 4: Graph of the distance function with the molar fraction of water.

In both graphs "XH<sub>2</sub>O" means the molar fraction of water. These results are compatible with the absorbance data presented in Table 2. Samanta *et al.* (2014) observed a similar behavior with the PEG 200 and PEG 400 that the interactions with HSA change from a non-interaction or weakly repulsive (like an osmolyte) to interact (like a nonspecific binder) as the PEG concentration increases. The polymer addition increases the protein compaction without negatively altering the tertiary structure, what can result in high equilibrium constant values also indicating the high stability of the rHSA:PEG complex formed.

#### 4. Conclusions

The obtained results showed that the water/PEG/rHSA mixtures analyzed are thermodynamically stable in the experimental conditions applied with an equilibrium constant value of complexation equal to  $K = 1.56 \times 10^8 \text{ M}^{-1}$ . The calculations based on UNIQUAC model for Gibbs energy of mixture, as well as for distance function, corroborate with the experimental observations. Diluted protein-PEG solutions do not form a two-phase system especially with low molecular weight PEG. The experiments provided a preliminary view of the behavior of water, PEG, rHSA systems. However, it is important that future works can evaluate other experimental conditions to settle a range of stability, filling the lack of experimental data in literature.

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#### References

- Agena SM, Bogle ID, Pessoa FL. An activity coefficient model for proteins. *Biotechnol Bioeng.* 1997 Jul 5;55(1):65-71
- Bekale, L., Agudelo, D., Tajmir-Riahi, H. A., 2015, The role of polymer size and hydrophobic end-group in PEG-protein interaction, *Colloids and Surfaces B: Biointerfaces*, Vol. 130,141–148.
- Walker, J. M.(Ed) ,2002, *The Protein Protocols Handbook*, Humana Press Inc, New Jersey, USA
- Fanali, G., Di Masi, A., Trezza, V., *et al.*, 2012, Human serum albumin: From bench to bedside", *Molecular Aspects of Medicine*, Vol. 33, 209–290
- Farruggia, B., García, G., D'angelo, C., *et al.*,1997, Destabilization of human serum albumin by polyethylene glycols studied by thermodynamical equilibrium and kinetic approaches", *International Journal of Biological Macromolecules*, Vol. 20, 43–51,
- Guo, M., Narsimhan, G. A,1996, Model for the Prediction of Precipitation Curves for Globular Proteins with Nonionic Polymers as the Precipitating Agent, *Separation Science and Technology*, 1777-1804,
- Harris, J. M., & Chess, R. B. Effect of pegylation on pharmaceuticals, 2003, *Nature Reviews Drug Discovery*, Vol. 2, 214–221,.
- Perez, B., Malpiedi, L. P., Tubío, G., *et al.*, 2013, Experimental determination and thermodynamic modeling of phase equilibrium and protein partitioning in aqueous two-phase systems containing biodegradable salts, *Journal of Chemical Thermodynamics*, v. 56,136–143
- Ragi, C., Sedaghat-Herati, M. R., Ouameur, A. A., *et al.*, 2005, The effects of poly(ethylene glycol) on the solution structure of human serum albumin, *Biopolymers*, vol. 78, 231–236.
- Samanta, N., Mahanta, D. Das, Hazra, S., *et al.*,2014, Short chain polyethylene glycols unusually assist thermal unfolding of human serum albumin, *Biochimie*, v. 104, 81–89.