Endotoxin Removal in Some Medicines and Human Serum Albumin Solution by Affinity Membranes

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Abstract: Three kinds of affinity membranes utilized for endotoxin removal, namely, chitosan affinity membrane (KFCC517), hydrophobic and cationic charged membrane (KFCG316) and metal chelate affinity membrane (KFCM402) were prepared by using cellulose membrane as support. The adsorbability and applications of the membranes in endotoxin removal were investigated. The results showed that all of these affinity membranes could be utilized for endotoxin removal in several kinds of solutions. The factors affecting endotoxin removal such as ionic strength, pH value and flow rate were investigated, and the results were discussed. Three cartridges of membranes KFCC517, KFCG316 and KFCM402 were utilized for endotoxin removal in human serum albumin solution. The cartridge of membrane KFCC517 also was utilized in medicine injections, such as hydrocortisone, puerarin, tetracaine hydrochloride and dextran 40 glucose. Under the optimal conditions, the removal efficiencies of endotoxin were over 80% and the recoveries of desirable substances were over 95%.

Key words: endotoxin; removal; affinity membrane; human serum albumin; medicine
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亲和膜用于去除医药及人血清白蛋白溶液中的内毒素

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摘要：以纤维素膜为基质材料，制备了 3 种用于去除内毒素的亲和膜，分别为壳聚糖亲和膜（KFCC517），疏水阳离子亲和膜（KFCG316）和金属螯合物亲和膜（KFCG402）。研究了亲和膜对内毒素的吸附容量及在内毒素去除方面的应用，结果表明这 3 种亲和膜都可以用于多种溶液中内毒素的去除。考察了离子强度、pH 值以及流速对去除效果的影响，并分析了原因。KFCC517，KFCG316，KFCG402 都可用于人血清白蛋白溶液中内毒素的去除，KFCC517 还可用于医药制剂如氢化可的松、葛根素、盐酸丁卡因和右旋糖苷 40 葡萄糖注射液中内毒素的去除，在所选择的条件下，内毒素的去除效率高于 80%，有用物质的回收率为 95%以上。

关键词：内毒素；去除；亲和膜；人血清白蛋白；医药

Endotoxin, the ingredient of the cell wall of gramnegative bacteria, possesses potential biological toxicity that causes pyrogenic and shock reactions in mammals when intravenously injected even in a very small amount[1]. Its main toxic moiety is lipid A, which is composed of a diglucoamine that is highly substituted with amide- and ester-linked long-chain fatty acids and negatively charged phosphate groups[2,3].

A number of methods, such as decomposition by acids, alkalis or oxidizing agents[4], ultrafiltration and ion exchange[5], have been extensively utilized to remove endotoxins, but these methods display lower selectivities to the target components. In the last decade, the adsorbents with immobilized affinity ligands such as histidine and polymyxin have been investigated for endotoxin removal[5-11], but these methods still show some limitations.

This work mainly deals with the preparation, adsorbability and applications of affinity membranes utilized for endotoxin removal. Three kinds of membranes, namely, chitosan affinity membrane

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(KFCC517), hydrophobic and cationic charged membrane (KFCG316) and metal chelate affinity membrane (KFCM402) were prepared with cellulose-based filter paper as support. All of these membranes prepared can be utilized for endotoxin removal in solutions, and the removal efficiencies were over 80%. The endotoxin adsorption capacity of the membrane KFCC517 is higher than 2.4×10^6 EU/g. These affinity membranes can be utilized to treat several kinds of sample solutions.

1 Experimental

1.1 Material and equipment

Cellulose-based filter paper (initial membrane) were purchased from Hangzhou Xinhua Paper Manufactory (Hangzhou, China). Glycidyl methacrylate (GMA), 1-vinylimidazole, *N*, *N*, *N*′, *N*′-tetramethylethylenediamine and 1,6-dichlorohexane were from Fluka (Buchs, Switzerland). The endotoxin standard sample was from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). A Tachypleus Amebocyte Lysate (TAL) test kit for endotoxin determination was from Marine Organism Product Factory (Zhanjiang, China). Human serum albumin (HSA) and all the medicine injections were kindly supplied by The First Affiliated Hospital of Dalian Medical University (Dalian, China). All other chemicals were of analytical grade.

A BET-16 bacterial endotoxin test apparatus was from Electronic Instrument Factory of Tianjin University (Tianjin, China). A BT01-100 peristaltic pump was from Lange Constant Flow Pump Corporation (Baoding, China).

1.2 Membrane preparation

1.2.1 Chitosan affinity membrane

First, cellulose-based filter paper was cut to round pieces with diameter of 47 mm. Then 40 sheets of this round cellulose membranes were immersed in a solution of 1.5 g of potassium periodate in 100 mL of 10% (*V*/*V*) sulfuric acid solution. The mixture obtained was placed to react for 12 h at room temperature with occasional stirring. After that, the oxidized membranes were washed with 1 000 mL deionized (D.I.) water.

Secondly, the oxidized membranes obtained from the last step were immersed in a solution of 0.9 g of chitosan in 100 mL of sodium acetate buffer (pH 6.0). The mixture was placed to react for 12 h at room temperature with occasional stirring. After that, the membranes were washed with 100 mL of 1% (*V*/*V*) acetic acid solution and 1 000 mL D.I. water.

Finally, the membranes grafted with chitosan were immersed in a solution of 0.3 g potassium tetrahydroborate in 100 mL of 0.1 mol/L sodium carbonate buffer (pH 9.0) and placed to react for 12 h at room temperature with occasional stirring. The final membrane was taken out from the reaction solution, washed with 1 000 mL of D.I. water, and dried by air. The chitosan affinity membranes obtained were marked as KFCC517 (see Table 1).

1.2.2 Hydrophobic and cationic charged affinity membrane

For grafting GMA onto start cellulose membrane, the membrane was first immersed in 100 mL of D. I. water and heated to 70 ℃ with occasional stirring. Then 8 mL of GMA, 0.4 g of ammonium persulfate (APS) and 0.6 g of sodium thiosulfate (STS) were added successively into the reaction mixture and the reaction was allowed to proceed for 1 h. Then the mixture was cooled to room temperature. The supernatant was decanted and the membrane was washed with 1 000 mL of D.I. water and air-dried.

After grafted, the membranes were immersed in a solution of 8 mL of 1-vinylimidazole and 100 mL of D.I. water in a reactor. The reaction was allowed to proceed at 70 ℃ for 1 h – 3 h. After the membranes were taken out and washed with D.I. water, they were immersed in 100 mL of D.I. water (70 ℃) again. Ten mL of *N*, *N*, *N*′, *N*′-tetramethylethylenediamine and 10 mL of 1,6-dichlorohexane were added into the reactor and the reaction was allowed to proceed overnight with occasional stirring. Then the reactor was cooled to room temperature. The supernatant was decanted and the membranes were washed with D.I. water and air-dried. The membranes marked KFCG316 were obtained (see Table 1).

1.2.3 Metal chelate affinity membrane

A solution of 100 mL of 0.1 mol/L sodium carbonate (pH 8.0) containing 4.0 g of iminodiacetic acid (IDA) was heated to 50 ℃. The cellulose membranes (20 sheets) grafted with GMA as described in

section 1.2.2 were immersed in the solution and allowed to stand at 50 ℃ – 60 ℃ for 6 h. Finally, the membranes were taken out and washed with 1 000 mL of D.I. water and air-dried.

The membranes obtained above were loaded into a membrane cartridge designed in our laboratory[12]. A volume of 250 mL of 0.05 mol/L zinc chloride solution was pumped through the membrane cartridge at a flow rate of 0.5 mL/min. Then the cartridge was washed with 1 000 mL of D. I. water. So we obtained the cartridge of the membrane KFCM402 (see Table 1).

Table 1 Label and composition of the membranes

Membrane	Matrix	Ligand
KFCC517	cellulose	-chitosan
KFCG316	cellulose-GMA	-vinylimidazole-dichlorohexane-tetramethylethyldiamine
KFCM402	cellulose-GMA	-IDA-Zn^{2+}

1.3 Endotoxin adsorption

Sample solutions containing endotoxin were prepared by putting a certain amount of endotoxin standard into the sample solutions and shaking to make uniformity.

Dynamic test: 20 sheets (about 3 g) of membranes prepared above were packed into a cartridge as described elsewhere[12]. A sample solution containing endotoxin was pumped through the cartridge at a flow rate of 2 mL/min at room temperature for endotoxin removal. The filtrate was collected in fractions and the endotoxin content of each fraction was determined by the BET-16 apparatus.

Static test: the weighed amounts of membranes were dispersed into the solutions of endotoxin in sterile tubes at room temperature while agitating occasionally for 2.5 h. The endotoxin content in the supernatant was assayed after the membranes were settled down.

Regeneration of the membrane cartridges: the membrane cartridges were regenerated by flushing successively with 100 mL of depyrogen water and the following solutions, 20 mL of 0.2 mol/L sodium hydroxide, 50 mL of 5 g/L deoxycholic sodium, 160 mL of 0.2 mol/L sodium hydroxide, and 250 mL of 1.5 mol/L sodium chloride, finally 250 mL of depyrogen water.

1.4 Substance assay

Endotoxin content was assayed by a kinetic-turbidimetric method. The turbidity produced by reaction of TAL with endotoxin can be inspected by the BET-16 apparatus at 660 nm. The data obtained were processed by a software automatically. The minimum detection limit of the BET-16 apparatus was 0.001 EU/mL. The endotoxin standards were utilized for making a calibration curve. The endotoxin contents of each fraction were obtained by comparing with the calibration curve.

HSA was determined by the absorbance measurement at 280 nm by a spectrophotometer. According to the Beer's Law ($A = abC$), the recovery (C_i/C_0) was obtained by calculating A_i/A_0.

Hydrocortisone was determined by the absorbance measurement at 242 nm by a spectrophotometer.

Tetracaine hydrochloride was assayed by titration with silver nitrate solution. Then the recovery of tetracaine hydrochloride was calculated.

The recovery of dextran 40 glucose was determined by polarimetric analysis with an automatic polarimeter.

Puerarin content was determined by the absorbance measurement at 250 nm by a spectrophotometer.

2 Results and discussion

2.1 Results of endotoxin removal with the affinity membranes

2.1.1 Efficiencies of three types of membranes in endotoxin removal

Three types of membranes were packed into three membrane cartridges (20 sheets of the membrane for each cartridge). NaCl solution containing endotoxin was prepared by putting 120 EU endotoxin standard into 120 mL normal saline. Then the solution was pumped through the cartridge at a flow-rate of 0.5 mL/min. The filtrate from the outlet of the cartridge was collected at 30 min, 90 min, 150 min and 210 min respectively and 2 mL for each time was collected. The Endotoxin content of each fraction was determined by the BET-16 apparatus.

Fig.1 shows that the removal efficiencies of endotoxin in NaCl solutions are all over 92% by three

kinds of membranes. The best one is the affinity membrane KFCC517 modified with chitosan, and the second is the hydrophobic and cationic charged membrane KFCG316. Compared with the membranes KFCC517 and KFCG316, the metal chelate affinity membrane KFCM402 is less efficient. This is probably because either chitosan grafted on the membrane KFCC517 or lipid A of endotoxin has the similar structure of amino glucose and it causes good biological compatibility between them. Otherwise, the partially positively charged amino group of chitosan may attract the negatively charged phosphate ester groups of endotoxin, so the affinity membrane KFCC517 can effectively remove endotoxin. The membrane KFCG316 also can remove endotoxin because both of its two parts, i.e. hydrophobic and cationic charged parts can interact with the long-chain fatty acids and negatively charged phosphate groups of endotoxin respectively. However, as a general ligand, the metal chelate affinity ligand is often utilized for the separation of many biological molecules, but the membrane KFCM402 has weak cationic charge and short alkyl chains, so it is not good enough to adsorb much endotoxin.

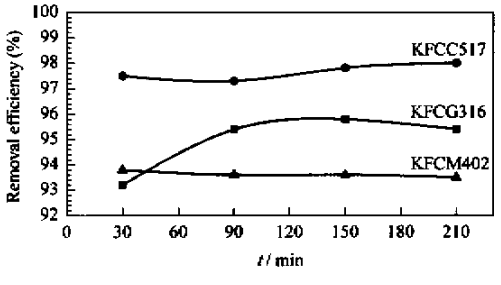

Fig.1 Endotoxin removal by three type membranes

Endotoxin initial concentration: 1.0 EU/mL; ionic strength: 0.15 mol/L, pH 6.0; flow rate: 0.5 mL/min.

2.1.2 Adsorption capacity

The adsorption of endotoxin onto the affinity membranes was investigated with 2.0 mg of membrane KFCC517 by static test. The initial solution was prepared by putting different amounts of endotoxin standard into normal saline. The results are shown in Fig.2. As the high concentration of endotoxin was not available, the adsorption capacity of the membrane did not reach saturation and saturated capacity was not calculated owing to the lack of data. However, the adsorption capacity calculated could be higher than 2.4×10^6 EU/g according to the content of endotoxin in the supernatant of the original standard with high concentration in this test. The value was similar to some other adsorbents reported in literature[2,9,10].

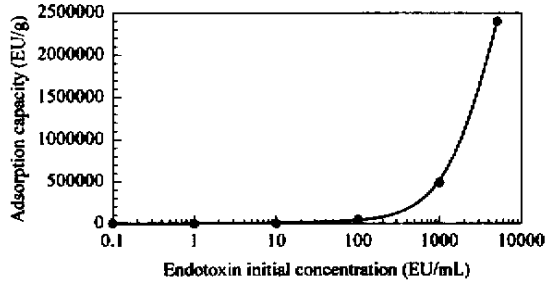

Fig.2 Equilibrium isotherms of endotoxin onto the membrane KFCC517

NaCl solution, room temperature; ionic strength: 0.15 mol·kg^{-1}, pH 6.0; adsorption time: 2 h.

2.2 Effects of experimental conditions

Affinity interaction mechanism is a complicated process, so the experimental conditions, such as ionic strength, pH value and flow rate can all affect the removal efficiency of endotoxin. The effects of some factors were investigated as follows.

2.2.1 Effect of ionic strength

The effect of ionic strength on the efficiency of endotoxin removal are shown in Fig.3. It can be seen that ionic strength has only minimal influence on the removal of endotoxin in the range of 0.05 mol/kg – 0.6 mol/kg. As endotoxin is negatively charged in addition to hydrophobic character, normally, there are both ionic force and hydrophobic interaction existed between endotoxin and the affinity membrane. Such interactions lead to opposite effects with the change of ionic strength, at the synergic interaction of ionic force and hydrophobic force; the change of the removal efficiency is minimized.

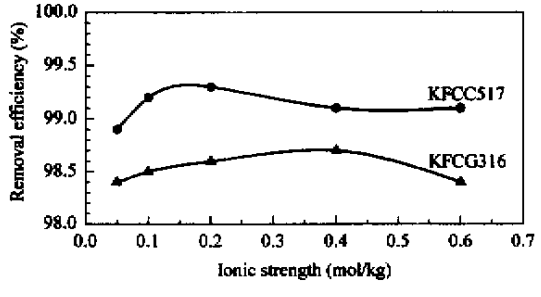

Fig.3 Effect of ionic strength on endotoxin removal

Affinity membrane: 5.0 mg; room temperature; endotoxin initial concentration: 5.0 EU/mL; pH 6.0; adsorption time: 2 h.

2.2.2 Effect of pH

The effect of pH values on the efficiency of endotoxin removal are shown in Fig.4. It can be seen that the membrane KFCG316 is not severely affected by pH value. On the one hand, with the increase of pH the positive charge of the membrane was decreased, so the ionic interaction between the affinity membrane and endotoxin was reduced. However, on the other hand, the hydrophobic interaction could make up the decrease of ionic force since there was hydrophobic action between the membrane and endotoxin. Therefore, the membrane still maintained its adsorption to endotoxin.

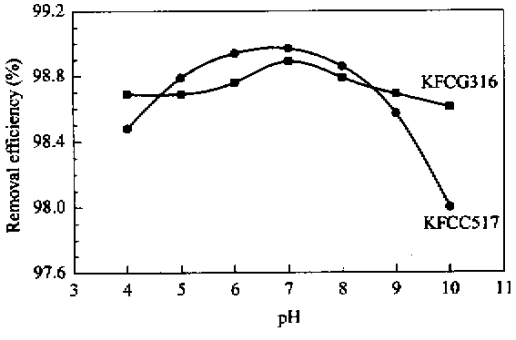

Fig.4 Effect of pH on endotoxin removal

Affinity membrane: 5.0 mg; room temperature; endotoxin initial concentration: 5.0 EU/mL; ionic strength: 1.5 mol/kg; adsorption time: 2 h.

It also can be seen that the removal efficiency of the membrane KFCC517 was affected a little more severely than that of the membrane KFCG316. The highest adsorption occurred at pH values between 6 – 8. The removal efficiency decreased obviously at pH 10. This is probably because, after pH value greater than 8, the increase of the concentration of hydroxide ion inhibited the ionization of the amino group of chitosan. Therefore the cationic charge of the affinity ligand was cut down, and the interaction between the affinity membrane and endotoxin fell down. Thus, the removal efficiency reduced considerably.

2.2.3 Effect of flow rate

The cartridge with 20 sheets of the membrane KFCG316 was utilized to remove endotoxin from endotoxin-containing water (2.24 EU/mL) at different flow rates in dynamic tests. The results are displayed in Fig.5. They show clearly that the removal efficiency of the cartridge decreases not much with the increase of flow rates.

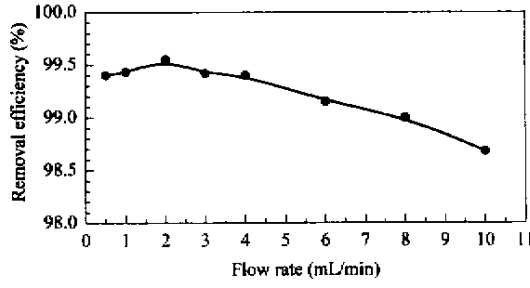

Fig.5 Effect of flow rate on endotoxin removal

2.3 Applications of affinity membranes

Endotoxin in medicine injections is harmful to human's health. Thus, the membranes prepared were utilized for endotoxin removal in several medicine injections. The results are shown as follows.

2.3.1 Endotoxin removal in some medicine injections

The sample solutions were prepared by adding 60 EU of endotoxin standard into 60 mL of the medicine injections and pumped all the solutions through the membrane cartridges. The endotoxin contents in the filtrates from the cartridges were determined.

The results of the membrane KFCC517 cartridge utilized for endotoxin removal in hydrocortisone, puerarin, tetracaine hydrochloride, dextran 40 glucose injections are shown in Table 2.

Table 2 The results of the membrane KFCC517 used for endotoxin removal in several medicine injections

Injection	Endotoxin concentration (EU/mL)		Endotoxin removal efficiency (%)	Main component recovery (%)
	sample	filtrate		
Hydrocortisone	1.0	0.059	94.1	100
Dextran 40 glucose	1.0	0.080	92.0	97.8
Puerarin	1.0	0.031	96.9	97.2
Tetracaine hydrochloride	1.0	0.420	58.0	100

It can be seen from Table 2 that the recoveries of hydrocortisone, puerarin, dextran 40 glucose injections are all over 95% and the removal efficiencies of endotoxin are over 92%. However, for tetracaine hydrochloride injection, the recovery of tetracaine hydrochloride was 100% while endotoxin removal efficiency was only 58%. This is reasonable if we think over the interaction between the medicines (see Fig.6) and endotoxin. For hydrocortisone, puerarin and dextran 40 glucose, there is little interaction between them and endotoxin because there are not so

many cationic charges in their structures. However, for tetracaine hydrochloride it was positively charged at pH 6 because there are two amino groups in its structure so it can react with the negatively charged phosphate group of endotoxin molecule. Such interaction disturbs the interaction between the membrane and endotoxin. Therefore, its efficiency of endotoxin removal was lower than those of other medicine injections.

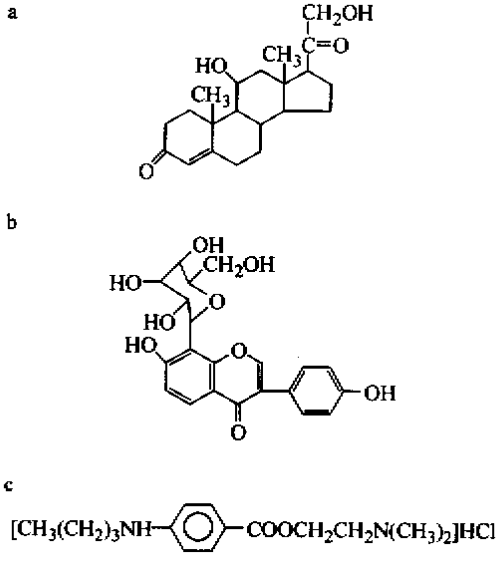

Fig.6 Structures of the medicines
a. hydrocortisone; b. puerarin; c. tetracaine hydrochloride.

When pH was adjusted from 6 to 7, less amino groups in tetracaine hydrochloride were positively charged, the removal efficiencies by both the membrances KFCC517 and KFCG316 were increased to 81.15%, and the recovery of tetracaine hydrochloride still remained at 100%. The results are shown in Table 3 in detail.

Table 3 Effect of pH on endotoxin removal in tetracaine hydrochloride

Membrane	pH	Endotoxin concentration (EU/mL)		Removal efficiency (%)
		sample	filtrate	
KFCC517	6	1.0	0.435 8	56.42
	7	1.0	0.188 5	81.15
KFCG316	6	1.0	0.344 2	65.58
	7	1.0	0.188 5	81.15

2.3.2 Endotoxin removal in HSA solutions

The membrane cartridges of KFCC517, KFCG316 and KFCM402 were utilized for endotoxin removal in HSA solutions and the results are shown in Table 4.

Table 4 Removal efficiencies of Endotoxin and HSA recoveries in HSA solutions

Membrane	Removal efficiency (%)	HSA recovery (%)
KFCC517	94.8	99.2
KFCG316	94.8	98.9
KFCM402	83.3	98.4

HSA solutions containing endotoxin were prepared by adding 60 EU endotoxin standard into 100 mL HSA solutions with HSA concentration of 2.0 g/L.

Table 4 shows that the membranes KFCC517 and KFCG316 can remove endotoxin effectively while KFCM402 only can remove 83.3% of endotoxin in HSA solutions. By comparing the removal efficiencies of endotoxin in Table 4 and Fig.1, it can be seen that removing endotoxin in HSA solution is more difficult than that in NaCl solution. This is probably because endotoxin can conjugate with HSA. The membrane KFCG316 cartridge has also been utilized for endotoxin removal in patient ascites[13], the removal efficiency was 82.8%, and the recoveries of useful substances were over 95%.

3 Conclusion

All three kinds of membranes prepared in this study could be utilized for endotoxin removal in solutions. The adsorption capacity for the membrane KFCC517 was higher than 2.4×10^6 EU/g. The Effects of ionic strength, pH value and flow rate on the efficiency of endotoxin removal were not severe. These affinity membranes were utilized to treat HSA solutions and some medicine injections such as hydrocortisone, puerarin, dextran 40 glucose and tetracaine hydrochloride. Under the optimal conditions, the removal efficiencies of endotoxin were over 80% and the recoveries of target substances were over 95%.

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会 议 通 知

第五届全国毛细管电泳及相关微分离分析学术报告会(CCE2002)和第四届亚太毛细管电泳及相关微分离分析学术报告会(APCE2002)将分别于 2002 年 10 月 8 日 ~ 10 日和 2002 年 10 月 11 日 ~ 14 日在上海召开。会议讨论的相关议题包括毛细管电泳、芯片毛细管电泳、芯片实验室及其他微分离分析技术。

大会已开始征稿。凡关于这一领域的技术、应用、仪器和附件的研制及基础理论研究等方面尚未公开发表的论文均可应征,应征稿件请于 2002 年 8 月 1 日前寄出,提交大会学术委员会审定。

被接受为 CCE2002 的稿件将编入本届报告会文集“毛细管电泳进展(第五卷)”;APCE2002 的会议论文集将以专刊的形式发表于 Electrophoresis 杂志。

会议热忱欢迎在学博士、硕士研究生参加,并将根据财力状况在费用上给予尽可能多的照顾。会议也热忱欢迎无论文者参加。相关详情请见会议主办单位主页:http://www.chip-bioanalysis.com

第五届全国微分离分析学术报告会(CCE2002)日期:2002 年 10 月 8 日至 10 日
第四届国际微分离分析学术报告会(APCE2002)日期:2002 年 10 月 11 日至 14 日

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