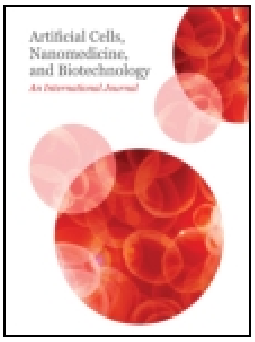

Artificial Cells, Nanomedicine, and Biotechnology
An International Journal

ISSN: 2169-1401 (Print) 2169-141X (Online) Journal homepage: informahealthcare.com/journals/ianb20

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To cite this article: Wentao Zhou, Shen Li, Shasha Hao, Jiaxin Liu, Hong Wang & Chengmin Yang (2015) An optimal polymerization process for low mean molecular weight HBOC with lower dimer, Artificial Cells, Nanomedicine, and Biotechnology, 43:3, 148-151, DOI: 10.3109/21691401.2014.934455

To link to this article: https://doi.org/10.3109/21691401.2014.934455

Artificial Cells, Nanomedicine, and Biotechnology, 2015; 43: 148–151
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ISSN: 2169-1401 print / 2169-141X online
DOI: 10.3109/21691401.2014.934455

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An optimal polymerization process for low mean molecular weight HBOC with lower dimer*

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Abstract

The new research tried to improve the distribution of molecular weight of Hb-based oxygen carriers (HBOC), a bottleneck of glutaraldehyde (GDA)-polymerization process. The orthogonal experiments were done on the basis of the early study of human placenta Hemoglobin (Hb)-crosslinked-GDA and three factors were selected including the molar ratio of GDA and Hb, Hb concentration, and the rate of the feeding GDA. The optimal match condition of polymerization process prepared for the purpose of lower mean molecular weight, content of super-weight molecule, and the content of dimer. The results showed that the molar ratio of GDA and Hb was the greatest influencing factor on the molecular weight distribution of polymerized-Hb, followed by the Hb concentration, and the last is the rate of feeding GDA. The optimum matching conditions had reached the objective that the mean molecular weight with 155.54 ± 5.79, the content of dimer with 17.23 ± 3.71, and content of super-weight molecule with 0.17 ± 0.09, and the results can be repeated in the 30 times expansion experiments.

Keywords: human placenta Hb, Hb concentration, molar ratio of GDA and Hb, orthogonal experiment, rate of feeding GDA

Preface

Although it is a milestone of 20th century medicine that the understanding of the development of blood storage and testing technology that have enabled the routine transfusion of blood and immunologic matching requirements, there also exists some risks like infectious disease by transmission. Therefore, when blood is not available in certain situations, alternative hemoglobin based oxygen-carrying solutions (HBOC) could be lifesaving in the therapy of hemorrhagic shock and/or the temporary maintenance of patients with rare blood types. The potential benefits of HBOC include universal compatibility without the need for crossmatching of donated blood, availability, lack of infection, and long-term storage HBOC is especially suitable for hospital trauma first aid at the scene. However, mean arterial pressure and vascular resistance increase, the microcirculation blood flow decrease, the organization hypoxemia and immunogenicity increase, and other adverse reactions emerge when HBOC is used in animals or in clinical trials (Estep et al. 2008, Philbin et al. 2005). There are many reasons, but the distribution of molecular weight of HBOCs is likely to be one of the main factors.

Theoretically, the lower average molecular weight of HBOC is, the better microcirculation will be. This idea has been verified in the preliminary experiments. However, the animal experiments also pointed out that slight hemoglobinuria may occur in the rats on treatment with the lower average molecular weight of HBOCs. This orthogonal experiment is based on single factor optimization study (Zhou et al. 2013) and selected three influencing factors including the molar ratio of glutaraldehyde (GDA) and hemoglobin (Hb), Hb concentration, and the rate of feeding GDA to optimize matching conditions. The main goal of this study is to try to reduce the content of dimer on the basis of lower average molecular weight. Finally, the results of the operation showed that the effect of improving molecular weight distribution was obvious and the expected results had been reached.

Materials and methods

Reagents and main instruments

Reagents

Human placental blood was obtained from Sichuan New Life Stem Cell Biotech Inc. Chengdu, Sichuan and Tianjin

*The research of this article is based on the original article of "Wentao Zhou, Lanzhen Zhao, Jinfeng Wang, et al. An Optimal Polymerization Conditions for Poly-Human Placenta Hemoglobin with Lower Mean Molecular Weight [J]. Artificial Cells, Nanomedicine, and Biotechnology, 2013; 41(5): 289–292", which was designed for decreasing the content of dimer of HBOC based on the lower average molecular weight, because higher levels of dimer of HBOC can led to the emergence of hemoglobinuria as experienced in previous study. So this research, which is a further study on the basis of the previous article, has a different purpose and different research method from that in previous article.

Supported by National High-tech R&D Program of China (863 Program) (Grant No. 2012AA021903);

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(Received 25 April 2014; revised 9 June 2014; accepted 10 June 2014)

Union Stem Cell Gene Engineering Co. LTD (Tianjin). GDA was purchased from Sigma (Sigma, AR). And all of other reagents were AR.

Main instruments

Instruments used in this investigation are High-capacity, low-temperature centrifuge (Beckman); CARY50 UV-Vis spectrophotometer (Beckman); Master Flex UF (Millipore); High performance liquid (Waters e2695/2489); Gel column Superdex 200 (GE); Blood cell analyzer (Mindray); Delsa Nano (Beckman Coulter), and Hemox analyzer (TCS Scientific Corp).

Methods

The Stroma-Free human placenta Hb was prepared as described previously (Yang et al. 2006)

Orthogonal experiment. Preliminary experiment research of the author showed that the related influencing factors of Hb and GDA polymerization: the approach of feeding GDA, Hb concentration, the mole ratio of GDA and Hb had been investigated. In this study, the reaction time as the influencing factor was investigated. From the experimental results, the appropriate reaction time was chosen, then three influencing factors including the Hb concentration(A), the rate of feeding GDA (B) (Bonsen et al. 1975, Sehgal et al. 1986), the molar ratio of GDA and Hb (C), and three level orthogonal experiments were carried out to reduce the content of dimer on the basis of lower average molecular weight.

The polymerization conditions

100 ml stroma-free human placenta Hb solution was reacted with GDA at 1.00% in sterile and pyrogen-free water for at 4 ± 2°C while gently stirring in a 250ml filter flask filled with N_2 for a specified time, followed by a reaction with $NaBH_4$ at 1.56% under the same conditions for 30 min. After the reaction, the pH was adjusted to 7.30 ± 0.05. The product was stored at 4 ± 2°C.

Detection of the molecular weight distribution (Zhou et al. 2013)

Statistics. Data are expressed as Mean ± SEM. Groups were compared using the analysis of unpaired students t-test, and statistical significance was $P < 0.05$.

Results

Reaction time

Polymerization conditions were: the approach of feeding GDA: titanium cartridge, concentration of Hb: 6.50 ± 0.30%, the ratio of GDA and Hb: 6.50 ± 0.30%, and reaction time was changed (30, 60, 90, 120 min).

Compared with the results of 30 min, the results of mean molecular weight(A), content of dimer (B), the super-weight molecular content (C) of poly-Hb at 60 min had statistically significant difference ($P < 0.01$); compared with the results of 60 min, there was no statistically significant difference between the results of mean molecular weight and the content of dimer of poly-Hb at 90 min and 120 min ($P > 0.05$), but there were significant differences in the super-weight molecular content between 60 min and 90 min/120 min ($P < 0.05$). Under these certain conditions, the reaction reaches equilibrium point was at about 60 min.

Shown in Figure 1, there were significant differences at 30 min and 60 min in the mean molecular weight, the content of dimer, the super-molecular content of poly-Hb ($P < 0.01$); while there was no significant difference ($P > 0.05$) in the mean molecular weight, the content of dimer at 60, 90, and 120 min. Under this condition, the reaction reached the equilibrium at 60 min, so the optimized reaction time was 60 min.

Orthogonal experiment

The mean molecular weight

From the analyzed results as shown in Table I, it can be concluded that the degree of influence on the mean molecular weight in orthogonal experiment was in the order: the molar

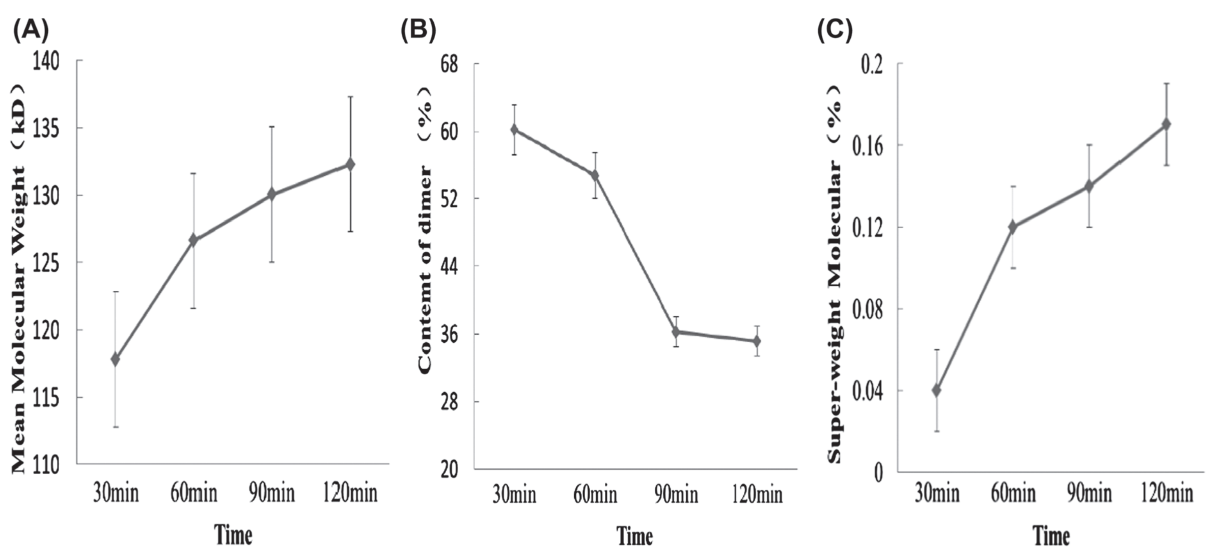

Figure 1. Effects of poly-Hb with the reaction time (n = 3).

Table I. Effects of mean molecular weight (kD) of poly-Hb.

Group	A (%)	B(ml/min)	C	1	2	3	Average
1	1	1	1	110.33	114.45	110.34	111.73
2	1	2	2	130.43	138.07	129.76	132.77
3	1	3	3	148.87	162.65	159.65	157.00
4	2	1	3	152.32	149.67	149.74	150.54
5	2	2	1	117.21	117.98	115.54	116.92
6	2	3	2	166.43	156.98	158.87	160.73
7	3	1	2	119.76	120.98	121.43	120.74
8	3	2	3	161.10	165.11	162.65	162.92
9	3	3	1	141.73	138.96	139.65	140.02

Ave1 133.83 127.67 122.89
Ave2 142.73 137.54 138.08
Ave3 141.23 152.58 156.82
Ex D 8.90 24.91 33.93

ratio of GDA and Hb > the rate of feeding GDA > the Hb concentration. To obtain the lower mean molecular weight of poly-Hb, the optimization group is A1B1C1.

The super-weight molecular content

From the analyzed results as shown in Table II, we can conclude that the degree of influence on the content of super-weight molecules in orthogonal experiment was in the the order: the molar ratio of GDA and Hb > the Hb concentration > the rate of feeding GDA. To obtain the lower content of super-weight poly-Hb, the optimization group is A1B2C1.

The effective polymerized ratio

From the analyzed results as shown in Table III, we can conclude that the order for degree of influence on the content of dimer in orthogonal experiment was as follows: the molar ratio of GDA and Hb > the Hb concentration > the rate of feeding GDA. To obtain the lower content of dimer of poly-Hb, the optimization group is A2B3C2.

Comprehensive analysis

The main goal of this orthogonal experiment was to reduce mean molecular weight and content of dimer of GDA-PolyPHb, but under the same conditions, the lower mean molecular weight and content of dimer were contradictory, so the exact optimal matching group can't get through the analysis of orthogonal experiment. Then on comprehensive analysis of the mean molecular weight, the super-weight molecular content, and the effective polymerized ratio, the optimal matching group is third group of nine groups.

Table II. Effects of the content of super-weight molecular (%) of poly-Hb.

Group	A (%)	B (ml/min)	C	1	2	3	Average
1	1	1	1	0	0	0	0
2	1	2	2	0	0.42	0.11	0.18
3	1	3	3	1.04	2.21	1.09	1.45
4	2	1	3	0.07	0.29	0.06	0.14
5	2	2	1	0	0	0	0
6	2	3	2	1.53	0.98	1.32	1.28
7	3	1	2	0	0	0	0
8	3	2	3	0.19	0.77	1.21	0.72
9	3	3	1	0	0.06	0	0.02

Ave1 0.05 0.67 0.01
Ave2 0.30 0.11 0.49
Ave3 0.92 0.48 0.77
Ex D 0.87 0.554 0.76

Table III. Content of dimer(%) of poly-Hb.

	A (%)	B (ml/min)	C	1	2	3	Average
1	1	1	1	35.14	34.68	33.69	34.50
2	1	2	2	27.08	26.75	27.81	27.21
3	1	3	3	19.70	16.87	18.93	18.50
4	2	1	2	25.53	25.31	25.68	25.51
5	2	2	1	28.94	27.29	26.50	27.58
6	2	3	3	14.36	18.47	15.65	16.16
7	3	1	3	30.13	27.02	22.63	26.59
8	3	2	1	31.76	30.86	30.80	31.14
9	3	3	2	35.06	32.10	35.03	34.06

Ave1 26.74 28.87 32.05
Ave2 23.08 28.64 23.32
Ave3 30.60 22.91 25.05
Ex D 7.51 5.96 8.73

Expansion experiments

The optimal condition was chosen based on the above experiments, the experiments were implemented after expanding 30 times (3000 ml) as the given conditions. The poly-Hb was purified by ultrafiltration with a 100 kD ultrafiltration membrane reactor (Millipore) and the pH was adjusted to 7.35 ± 0.05, and then the product was bulk-filtered with a 0.8-μm filter followed by 0.2-μm sterile filtration steps. The final product was concentrated to 6.0 g/dl in 10 ml sterile glass bottles. The product was stored at −40°C.

Table IV shows the comparison of the molecular weight distribution of poly-Hb and final products based on optimal process with the internal standards of Institute of Blood Transfusion, Chinese Academy of Medical Science.

Figure 2 shows the comparison of the circular dichroism of final products with the stroma-free human placenta Hb. This can be very obvious explanation in the process of cross-linking without disrupting the secondary structure.

Discussion

The purpose of this study is to select optimum matching condition to obtain further lower mean molecular weight, the content of super-weight molecule, and lower content of dimer of HBOC. The data indicates that the desired effect has been achieved, and final product accorded with the internal standards of Institute of Blood Transfusion, Chinese Academy of Medical Sciences.

It is obvious that order of the degree of influence on the molecular weight distribution of poly-Hb in orthogonal experiment is as follows: the molar ratio of GDA and Hb > the Hb concentration > the rate of feeding GDA. According to the chemical reactions and chemical equilibrium theory of mass action law, under certain conditions, the lower substrate concentration may decrease the reaction rate in the reaction system Also, it is conducive to the negative direction

Table IV. The molecular weight distribution of poly-Hb and final products based on optimal process ($n = 3$).

Detection index	Poly-Hb	Final products
Average molecular weight (kD)	155.54 ± 5.79	231.57 ± 15.72
Content of dimer (%)	17.23 ± 3.71	8.45 ± 2.19
Content of super-weight molecular (%)	0.17 ± 0.09	1.09 ± 0.74

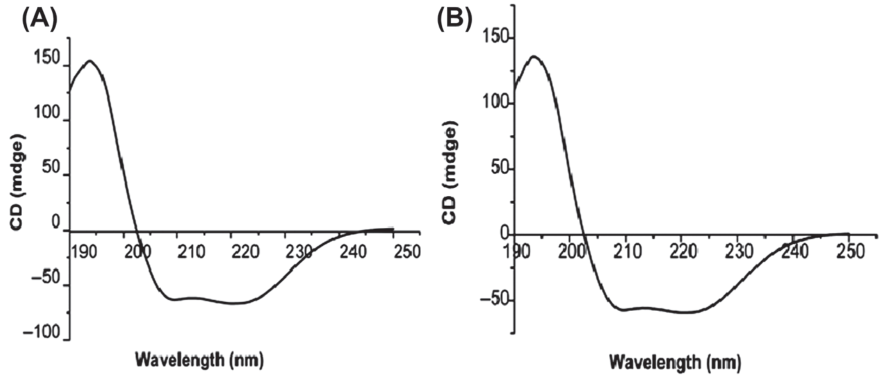

Figure 2. Secondary structure of final products with circular dichroism (A). Compared with the secondary structure of the stroma-free human placenta Hb (B), the secondary structure of final products is consistent.

in chemical equilibrium reactions. The molar ratio of GDA and Hb is not only influencing the reaction rate by affecting the concentration of GDA, but also by affecting the amount of substrate. On the lowering of the rate of feeding of GDA, the GDA dispersed more quickly and uniformly into the Hb solution, which can increase the dispersion of the crosslinking reaction and orderliness.

From this orthogonal experiment, the groups can get, with lower mean molecular weight, lower content of superweight molecule and lower content of dimer, respectively. But under the same conditions, the lower mean molecular weight and higher effective polymerized ratio are contradictory in one experiment, so the exact optimal matching group can't get through the analysis of orthogonal experiment. Therefore, we only choose the optimum matching condition in these nine experiments.

Conclusion

In summary, on the basis of without reducing the effective conversion rate of HBOC, this study had not only maintained a lower mean molecular weight (155.54 ± 5.79 kD), but also reduced the content of two dimer($17.23 \pm 3.71\%$) of HBOC, and the results can be repeated in the 30 times expansion experiments, which suggested the desired results were really achieved.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

References

Bonsen P, Los Alton, CA.; Blaver M, Weston, Mass.; Morris KC, Mountain View, Calif. 1975. US Patent 4001200.

Estep T, Bucci E, Farmer M, Greenburg G, Harrington J, Kim HW, et al. 2008. Basic Science focus on blood substitutes: a summary of the NHLBI Division of Blood Diseases and Resources Working Group. Workshop, March 1. 2006. Transfusion. 48:777–780.

Philbin N, Rice J, Gurney J, McGwin G, Arnaud F, Dong F, et al. 2005. A Hb-based oxygen carrier, bovine polymerized Hb(HBOC-201) versus hetastarch (HEX) in a moderate severity hemorrhagic shock swine model with delayed evacuation. Resusciatation. 66:367–378.

Sehgal LR; De Woskin RE, both of Cook Country; Moss GS; Gould GA, both of Lake Country; Rosen AL; Sehgal H, both of Cook Country, all of III. (1986) US Patent 4826811.

Yang C, Wang H, Zeng M, Jiaxin L, Limin L, Xiaojin W. 2006. CN Patent 1238380C.

Zhou W, Zhao L, Wang J, Li S, Chen G, Liu J, Yang C. 2013 An optimal polymerization conditions for poly-human placenta Hb with lower mean molecular weight. Artif Cells Nanomed Biotechnol. 41:289–292.