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IMMOBILIZATION OF UREASE INTO CARBOXYMETHYLCELLULOSE - GELATINE SYSTEM

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ABSTRACT

In the present work carboxymethylcellulose (CMC) and CMC-gelatin were used as carrier systems for urease immobilization. Immobilization was based on the formation of insoluble salts of CMC and gelatin with chromium(111) ions. Chromium(lll) acetate (CA) and chromium(lll) sulfate (CS) were used for this purpose and their effect on urease activity was investigated. The activities of immobilized urease using pure and CMC-gelatin carrier systems were compared. Urease activity was determined by using Berthelot method. Reuse number, pH, enzyme and cross linker concentrations and the incubation period were the factors taken into account in this investigation. Activity of immobilized enzymes were found to be stable for at least 2 months and 16-24 usage. Immobilization percentage obtained under optimum conditions was 40%.

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INTRODUCTION

Immobilized enzymes are used in food technology, biotechnology, biomedicine and analytical chemistry because of their various advantages [1,2].

Urease enzyme catalyses the hydrolysis of urea to ammonium and carbondioxide. Since one of the main objectives of artificial kidney machines is removal of urea from blood for detoxification; researches has been carried on the usage of immobilized urease for these applications by various investigators [3-5]. Most commonly used immobilization techniques were covalent binding and micro encapsulation with different carrier synthetic or natural macromolecular compounds [6-10].

Sodium carboxymethylcellulose is most commonly called CMC . In food industry, the term cellulose gum is used to designate purified CMC suitable for use in foods since CMC is physiologically inert. It is the most widely used water soluble cellulose derivative. Salts of CMC with heavy metal ions such as silver, copper, lead, and zirconium are insoluble in water and can be formed by adding a water soluble salt of the metal to an aqueous CMC solution. The current uses of CMC are in detergents, foods, drilling muds, textiles, paper, pharmaceuticals and paints etc [11,12].

Gelatin is a water soluble protein resulting from the partial hydrolysis of collagen. The most characteristic physical property of gelatin is its ability to form reversible elastic gels when its aqueous solutions are chilled. Gelatin is used in foods, photographic films, pharmaceuticals etc. Gelatin hardens when reacted with chromium salts and aldehydes [13].

The scope of this research is on the use of two biocompatible ingredients CMC and gelatine as support material in urease immobilization. CMC and CMC-gelatin systems were compared with each other with respect to immobilization percentages. Various experiments were made with different concentrations of cross linkers CA and CS to obtain the most suitable immobilization conditions. pH, incubation time, reuse number were some of the factors considered for the comparison of CMC and CMC-Gelatin carrier systems.

Further studies can be made on the usage of urease enzyme, immobilized by this method in artificial kidney systems.

MATERIALS

Urease enzyme was purchased from Merck (Art 8489)

Urea was obtained from Merck

Granular photographic gelatin was obtained from Croda Gelatin Co.

Polyester films were purchased from Dupont De Nemours which was precoated with photographic gelatin (100 μm).

Carboxymethylcellulose was purchased from Sigma (Low viscosity C-8758)

Cellulose paper was 75g/m^2 and obtained from Toprak Paper Co. Other chemicals and buffers were analytical grade and no further purification was made.

APPARATUS

Spectronic 20 model spectrophotometer was used for ammonium determinations.

METHODS

Immobilization of urease

CMC System

0.450 g CMC was dissolved in 10 ml of phosphate buffer (pH 6.5) and 1 ml of urease solution (40 mg/ml - 80 U) was added to this solution. Then required amounts of CA or CS were added as cross linker. 0.1 ml Aliquots taken from this solution were placed on paper which was precoated by 4.5% w/v CMC and insolubilized by CA or CS.

CMC-Gelatin System

0.225 g CMC and 0.225 g gelatin were dissolved in 10 ml of phosphate buffer (pH 6.5) and 1 ml of urease solution (40 mg/ml - 80 U) was added. Later cross linkers were added to the solutions and 0.1 ml samples were taken and were placed on polyester films as described earlier [14,15].

All the immobilization processes were performed at 32°C.

Urease activity assay

Phenol-hypochloride method was used to determine urease activity [16]. Experiments were carried out parallelly by using blanks and test samples. 1.5 ml Phosphate buffer (pH 6.5) was transferred in each test tube and immobilized urease was immersed in these solutions. Solutions were preincubated 2 minutes at 37°C and 0.2 ml urea solution (0.2 mg/ml) was added, test tubes were kept in water bath at 37°C for 15 minutes and reactions were ended by removing immobilized urease. Phenol-sodium nitropurisside solution (0.106M-0.170 mM) and sodium hydroxide-hypochloride solution (11 mM - 0.125 M) were added then tubes were stirred for 30 minutes in water bath at 37°C. Absorbances were determined at 555 nm by using UV-Spectrophotometer. All readings were made against blanks.

RESULTS AND DISCUSSION

To investigate the type of immobilization enzyme leakage tests were performed. Immobilized urease containing strips (paper or polyester film) were washed by phosphate buffer (pH 6.5) for 17 minutes. Enzymatic activity in the washing solutions were assayed and the results obtained are given in Table 1.

The results showed that the enzyme leakage was negligible, thus supporting the chemical cross linking rather than physical entrapment for the type of immobilization.

TABLE 1. Absorbance values[a] obtained from the washing solutions of immobilized urease.

SUPPORT & CROSS LINKERS	NUMBER OF WASHINGS			
	1	2	3	4
CMC [CA]=0.10M	0.074	0.020	0.012	0.010
CMC [CA]=0.15M	0.053	0.004	0.000	0.000
CMC [CA]=0.20M	0.035	0.000	0.000	0.000
CMC [CA]=0.25M	0.025	0.001	0.000	0.000
CMC [CA]=0.30M	0.020	0.001	0.000	0.000
CMC-Gelatin [CA]=0.05M	0.051	0.000	0.000	0.000
CMC-Gelatin [CA]=0.10M	0.041	0.011	0.000	0.000
CMC-Gelatin [CA]=0.15M	0.039	0.009	0.000	0.000
CMC-Gelatin [CA]=0.20M	0.030	0.014	0.001	0.000
CMC-Gelatin [CA]=0.25M	0.027	0.008	0.000	0.000
CMC-Gelatin [CS]=0.0050M	0.092	0.002	0.000	0.000
CMC-Gelatin [CS]=0.0075M	0.070	0.009	0.000	0.000
CMC-Gelatin [CS]=0,0100M	0.040	0.008	0.000	0.000

a: Value obtained for the same quantity of free enzyme (2 U) used in immobilization was 0.484.
CMC:carboxymethylcellulose, CA: chromium acetate, CS: chromium sulfate

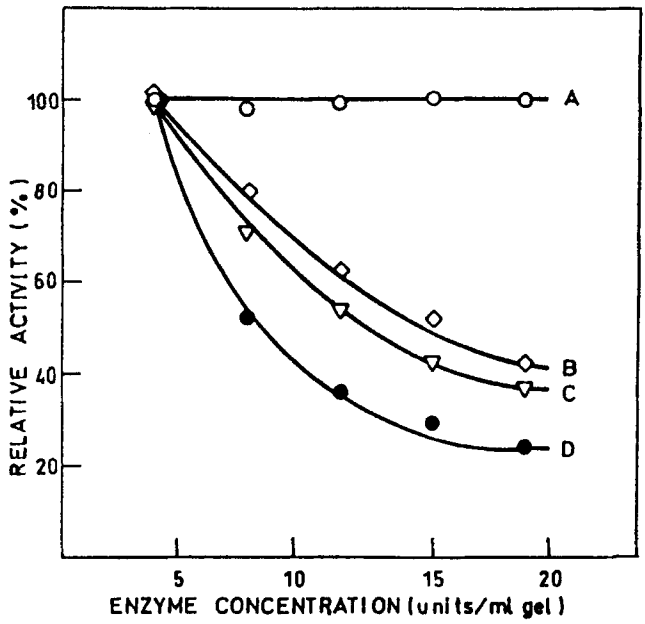

Figure 1. Effect of **enzyme concentration** on free and immobilized urease activity. A:Free, B:CMC-Gelatin-CA system, C:CMC-CA system, D:CMC-Gelatin-CS system CMC: carboxymethylcellulose, CA: chromium acetate, CS: chromium sulfate

Immobilization was carried out for different urease concentrations for both of the carrier systems. The results are given in Figure 1. As seen in Figure 1 although relative activity of free enzyme does not change, relative activities obtained for immobilized samples decrease by increasing urease concentration. This can be explained by increasing enzyme-enzyme cross linking thus causing a higher degree of enzyme inactivation.

Effect of pH on relative activities of free enzyme and immobilized enzyme with different cross linkers within different carrier systems were investigated. Results are given in Figure 2. The optimum pH value found for free enzyme was 8.0. In

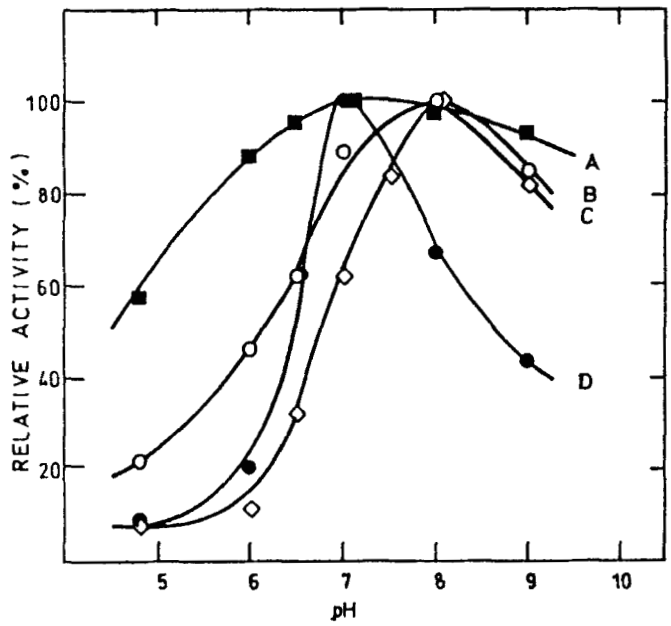

Figure 2. Effect of **pH** on free and immobilized urease activity. A:CMC-CA system, B:CMC-Gelatin-CA system, C:Free, D:CMC-Gelatin-CS system
CMC: carboxymethylcellulose, CA: chromium acetate, CS: chromium sulfate

the case for CMC-CA optimum value shifts to pH 7.0 and the effect of pH variations seem to be negligible between pH 6.0 and 9.0, which is an advantage of CMC-CA system. A similar effect can be seen in CMC-Gelatin-CA system but pH interval is narrower for high relative activity (pH 7.0-8.5) and no shift is observed for the optimum pH value. For CMC-Gelatin-CS system a shift to pH 7.0 is observed however the trend is similar to that of free enzyme. The pH investigations showed that CMC-CA system is better than other tested systems, because it is possible to work with it in a pH range of 6.0 to 9.0 and obtain a high relative activity.

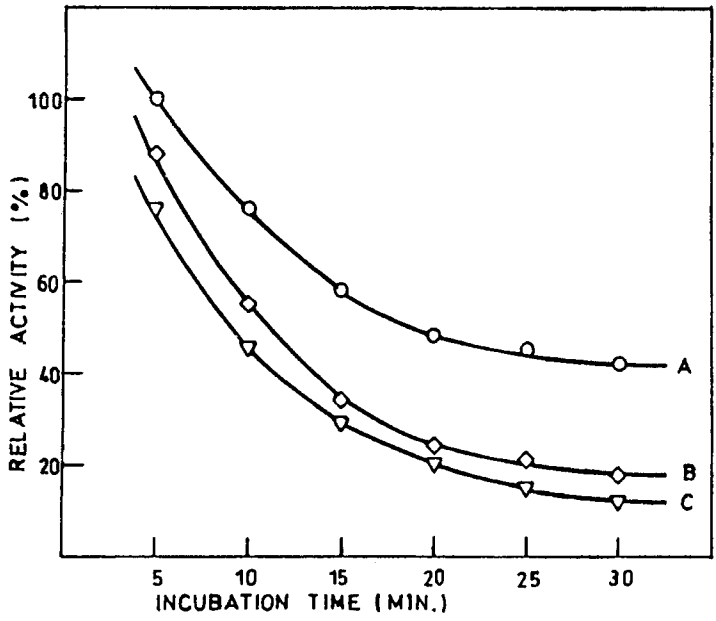

Figure 3. Effect of **incubation time** on free and immobilized urease activity. A:Free, B:CMC-CA system, C:CMC-Gelatin-CS system. CA: chromium acetate CMC: carboxymethylcellulose, CS: chromium sulfate

To analyze the effect of physical structure of immobilized enzymes on reaction yield, incubation period was tested. Free enzyme and cross linker CA with both carrier systems were investigated and the results are presented in Figure 3.

The decline in the relative activity of free enzyme is less than the decline in the relative activities of immobilized enzymes as expected, however the results showed that diffusion limitation caused by physical structure of immobilized enzyme is not so important. Thus immobilization of urease with either of the carrier systems is successful with respect to this parameter. There is not much difference between the relative activities of CMC-CA and CMC-Gelatin-CS systems due to diffusion

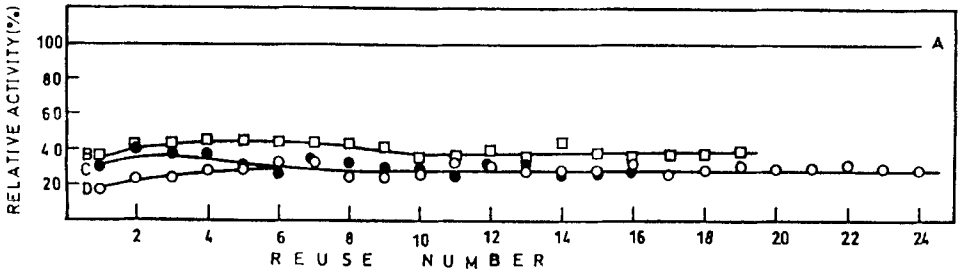

Figure 4. Variation of immobilized urease activity by **reuse number** for different cross linker concentrations in **CMC-Gelatin-CA** system. A:Free, B:0.10M CA, C:0.25M CA D:0.05M CA.
CMC: carboxymethylcellulose, CA: chromium acetate

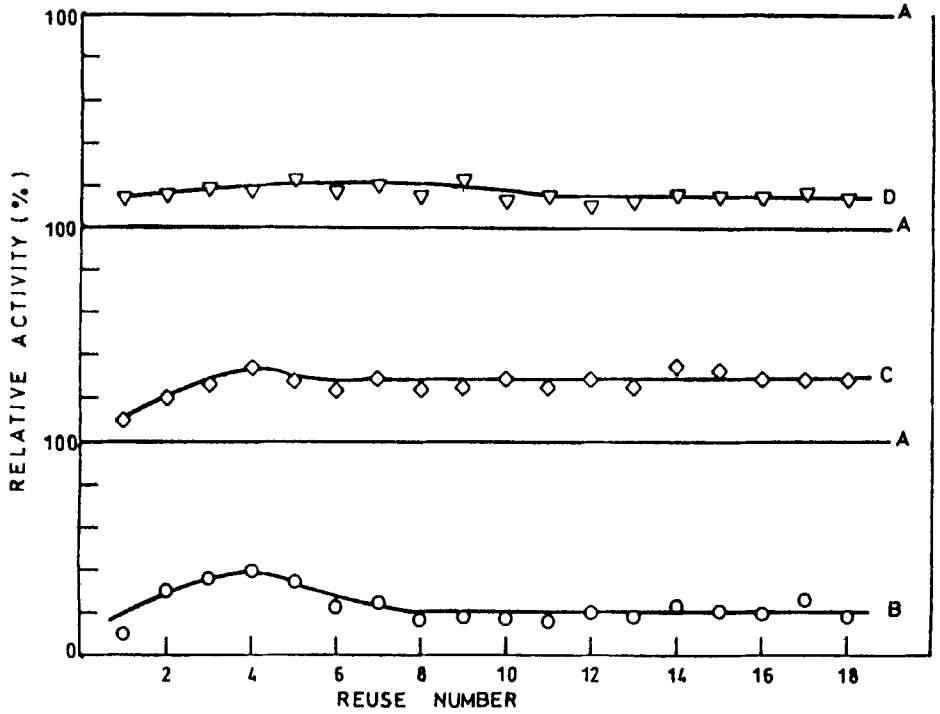

Figure 5. Variation of immobilized urease activity by **reuse number** for different cross linker concentrations in **CMC-Gelatin-CS** system. A:Free, B:0.005M CS, C:0.0075M CS, D:0.010M CS
CMC: carboxymethylcellulose, CS: chromium sulfate

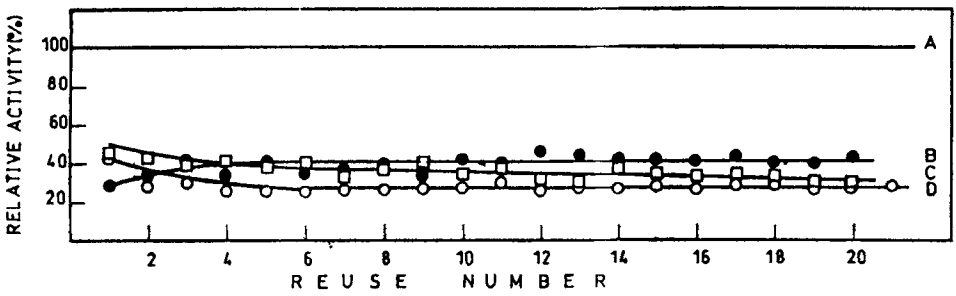

Figure 6. Variation of immobilized urease activity by **reuse number** for different cross linker concentrations. in **CMC-CA** system. A:Free, B:0.20M CA, C:0.10M CA, D:0.15M CA.
CMC: carboxymethylcellulose, CA: chromium acetate

limitations which is assumed to increase with incubation period.

To investigate the effect of cross linker concentration on the activity of immobilized enzyme, [CA] was varied from 0.05M to 0.30M and used with both carrier systems. On the other hand effect of [CS] changes were only analyzed for CMC-Gelatin carrier system because immobilization percentage obtained with this cross linker was lower. To make a better comparison, experiments were carried on up to 18-24th use for each different cross linker concentration. Results are given in Figures 4-6.

The results obtained show that immobilizations with the cross linkers and the carrier systems used are successful. There is no significant difference on the relative activities of immobilized enzymes between first use and the final use. Another important point which should be taken into consideration is that the activity of free enzyme in a 32 day period decreases 70% however the decrease in a ctivity observed for immobilized enzymes in this period is negligible. The difference between the relative activities of CMC-CA system and CMC-Gelatin-CA system is insignificant. When CS is used as cross linker, the relative activity

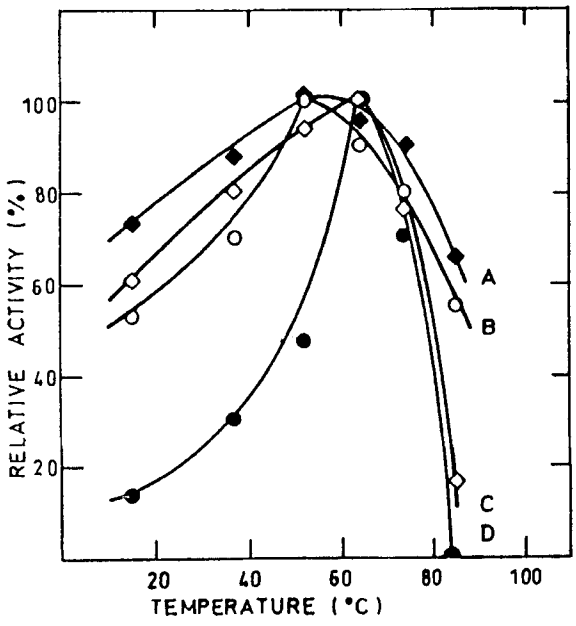

Figure 7. Effect of **temperature** on free and immobilized urease activity. A:CMC-CA system, B:Free, C:CMC-Gelatin-CA system, D:CMC-Gelatin-CS system
CMC: carboxymethylcellulose, CS: chromium sulfate, CA: chromium acetate

(immobilization %) is lower for CMC-Gelatin-CS system compared to chromium acetate. Immobilization percentages obtained in reuse number tests with CMC-Gelatin-CA carrier system for 0.05M, 0.10M, and 0.25M CA concentrations were 28, 38, and 28% respectively (Figure 4). The decrease in immobilization percentage , for the cross linker concentrations above 0.10M was accounted for enzyme deactivation and increased diffusion limitations due to increasing CA concentration. Similar results were observed in CMC-Gelatin-CS system as 20, 28 and 14% immobilization for 5.0×10^{-3}M, 7.5×10^{-3}M and 10×10^{-3}M CS concentrations respectively (Figure 5). No enzymatic activity was observed for the concentrations above 10×10^{-3}M. These results show that CA is a better cross linker than CS for urease immobilization.

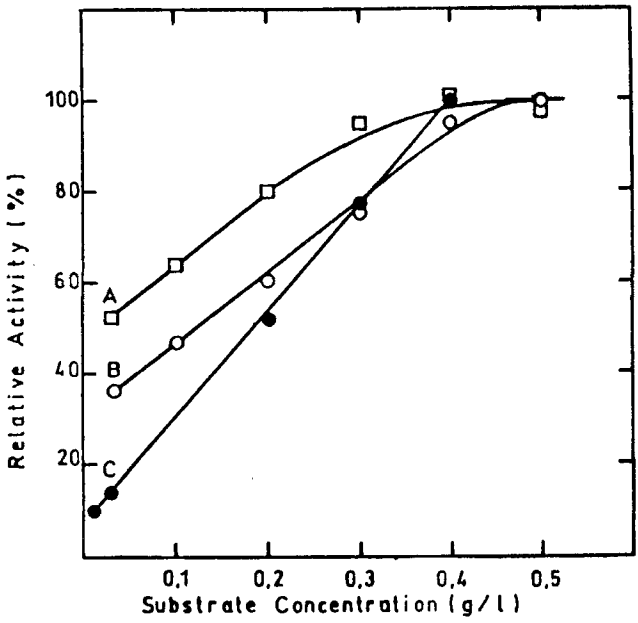

Figure 8. Effect of **substrate concentration** on free and immobilized urease activity. A:CMC-Gelatin-CA system, B:CMC-CA system, C:free. CA: chromium acetate CMC: carboxymethylcellulose.

To find out the effect of CA concentration in CMC carrier system for urease immobilization 0.10M, 0.15M, and 0.20M CA concentrations were used (Figure 6). Immobilization percentages obtained for these concentrations in the reuse experiments were 31, 27, and 40. The comparably high immobilization percentage obtained for 0.10M CA concentration can be explained by the physically unstable nature of the immobilized urease for this concentration and possible enzyme leakage due to deficient use of cross linker. It was observed that concentrations above 0.20M has negligible effect on the immobilization percentage and thus 0.20M CA was used for the rest of the work.

To investigate the effect of temperature on the activity of free enzyme and immobilized enzyme

experiments were performed in the range 15° C to 85° C and results obtained are given in Figure 7. Maximum activities obtained for free enzyme and CMC-CA system were at 52° C and for CMC-Gelatin-CA and CMC-Gelatin-CS systems were 64° C. The activities obtained within this range show that CMC-CA system is not effected much by temperature variations and a relative activity over 70% can be obtained in the range of 15° C to 84° C.

Effect of substrate concentration on the relative activities of free enzyme and immobilized enzymes were analyzed and results are given in Figure 8. 0.03-0.50 g/l Urea concentrations were used in these experiments. In the case of free enzyme relative activity changes linearly with increasing urea concentration (Figure 8C.). For CMC-Gelatin-CA system maximum relative activity was reached at 0.40 g/l and stayed constant up to 0.50 g/l (Figure 8A). Finally for CMC-CA system maximum activity was obtained at 0.50 g/l urea concentration (Figure 8B.). Results of these experiments show that substrate concentrations above; 0.40 g/l for CMC-Gelatin-CA system and 0.50 g/l for CMC-CA system does not effect relative activities considerably.

In summary following conclusions can be drawn from the present work.

CMC-CA system can be recommended because of its wider (high relative activity) pH range compared to CMC-Gelatin-CA system. Immobilizations with either method result with considerable savings because of low costs of carrier systems and high reuse numbers. In addition to the previously mentioned criteria biocompatible nature of CMC and gelatin permits the use of immobilized urease with these carrier systems in biomedical applications. In future paper or polyester film rolls can be coated by immobilized urease on both sides which will have large surface area, and can be used in artificial kidney machines successfully. Coating technology can be similar to photographic film coating procedure. The principle of photographic film developing can be used in the artificial kidney machines.

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