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Properties of several glycidyl methacrylate–triallyl isocyanurate based affinity adsorbents for removing circulating immune complexes

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Abstract

Biocompatible affinity adsorbents prepared from macroporous glycidyl methacrylate–triallyl isocyanurate copolymer (GT) has been used for removing circulating immune complexes (CICs). In this work, adsorption of circulating immune complexes on GT-based affinity adsorbents has been studied by using batch and hemoperfusion studies. In batch mode, the equilibrium adsorption level was determined to be a function of the contact time, temperature, initial CIC concentration and adsorbent dosage. In animal hemoperfusion trials, removal of CICs is efficient and rapid. IgG, IgM and complement C3, C4 are minimally affected. There are negligible decreases in RBC, WBC, PLT and HB. Acid–base equilibrium, electrolytes and plasma proteins also are minimally affected.

Keywords: Adsorbents; Circulating immune complexes; Glycidyl methacrylate; Triallyl isocyanurate

1. Introduction

Circulating immune complexes (CICs) are thought to be involved in the pathogenesis of many immune diseases, such as systemic lupus erythematosus, autologous immune complex nephritis, etc. [1–3]. Deposition of circulating immune complexes along vascular membranes may cause immunologic injury to varying degrees. For instance, CICs can lodge in vulnerable tissues such as glomeruli and incite an inflammatory response that may ultimately lead to end-stage renal damage [4,5]. The presence of Fc receptor in glomerular mesangial cells and C3b receptor on the surface of glomerular visceral epithelial cells promotes the formation of glomerular immune deposits. Furthermore, small caliber and low-circulating speed in glomerular capillary are associated with increase in circulating immune complexes captured by glomerular capillary endothelial cells. Development of techniques to remove CICs in blood and cure immunologic disease has constantly been a topic deeply concerned. There are no effective treatments for immune complex diseases at present. The widespread methods in the clinic are hormone, immunosupressant therapy, etc. [6–8]. Hormone and immunosupressant therapy usually results in immunosuppressive effects and cannot

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effectively eliminate pathological material in vivo and the side effects of the therapy is prevalent [9,10]. As is known to all, blood perfusion has been successfully applied in the clinic to treat heterointoxication and endointoxication in recent years [11]. However, there are rather few reports about hemoperfusive removal of pathologic circulating immune complexes directly from whole blood.

In the present study, several affinity adsorbents based on macroporous glycidyl methacrylate–triallyl isocyanurate copolymer (GT) were used as matrix for hemoperfusion, the one which has highest specific affinity for circulating immune complexes was sorted out. The influence of adsorbent dosage, contact time, temperature and initial concentration of CICs on adsorption efficiency and adsorptive capacity has been studied. New Zealand white rabbits were chosen to establish animal model systems of high CICs level. Hemoperfusion treatment successfully removes circulating immune complexes in animal models. Further experiments demonstrate that GT–Tyr possesses fine blood compatibility; experimental animals are alive and healthy, keep stable vital signs, and have no complications such as hemorrhage, psychosis, etc. after hemoperfusion.

2. Experimental

2.1. Material

Glycidyl methacrylate and polyvinyl alcohol was purchased from Beijing Organic Chemical Plant. Triallyl isocyanurate was purchased from Dongfeng Chemical Plant, Hefei. Azobisisobutyronitrile (AIBN) was of chemical grade and purified by recrystallization from ethanol. *N*-heptane was purchased from First Chemical Reagent Co., Tianjin. Butyl acetate and Sodium chloride was of analytical grade and used without further purification. L-phenylalanine, L-tryptophan, L-tyrosine, L-proline and L-hydroxyproline were purchased from Sigma (St. Louis, MO).

Horse serum was purchased from Sigma (St. Louis, MO). Radial immunodiffusion kit was from The Binding Site (Birmingham, UK).

Adult male New Zealand white rabbits, 4-months-old, 2.3–2.5 kg in weight, were obtained from Institute of Zoology, First Military Medical University.

2.2. Preparation and characterization of affinity adsorbents

From an aqueous solution containing sodium chloride and polyvinyl alcohol, macroporous glycidyl methacrylate–triallyl isocyanurate copolymer was synthesized by suspended polymerization procedure [12,13]. The system using AIBN as the initiator, butyl acetate and *n*-heptane as pore-forming materials. The porous copolymer was formed by heating to 60 °C for several hours in N_2 atmosphere. Porous microstructure of copolymer can be controlled by varying the dosage of cross linking agent and the ratio of butyl acetate to *n*-heptane. The porous polymerpellets were chemically modified, and different amino acid ligands were immobilized on the pellets by covalent coupling. In this study, the mass ratio of GT to amino acid in the immobilize reaction was fixed on 1:1.6 [14]. The resulting affinity adsorbents were swelled fully in anhydrous alcohol, washed with saline and dried in vacuum. According to the immobilized amino acid ligands, they were named GT–Phe, GT–Trp, GT–Tyr, GT–Pro and GT–HyPro, respectively.

The specific surface area of affinity adsorbents was measured by adsorption of N_2 gas with a surface area analyzer (Quantasorb, Syosset, NY) and were calculated by a single-point Brunauer–Emmet–Teller (BET) method [15]. Average pore radius and pore volume were determined from mercury porosimetry measurements, carried out on a Micromeritics Poresizer 9320 (Gosford, Australia).

2.3. Determination of circulating immune complex (CIC) levels in serum

C1q enzyme-linked immunosorbent assay (ELISA) method [16] was used to evaluate CIC levels. Testing kits were provided by Sino-American Biotechnology Co.

The CIC levels of 30 healthy rabbits were detected. The average value of blood CIC concentrations in the healthy subjects was 44.37 ± 8.16 μg/ml and was used as normal control.

2.4. Animal model

High CICs model was established through injection of horse serum [17]. Horse serum at 3 ml every fourth day, was given by three-point subcutaneous injections seven times to the back of experiment rabbits. Phagocytic activity in the reticuloendothelial systems of rabbits was depressed by intravenous injection of *E. coli* endotoxin on the 24th day (0.15 μg every rabbit), in this way, immune complexes will not be removed effectively by reticuloendothelial systems. On the 24th day or thereafter, the concentrations of CICs in animal models reaches 190.28±14.63 μg/ml, more than four times of normal control group. Pathological, electron microscope examination confirm that there are dispersive granular immune complex deposits along glomerular basement membrane.

2.5. Adsorption experiments in vitro

In a 25-ml Erlenmeyer flask which was sealed with a solid rubber stopper, certain amount of adsorbent was immerged in serum, whose CIC level has already been measured. The Erlenmeyer flask was incubated in a water bath at constant temperature, and was then stirred from the outside by using a vibrator. The stirring was stopped, and the change of CIC level in serum was observed.

The adsorption efficiency (%) of adsorbents was assessed according to the following formula:

$$\text{adsorption efficiency}\,(\%) = (C_0 - C)/C_0 \times 100\% \quad (1)$$

The adsorption capacity (in micrograms per gram) was calculated according to the following formula:

$$\text{adsorption capacity}\,(\mu g/g) = (C_0 - C)/M \times V \quad (2)$$

where C_0 and C are the CIC level in serum at the beginning and at the closing of adsorption experiment, respectively. M is the amount of adsorbent (in grams). V is the volume of the serum (in ml).

2.6. Blood perfusion

Under aseptic conditions in a laminar airflow cabinet, 200 g affinity adsorbents were packed into a polytetrafluoroethylene perfusion columns. The adsorbent in the column was pre-equilibrated with physiological saline twice. Seal off the affinity chromatography column at both end and sterilize by autoclaving. During hemoperfusion, an constant current with velocity of 80~1:2:0 ml/min was maintained using a constant current pump HL-2 (Pengdai Industry Crop, Shanghai, China).

The rabbits were initially preanesthetized with ketamine (4 mg/kg body wt im), then anesthetized with 100 mg ketamine and 10 mg diazepam, placed on an operating table maintained at body temperature (37 °C). Polyethylene catheters were inserted into the right femoral artery and the right femoral vein.

In the process of blood perfusion, arterial blood samples were collected from the catheter in the femoral artery for determination of blood CIC levels with C1q ELISA, for measurement of Immunoglobulins IgG, IgM, complement C3, C4 concentrations with single radial immunodiffusion assay, and for blood routine examination, plasma proteins and blood gas analysis. All the examinations mentioned above were carried out within 2 h after sampling.

3. Results and discussion

3.1. Porous microstructure of affinity adsorbents

Fig. 1 is a correlation diagram displaying the relative microstructure characters of adsorbents studied in this paper. The largest specific surface area was present in GT–Tyr (106.57 m^2/g), and then GT–Pro, GT–HyPro, GT–Phe and GT in descending stairs, the smallest was present in GT–Trp (59.12 m^2/g). The largest average pore radius was observed in GT–Trp (261 Å), and then in descending order were GT–Phe, GT, GT–HyPro, GT–Pro, GT–Tyr (46 Å). Pore volume has orders similar to average pore radius.

3.2. Adsorption properties of affinity adsorbents for CICs in serum

Adsorption experiments in vitro demonstrate that five kinds of adsorbents have different adsorption efficiency and adsorption capacity for CICs in serum

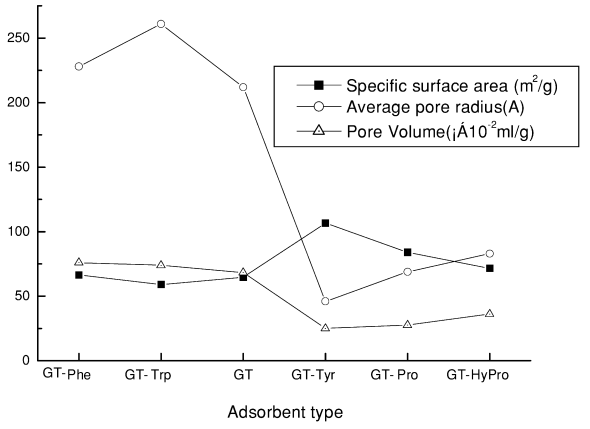

Fig. 1. Correlation diagram of the specific surface area, average pore radius and pore volume for five kinds of affinity adsorbents, GT matrix was used as contrast.

(Fig. 2). GT–Tyr hold the highest adsorption efficiency and adsorption capacity for CICs, that is 39.8% and 140.0 μg/g, respectively, when C_0 = 88.02 μg/ml. The second highest adsorption efficiency and adsorption capacity for CICs goes to GT–Trp, 35.1% and 123.4 μg/g, respectively. The rest adsorbents, from high adsorption efficiency and capacity to low, are GT–Phe>GT–Pro>GT–HyPro. Adsorption efficiency and capacity of these five kinds of affinity adsorbents with immobilized amino acid ligands are all higher than GT matrix. There is few literature discussing the CICs adsorption mechanism on affinity adsorbents. We hypothesize it might be connected with porous microstructure of adsorbents, the performance of immobilized ligands, and the structure of immune complexes. Further investigations are required to elucidate the nature of the adsorption mechanism. In the present work, further study focused on GT–Tyr, which hold the highest adsorption efficiency and capacity.

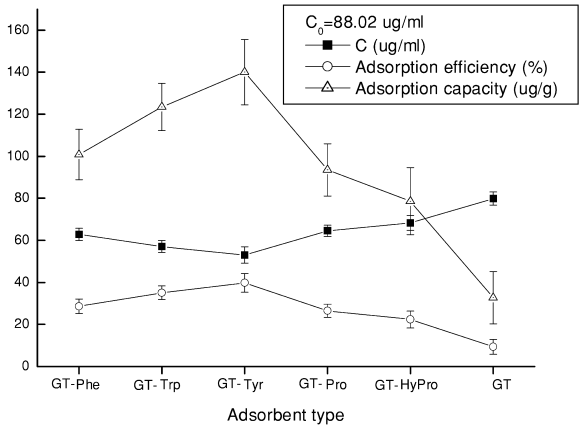

Fig. 2. Adsorption property of the five kinds of adsorbents to CICs in serum. GT matrix was used as contrast. Experiment conditions: sample size, n = 10; adsorbent dosage, 0.5 g; initial CIC concentration, 1 ml serum whose CIC level is 176.04 μg/ml was diluted with 1 ml physiological saline; water bath temperature, 3.7 °C, stirred for 2 h. P < 0.05.

3.3. CIC adsorption on GT–Tyr

3.3.1. Effect of contact time on CIC adsorption

Fig. 3 shows how GT–Tyr adsorption efficiency and capacity vary with the contact time. The system reaches adsorption equilibrium after 90 min. When the initial concentration of CICs is 88.02 μg/ml, equilibrium adsorption efficiency and capacity are 39.8% and 140.16 μg/g, respectively.

3.3.2. Effect of temperature on CIC adsorption

The influence of temperature on the adsorption of CICs on GT–Tyr is displayed (Fig. 4). A large increase in equilibrium adsorption capacity and efficiency of GT–Tyr for 27 °C relative to 7 and 17 °C was observed. Above 27 °C, the slope of the temperature–capacity and efficiency curve tend to decrease. The increase pace of adsorption capacity

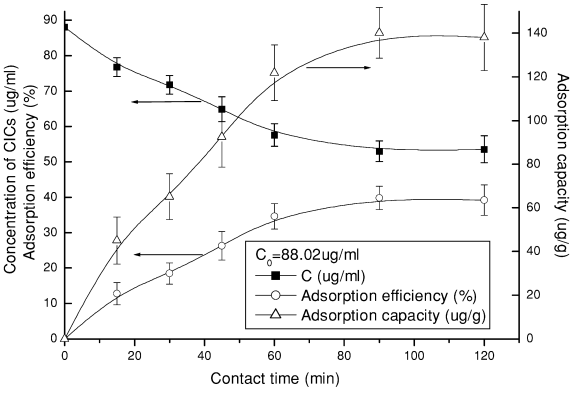

Fig. 3. The time-dependent adsorption of CICs on GT–Tyr. Experiment conditions: sample size, n = 10; adsorbent dosage, 0.5 g; initial CIC concentration, 1 ml serum whose CIC level is 176.04 μg/ml was diluted with 1 ml physiological saline; water bath temperature, 3.7 °C. P < 0.05.

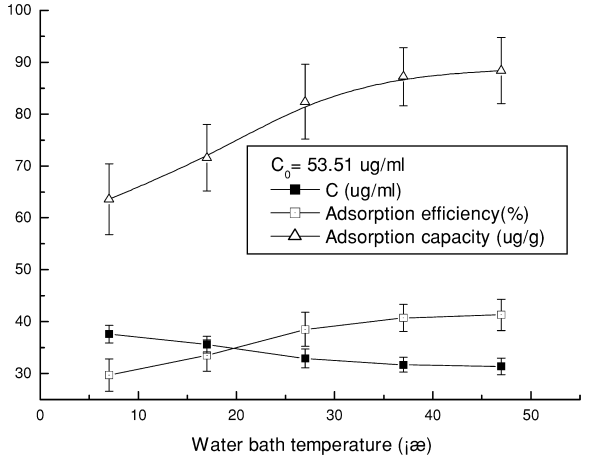

Fig. 4. Effect of temperature on the equilibrium adsorption of CICs on GT–Tyr. Experiment conditions: sample size, $n=10$; adsorbent dosage, 0.5 g; initial CIC concentration, 2 ml serum whose CIC level is 53.51 μg/ml, stirred for 2 h. $P<0.05$.

and efficiency for 47 °C relative to 37 and 27 °C was much slower.

3.3.3. Effect of initial CIC concentration on CIC adsorption

The effect of initial CIC concentration on the equilibrium adsorption efficiency and capacity of GT–Tyr is illustrated in Fig. 5. As the initial CIC concentration increases, the equilibrium adsorption efficiency of GT–Tyr is slightly dropped and the equilibrium adsorption capacity of GT–Tyr is increased obviously.

3.3.4. Effect of adsorbent dosage on CIC adsorption

The equilibrium adsorption level was also a function of the adsorbent dosage, as illustrated in Fig. 6. As the dosage of GT–Tyr increases, the equilibrium adsorption efficiency of GT–Tyr is slightly increased and the equilibrium adsorption capacity of GT–Tyr is dropped drastically.

3.4. Evaluate GT–Tyr in animal hemoperfusion trials

GT–Tyr was evaluated during hemoperfusion in an rabbit model. Arterial blood samples were collected intermittently for CIC level, complement, serum immunoglobulin, plasma protein, blood gas and blood routine examination. The CIC level change in rabbit model during hemoperfusion was illustrated in Fig. 7. The experiment demonstrate that CIC level in rabbit decline from 184.6 to 161.2 μg/ml after 15 min of hemoperfusion, and drop to 134.0 μg/ml at 30 min (adsorption efficiency

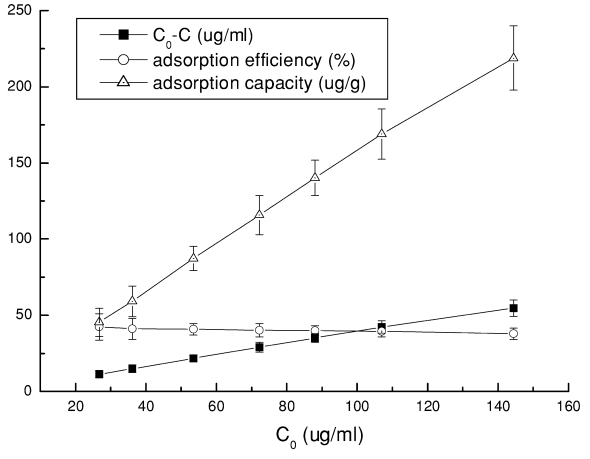

Fig. 5. Effect of initial CIC concentration on the equilibrium adsorption efficiency and capacity of GT–Tyr. Experiment conditions: sample size, $n=10$; adsorbent dosage, 0.5 g; initial CIC concentration, 2 ml serum whose CIC level vary according to abscissa; water bath temperature, 3.7 °C, stirred for 2 h. $P<0.05$.

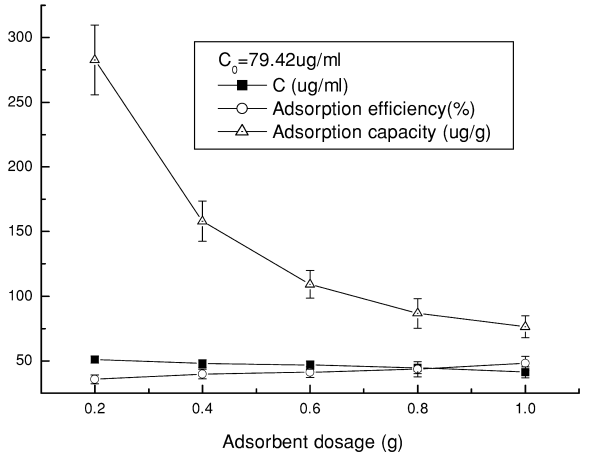

Fig. 6. Effect of adsorbent dosage on the equilibrium adsorption efficiency and capacity of GT–Tyr. Experiment conditions: sample size, $n=10$; adsorbent dosage vary according to abscissa; initial CIC concentration, 2 ml serum whose CIC level is 79.42 μg/ml; water bath temperature, 3.7 °C, stirred for 2 h. $P<0.05$.

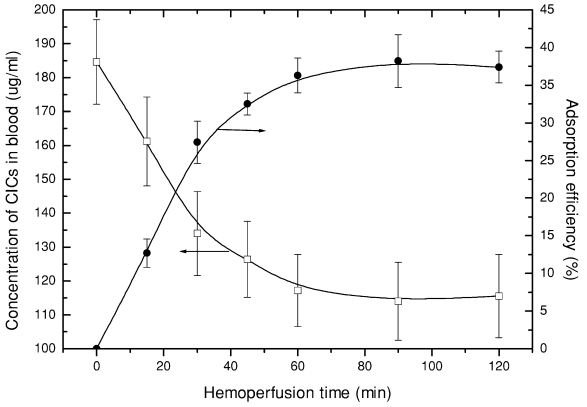

Fig. 7. Effect of hemoperfusion on CIC level in blood. Experiment conditions: constant hemoperfusive velocity 80~1:2:0 ml/min, the rabbits body temperature maintained at (37 °C), arterial blood samples were collected. Sample size, $n=10$. When hemoperfusion time $\leq$60 min, $P<0.01$. When hemoperfusion time $\geq$60 min, $P>0.05$.

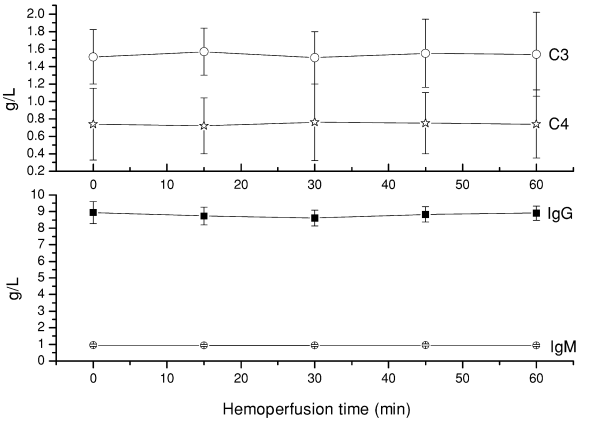

Fig. 8. Effect of hemoperfusion on the IgG, IgM, C3, C4 level in blood. Experiment conditions: constant hemoperfusive velocity 80~1:2:0 ml/min, the rabbits body temperature maintained at (37 °C), arterial blood samples were collected. Sample size, $n=10$. $P>0.05$.

27.4%), 126.4 μg/ml at 45 min (adsorption efficiency 32.5%), 117.2 μg/ml at 60 min (adsorption efficiency 36.3%). After 60 min, there is no noticeable decline in CIC level. The above data reveal that GT–Tyr can remove CIC via hemoperfusion efficiently and rapidly, the best hemoperfusion duration is 60 min.

In clinic, adsorbent should not only be able to remove harmful substances in vivo, but also have no blight on other components in blood. That is to say, ideal adsorbent should possesses fine biocompatibility. (1) IgG and IgM are the main antibody for type III allergic reaction. Constant amount of IgG and IgM are indispensable for normal function in vivo. Abnormal IgM and IgG quantity will result in various kind of diseases. IgG and IgM integrate with complement forming CIC. From the hemoperfusion results illustrated in Figs. 7 and 8, we know that GT–Tyr only show specific adsorption for antibody in CICs, leave antibody independent of CICs, i.e. normal IgG and IgM alone. (2) Complement C_3, C_4 are the most common complement components in vivo. Activation of C3 is necessary for classical and alternative pathway in innate immunity. C3 level falling indicate that there are complement activation and immunoreaction in vivo. Ideal adsorbent should not activate complement. As illustrated in Fig. 8, complement C3, C4 are minimally affected during hemoperfusion. It testify that GT–Tyr do not activate complement and immunologic injury. (3) Cell components in blood are essential for life maintenance. Any kind of cell trauma will be life-threatening. Hypoglobulia and thrombocytope will lead to hemorrhage, leukopenia make the patient susceptible. Ideal adsorbent should not adsorb or damnify blood cells. From Fig. 9 we know that there are no remarkable change in the amount of red-blood-cells (RBC), white-blood-cells (WBC) and hemoglobin (HB) during hemoperfusion. Only the amount of platelets (PLT) dropped about 10% at 30 min after hemoperfusion, this may result from the temperature difference between blood and adsorbent in perfusion column which destroy some platelets. The platelets amount recover to the level before hemoperfusion at 60 min after hemoperfusion. Thus, we can conclude that GT–Tyr do no harm to blood cells. (4) Body internal environment must keep homeostasis. Blood pH is the most important parameter for acid–base equilibrium. HCO_3^- concentration and carbon dioxide combining power indirectly reflect alkali reserve in vivo. The arterial partial pressure of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) reflect pulmon-

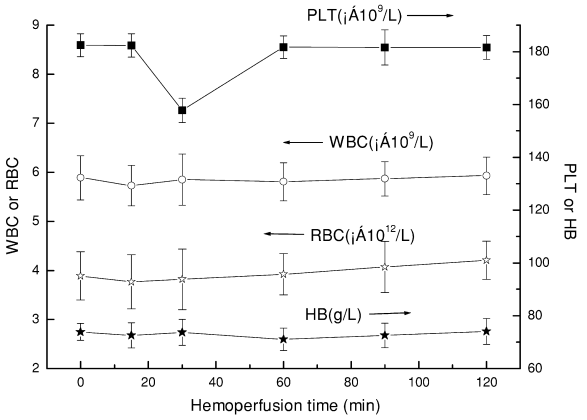

Fig. 9. Effect of hemoperfusion on the RBC, WBC, PLT, HB content in blood. Experiment conditions: constant hemoperfusive velocity 80~1:2:0 ml/min, the rabbits body temperature maintained at (37 °C), arterial blood samples were collected. Sample size, $n=10$. $P>0.05$.

ary gas exchange. In general, blood gas analysis is the criterion of acid–base equilibrium in vivo. Fig. 10 tell us that pH, PaO_2, $PaCO_2$, CO_2CP and HCO_3^- concentration keep stable in normal range during hemoperfusion. This prove that GT–Tyr do not disturb acid–base equilibrium in vivo. (5) Plasma protein and electrolyte balance are also indispensable for life maintenance. Ideal adsorbent should not have

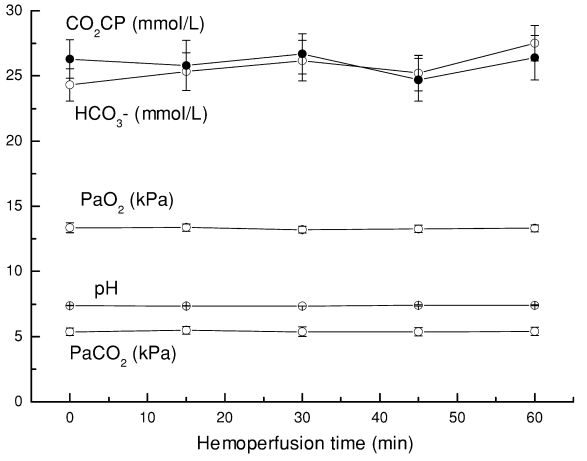

Fig. 10. Effect of hemoperfusion on blood gases and pH in blood. Experiment conditions: constant hemoperfusive velocity 80~1:2:0 ml/min, the rabbits body temperature maintained at (37 °C), arterial blood samples were collected. Sample size, $n=10$. $P>0.05$.

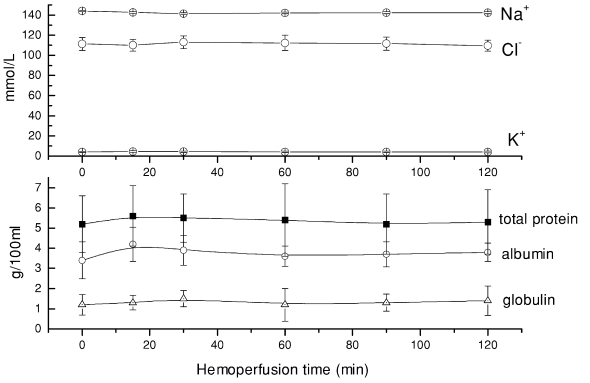

Fig. 11. Effect of hemoperfusion on plasma protein and electrolytes concentration in blood. Experiment conditions: constant hemoperfusive velocity 80~1:2:0 ml/min, the rabbits body temperature maintained at (37 °C), arterial blood samples were collected. Sample size, $n=10$. $P>0.05$.

side effects on plasma protein and electrolyte balance. The results in Fig. 11 show that there are negligible fluctuations of plasma proteins and electrolytes in normal range during hemoperfusion. It confirm that GT–Tyr do not have undesired impact on plasma protein and electrolyte. In summary, GT–Tyr is biocompatible.

4. Conclusion

Macroporous glycidyl methacrylate–triallyl isocyanurate copolymer based adsorbents (GT), which was immobilized with certain amino acid ligands, show specific affinity for circulating immune complexes. GT with tyrosine ligand (GT–Tyr), whose specific surface area, average pore radius and pore volume is 106.57 m^2/g, 46 Å and 0.25 ml/g, respectively, display the highest affinity for CICs. Batch study demonstrate that the adsorption level was mainly determined by adsorbent dosage, contact time, temperature and initial concentration of CICs. Hemoperfusion study in rabbit models confirm that GT–Tyr can remove CICs efficiently, and GT–Tyr own ideal biocompatibility. Therapy of immune disorders, i.e. systemic lupus erythematosus, immune complex nephritis, etc., is potentially available with this or its further improved versions.

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