JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 699–704

# Sterilization of heparinized Cuprophan hemodialysis membranes

H. W. M. TEN HOOPEN, W. L. J. HINRICHS, G. H. M. ENGBERS, J. FEIJEN
*Faculty of Chemical Technology and Institute for Biomedical Technology, University of Twente, PO Box 217, 7500 AE Enschede, The Netherlands*

The effects of sterilization of dry heparinized Cuprophan hemodialysis membranes by means of ethylene oxide (EtO) exposure, gamma irradiation, or steam on the anticoagulant activity and chemical characteristics of immobilized heparin and the permeability of the membrane were investigated. Sterilization did not result in a release of heparin or heparin fragments from heparinized Cuprophan. Sterilization of heparinized Cuprophan by means of EtO exposure and gamma irradiation induced a slight, insignificant decrease of the anticoagulant activity. In contrast, steam-sterilized heparinized Cuprophan showed a higher anticoagulant activity than unsterilized heparinized Cuprophan, which was most likely caused by cleavage of some of the covalent bonds between heparin and Cuprophan. The effects of sterilization on the permeability of unmodified Cuprophan and heparinized Cuprophan were compared. The permeability of unmodified Cuprophan for vitamin $B_{12}$ (Vit $B_{12}$) and sulfobromophthalein (SBP) was reduced by 20–35% after EtO exposure and gamma irradiation and was reduced by 90–95% after steam sterilization. The water permeability of unmodified Cuprophan remained the same after EtO exposure and gamma irradiation but also dramatically reduced after steam sterilization. These reductions were ascribed to the collapse of pores of the membrane. The permeability of heparinized Cuprophan was not affected by EtO exposure and gamma irradiation but dramatically reduced after steam sterilization, although to a lesser extent than in the case of unmodified Cuprophan. Apparently, the presence of immobilized heparin (partially) prevented the collapse of pores during sterilization. Gamma irradiation was recommended as the preferred method of sterilization for heparinized Cuprophan.

## 1. Introduction

Hemodialysis is nowadays a well established therapy for uremic patients. The majority of these patients is treated with membranes made of regenerated cellulose. The main disadvantage of the application of cellulosic membranes for hemodialysis is their relatively poor blood compatibility. An often applied strategy to improve the blood compatibility of blood-contacting material surfaces is the immobilization of heparin [1–3]. In a previous study we have shown that heparin can be covalently immobilized onto Cuprophan hemodialysis membranes by means of a N,N′-carbonyldiimidazole (CDI) activation procedure [4]. Cuprophan heparinized in this way indeed showed an improved blood compatibility. Furthermore, the heparinization procedure hardly affected the permeability of the membrane for model solutes with a molecular weight up to 12 000.

Prior to use, hemodialysis systems require sterilization. Although in some studies the effects of sterilization on the properties of non-immobilized heparin has been investigated [5–7], less information is available about the effects of sterilization on the properties of immobilized heparin [3, 8, 9]. Furthermore, in several reports it is shown that sterilization can adversively affect the permeability of hemodialysis membranes [10–14]. Therefore, the present study was undertaken to investigate the effects of sterilization by means of three commonly used methods, EtO exposure, gamma irradiation, and steam, on the chemical characteristics and biological activity of heparin immobilized onto Cuprophan. Non-immobilized heparin was used as a control. Furthermore, the effects of sterilization on the permeability of heparinized Cuprophan were investigated.

## 2. Materials and methods

### 2.1. Materials

Cuprophan 150 M was a gift from AKZO, Wuppertal, Germany. Formamide (synthesis grade, Merck, Darmstadt, Germany) was purified according to a method described by Verhoek *et al.* [15]. Heparin (sodium salt) from porcine intestinal mucosa (activity: 165–190 U/mg, Mn: 12 000 g/mol) was purchased from Diosynth, Oss, The Netherlands. Heparin also

0957–4530 © *1996 Chapman & Hall*

from porcine intestinal mucosa of which 55–60% of the sodium ions was substituted by benzyltrimethylammonium ions (heparin triton B, Mn: 16 000 g/mol) was purchased from HBG, Enschede, The Netherlands. Human antithrombin III (AT III, Sigma Chemical Company, St Louis, USA) was purified using a heparin-Sepharose column (Sigma) [16]. Bovine albumin (Alb), Vit $B_{12}$, and SBP were also purchased from Sigma. Chromogenic substrate $S_{2238}$ was obtained from Chromogenix, Mölndal, Sweden. Polyethylene glycol (6000 g/mol, PEG) was purchased from Fluka Chemie, Buchs, Switzerland. PTT-reagent was obtained from Boehringer, Mannhein, Germany. CPDA-1 plasma was purchased from the Rode Kruis Bloedbank Twente-Achterhoek, Enschede, The Netherlands. Phosphate buffered saline (pH: 7.4, PBS) was purchased from NPBI, Emmercompascuum, The Netherlands. CDI, NaCl, 1 N NaOH, 1 N HCl, $CaCl_2 \cdot H_2O$, toluidine blue O zinc chloride double salt (toluidine blue), and tris(hydroxymethyl)-ammonium methane (tris) (all analytic grade) were purchased from Merck. All chemicals were used without further purification except when mentioned.

## 2.2 Heparinization of Cuprophan

Cuprophan was heparinized as described earlier [4]. Briefly, heparin triton B dissolved in formamide was activated with CDI. Thereafter, the reaction mixture was transferred to Cuprophan membranes. After immobilization, the membranes were rinsed with demineralized water and then incubated in an aqueous 4 M NaCl solution. Thereafter the membranes were again rinsed with demineralized water, soaked in a 10 wt % solution of glycerol in demineralized water and subsequently dried by air exposure for 2 days.

The immobilization procedure yielded membranes with about 80 μg immobilized heparin per $cm^2$ membrane surface area [4].

## 2.3. Sterilization

Heparinized Cuprophan and as a control non-immobilized sodium salt of heparin were sterilized by means of EtO exposure, gamma irradiation, and steam using the following procedures. EtO sterilization (Griffith, Zoetermeer, The Netherlands): the samples were stored for 16 h at a relative humidity of 60% and a temperature of 35 °C. Subsequently, the pressure was reduced to 7.5 kPa. After one hour the pressure was elevated to 60 kPa with EtO (850 mg/l) at 45 °C. After 3 h, the pressure was reduced to 7.5 kPa at 40 °C and subsequently elevated to $10^5$ Pa with air. This air wash procedure was repeated once. Thereafter, the samples were stored for 3 days at 40 °C. Gamma irradiation (Gammaster, Ede, The Netherlands): the samples were exposed to a $^{60}Co$ source until the samples received 2.3 kGy. Steam sterilization (MST, Enschede, The Netherlands): the samples were autoclaved for 30 min at 120 °C.

All samples were sterilized in the dry state except for steam sterilization of non-immobilized heparin. In the latter case, heparin was dissolved in water. After sterilization, the solution was lyophilized.

Prior to further testing, the membranes were incubated in PBS at 4 °C for 16 h.

## 2.4. Complexation of heparin in solution with toluidine blue

A toluidine blue assay was developed to study the effects of sterilization on the degree of complexation of heparin in solution with toluidine blue. To 2 ml of a solution of sterilized heparin in aqueous 0.01 N HCl/0.2 wt % NaCl, 2 ml of a freshly prepared solution of 0.04 wt % toluidine blue in aqueous 0.01 N HCl/0.2 wt % NaCl, was added. The mixture was gently shaken for 4 h during which the formed heparin/toluidine blue complex precipitated. Subsequently, the mixture was centrifuged at 1000 g for 10 min after which the supernate was removed. Thereafter the precipitate was rinsed with aqueous 0.01 N HCl/0.2 wt % NaCl and then incubated in 5 ml of a 4/1 (v/v) mixture of ethanol and aqueous 0.1 N NaOH. In this medium the heparin/toluidine blue complex is broken and toluidine blue dissolves. The extinction of the resulting solution was measured at 530 nm against a 4/1 (v/v) mixture of ethanol and aqueous 0.1 N NaOH. Unsterilized heparin was used as a control.

## 2.5. Complexation of immobilized heparin with toluidine blue

A toluidine blue assay as described by Smith *et al.* [17] and modified for our purposes was used to study the effects of sterilization on the degree of complexation of heparin immobilized onto Cuprophan with toluidine blue. Heparinized and sterilized Cuprophan was cut in circular discs with a diameter of 7 mm. The immobilized heparin was stained by incubating the membrane in 5 ml of a freshly prepared solution of 0.04 wt % toluidine blue in aqueous 0.01 N HCl/0.2 wt % NaCl. After 4 h of gently shaking, the membrane was rinsed twice with demineralized water and then incubated into 5 ml of a 4/1 (v/v) mixture of ethanol and aqueous 0.1 N NaOH. After complete decolourization of the membrane, the extinction of the fluid phase was measured at 530 nm against a 4/1 (v/v) mixture of ethanol and aqueous 0.1 N NaOH. Unsterilized heparinized Cuprophan was used as a control.

## 2.6. Thrombin inactivation assay

The anticoagulant activity of sterilized non-immobilized heparin and heparin immobilized onto Cuprophan was investigated by means of a thrombin inactivation assay. A buffer solution consisting of an aqueous solution of 50 mmol/l tris, 1.0 g/l PEG, 1.0 g/l BSA, and 150 mmol NaCl adjusted to pH 8.4 was prepared. The buffer solution was used for the preparation of a substrate $S_{2238}$ (2.0 mg/ml)/AT III (70 mU/ml) solution, a thrombin solution (0.4 U/ml), solutions of sterilized heparin, and a series of solutions

of untreated heparin (0–20 mU/ml). To determine the anticoagulant activity of sterilized heparin, 40 µl of a solution of sterilized heparin was mixed with 150 µl of substrate/AT III solution. To determine the anticoagulant activity of immobilized heparin, membranes were cut into circular discs with a diameter of 5 mm and incubated in 150 µl of substrate/AT III solution and 40 µl of buffer solution. For the determination of a calibration curve, 40 µl of heparin solutions of known activities were mixed with 150 µl of substrate/AT III solution. The reactions were initiated by adding 50 µl of the thrombin solution. The reaction solutions were shaken for 10 min after which the reactions were quenched by adding 70 µl of 40 vol % acetic acid. Subsequently, the extinction of 200 µl of the resulting mixture at 405 nm was determined. The reactions were carried out at 20 °C. The calibration curve was used to determine the activity of heparin immobilized onto Cuprophan and sterilized non-immobilized heparin.

### 2.7. APTT assay

The anticoagulant activity of sterilized heparin was determined by means of an APTT assay. Fresh frozen human CPDA-1 plasma was thawed at 37 °C and stored on ice. To 50 µl of a solution of sterilized heparin in PBS, 50 µl of plasma and 50 µl of PTT reagents was added. The resulting suspension was mixed for three minutes at 37 °C. Thereafter, 50 µl of aqueous 20 mM $CaCl_2$ was added. The suspension was mixed again and the coagulation time was determined with a coagulatometer (LC-6, Lode, Groningen, The Netherlands). A calibration curve was obtained by measuring the coagulation time of solutions of heparin in PBS of known activities (0–0.7 U/ml). The calibration curve was used to calculate the anticoagulant activities of sterilized heparin.

### 2.8. Permeability for Vit $B_{12}$ and SBP

The effect of sterilization and heparinization on the permeability of Cuprophan for Vit $B_{12}$ and SBP was investigated using a Minitan-S ultrafiltration system (Millipore, Etten-Leur, The Netherlands). With a peristaltic pump, feed and filtrate were circulated in such a way that crossflow (400 ml/min) over the membranes was obtained. At the start of the experiment the feed consisted of a solution of 0.02 wt % Vit $B_{12}$ and 0.02 wt % SBP in PBS and the filtrate consisted of PBS. The experiments were performed at 37 °C. Samples of the filtrate were taken at different time intervals. The concentrations of Vit $B_{12}$ and SBP were determined as follows. Vit $B_{12}$: the extinctions of the samples were measured at 360 nm against PBS. SBP: the samples were 1/1 (v/v) diluted with aqueous 0.01 N NaOH after which the extinctions of the mixtures were measured at 578 nm against a 1/1 (v/v) mixture of PBS and aqueous 0.01 N NaOH. Calibration curves were used to determine the concentrations of the solutes.

The mass transfer coefficient, $K_0$, was calculated using the formula as derived by Smith *et al.* [18]:

$$-\ln(\Delta C_t/\Delta C_0) = K_0 * A * t * (1/V_1 + 1/V_2)$$

where $\Delta C_t$ and $\Delta C_0$ are the concentration differences of the solutes over the membrane at times $t$ and 0, respectively, $A$ is the membrane surface area, $t$ is the dialysis time, and $V_1$ and $V_2$ are the volumes of feed and dialysate, respectively.

### 2.9. Water permeability

The effect of sterilization and heparinization on the ultrafiltration characteristics of Cuprophan was investigated using an Amicon 8050 ultrafiltration cell (Amicon, Capelle aan de IJssel, The Netherlands). The ultrafiltration cell was supplied with the membrane under investigation and then filled with water. Subsequently, a pressure of $3 \times 10^5$ Pa was applied. After 1–2 h of equilibration, the water flow through the membrane was determined as a function of time during 2–4 h.

### 2.10. Statistics

The data were mutually compared by means of the Student $t$ test and were considered to be significantly different when $p < 0.05$.

## 3. Results and discussion

### 3.1. Complexation of heparin with toluidine blue

Sterilization by means of EtO exposure, gamma irradiation, or steam did not change the complexation characteristics of heparin immobilized onto Cuprophan or heparin in solution with toluidine blue (see Table I). Theoretically, EtO can react with most of the functional groups of heparin [3, 19]. These reactions can lead to a reduction of the number of negatively charged groups. It has been reported that gamma irradiation of dry heparin can result in a decrease of the molecular weight and a release of sulphate groups [5, 6]. Furthermore, Menzies *et al.* [7] showed that autoclaving heparin in solution at 121 °C for 50 min did not change the molecular weight of heparin or the complexation characteristics of heparin with azure A.

The toluidine blue assay is based on the complexation of toluidine blue with negatively charged groups of heparin. Therefore, a reduction of the number of negatively charged groups or chemical modification of

TABLE I Complexation of sterilized non-immobilized and immobilized heparin with toluidine blue as compared to unsterilized heparin (%)

| | EtO | Gamma | Steam |
|---|---|---|---|
| Nonimmobilized heparin ($n = 3$) | 96.8 ± 3.4 | 95.0 ± 5.3 | 98.6 ± 1.6 |
| Immobilized heparin ($n = 3$) | 105.7 ± 6.3 | 97.6 ± 3.6 | 89.1 ± 5.0 |

these groups will affect the complexation characteristics of heparin with toluidine blue. Depolymerization of heparin does not necessarily affect the complexation of heparin in solution with toluidine blue. However, depolymerization of immobilized heparin or cleavage of heparin–Cuprophan linkages may result in a release of heparin or heparin fragments from the surface causing less complexed toluidine blue.

The results show that the three different sterilization methods did not induce chemical modification of functional groups of heparin, degradation of heparin, or cleavage of the heparin–Cuprophan linkages to such an extent that complexation with toluidine blue was affected.

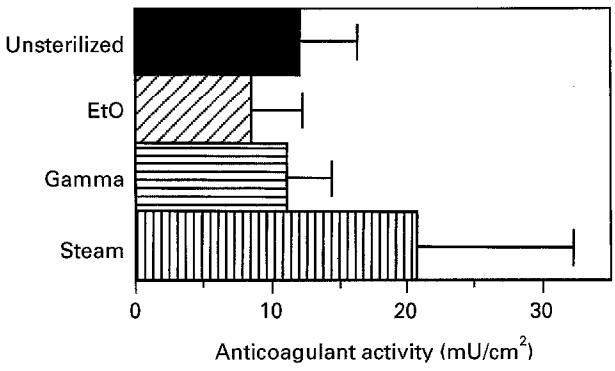


*Figure 1* The anticoagulant activity of unsterilized and sterilized heparinized cuprophan as determined by means of the thrombin inactivation assay.

## 3.2. Anticoagulant activity of heparin

The three different sterilization methods induced a small but insignificant decrease of the anticoagulant activities of non-immobilized heparin (see Table II). Chemical modification of functional groups or depolymerization can reduce the anticoagulant activities of heparin [20–22]. Therefore, the results of the thrombin inactivation and APTT assay confirm the conclusion of the results of the toluidine blue assay that these reactions did not or hardly took place during sterilization. Also Edwards *et al.* [6] found that the anticoagulant activities of heparin were hardly affected by gamma irradiation at a dose which is usually applied for sterilization. Furthermore, Menzies *et al.* [7] showed that autoclaving heparin in solution at 121 °C for 50 min did not affect the anticoagulant activities. To our knowledge, no adverse effects of EtO exposure on the anticoagulant activities of heparin have been reported [3].

After EtO and gamma sterilization, the anticoagulant activity of immobilized heparin was, like that of non-immobilized heparin, slightly but not significantly reduced (see Fig. 1). In contrast, steam sterilization significantly increased the anticoagulant activity. This increase can be explained as follows. The heparin–Cuprophan linkages established by means of the CDI-activation procedure may be susceptible to hydrolysis. Therefore, part of these linkages may be broken during steam sterilization. The results of the toluidine blue assay, however, revealed that steam sterilization induced no release of heparin from the membrane. Most likely, steam sterilization induced the hydrolysis of covalent linkages between heparin and Cuprophan but not to such an extent that heparin is released. As a consequence the number of covalent bonds between heparin and Cuprophan will be decreased after steam sterilization. When heparin is immobilized via less covalent bonds, the heparin chains will exhibit an enhanced mobility and more specific binding sites will be available for the interaction with AT III and factors of the coagulation cascade resulting in an increased anticoagulant activity [23].

TABLE II Anticoagulant activities of sterilized non-immobilized heparin compared to unsterilized heparin (%)

| | EtO | Gamma | Steam |
|---|---|---|---|
| Thrombin inactivation ($n = 8$) | 86.6 ± 8.7 | 84.8 ± 6.8 | 80.1 ± 17.2 |
| APTT ($n = 8$) | 91.3 ± 5.7 | 89.7 ± 5.2 | 91.4 ± 5.1 |

## 3.3. Permeability

Heparinization did not affect the permeability of Cuprophan for Vit $B_{12}$, whereas the permeability for SBP was substantially reduced (see Table III). This reduction can be ascribed to the negative charges of both SBP and heparin at physiological conditions by which the diffusion of the solute through the membrane was limited. The water permeability of Cuprophan increased after heparinization. Heparin is most likely not only immobilized at the surface but also in the pores of the membrane. Immobilization of heparin in the pores will lead to an increased swelling of the membrane in water and as a consequence to an increased water permeability. After sterilization by means of EtO exposure or gamma irradiation, the mass transfer coefficient of Vit $B_{12}$ and SBP decreased with 20–35% in the case of unmodified Cuprophan but remained the same in the case of heparinized Cuprophan. The net result was that after EtO exposure or gamma irradiation, heparinized Cuprophan had the same or a increased permeability for SBP and Vit $B_{12}$. Steam sterilization caused a dramatical decrease of the permeability of both types of membranes for the two solutes under investigation. This decrease was more pronounced for unmodified Cuprophan. The water permeability of both types of membranes was not affected by EtO exposure or gamma irradiation but dramatically decreased after steam sterilization (see Table IV). Also in this case, this decrease was more pronounced for unmodified Cuprophan.

The effects of different sterilization methods on the water permeability and the permeability for various solutes of cellulosic membranes have been studied by several investigators [10–14]. The results of these studies are not always consistent. Conflicting results can be ascribed to differences in pore characteristics, chemical structure, geometry (sheet, tubing, or fibre) of the membranes, experimental conditions of the permeability study, and whether the membranes are sterilized in the dry or wet state or acquired a special

TABLE III Permeability of Cuprophan, $K_0(\times 10^{-3}$ cm/min)

| | Unsterilized | EtO | Gamma | Steam |
|---|---|---|---|---|
| Vit $B_{12}$ ($n = 3$) | | | | |
| Unmodified | $1.74 \pm 0.07$ | $1.20 \pm 0.10$ | $1.13 \pm 0.09$ | $0.075 \pm 0.007$ |
| Heparinized | $1.78 \pm 0.17$ | $1.65 \pm 0.28$ | $1.55 \pm 0.04$ | $0.323 \pm 0.032$ |
| SBP ($n = 3$) | | | | |
| Unmodified | $3.93 \pm 0.20$ | $3.05 \pm 0.17$ | $2.76 \pm 0.21$ | $0.47 \pm 0.01$ |
| Heparinized | $2.92 \pm 0.20$ | $3.09 \pm 0.12$ | $2.99 \pm 0.10$ | $1.01 \pm 0.10$ |

TABLE IV Water permeability of Cuprophan in $\mu l/h \times cm^2$ at a pressure of $3 \times 10^5$ Pa ($n = 3$)

| | Unsterilized | EtO | Gamma | Steam |
|---|---|---|---|---|
| Unmodified | $300 \pm 4$ | $319 \pm 6$ | $300 \pm 8$ | $63 \pm 6$ |
| Heparinized | $385 \pm 11$ | $402 \pm 6$ | $352 \pm 14$ | $130 \pm 5$ |

pretreatment. In general, it has been observed that the permeability was not affected or maximally decreased with 35% after EtO or gamma sterilization of the membrane in the dry state as also found in our study. A decreased permeability can be related to the collapse of pores of the membrane resulting in a decreased porosity and swelling in water [10, 11, 13, 14]. A dramatical decrease of the permeability after steam sterilization of dry membranes has also been observed by Broek *et al.* [10]. In this case, a severe collapse of the pores took place during sterilization [10, 11]. Takesawa *et al.* [14] showed that the collapse of pores can be partially prevented by sterilization of the membrane in a wet state. For heparinized Cuprophan, however, steam sterilization of the membrane in a wet state cannot be recommended because it will result in a substantial hydrolysis of the heparin–Cuprophan linkages. The decrease of the permeability after steam sterilization can also be prevented by pretreatment of the membrane with highly concentrated aqueous glycerol solutions [11]. Filling of the pores with glycerol prevents collapse of the pores during sterilization.

After EtO and gamma sterilization, the permeability of unmodified Cuprophan for Vit $B_{12}$ and SBP was decreased whereas the permeability of heparinized Cuprophan for these solutes had not been changed. These results suggest that heparinization had a similar effect as high loading of the membrane with glycerol. As mentioned above, heparin was also immobilized in the pores of the membrane. Therefore, heparin most likely (partially) prevented the collapse of the pores. This effect of heparin was also evident for steam sterilization. After steam sterilization, the decrease of both the permeability for Vit $B_{12}$ and SBP and the water permeability was more pronounced for unmodified Cuprophan than for heparinized Cuprophan. However, for clinical applications, the decrease of the permeability of heparinized Cuprophan by steam sterilization is still unacceptable.

## 4. Conclusions

The effects of sterilization of dry heparinized Cuprophan by means of EtO exposure, gamma irradiation, or steam on the anticoagulant activity and chemical characteristics of heparin and the permeability of the membrane were investigated. Sterilization by means of the three different methods did not adversely affect the anticoagulant activity of immobilized heparin. After EtO exposure or gamma irradiation, the permeability of heparinized Cuprophan had the same or a increased permeability as compared to unmodified Cuprophan. A decreased permeability of unmodified Cuprophan was ascribed to the collapse of pores. In the case of heparinized Cuprophan, collapse of the pores was prevented most likely because heparin is immobilized in the pores. Steam sterilization caused a dramatical decrease of the permeability of both heparinized and unmodified Cuprophan rendering these membranes unsuitable for clinical hemodialysis treatment. Therefore, EtO exposure and gamma irradiation seem suitable methods of sterilization of heparinized Cuprophan. In numerous reports, however, it has been shown that EtO sterilized materials can induce severe toxic effects [24–27]. Remnants of EtO can give rise to high levels of anti EtO IgE antibodies resulting in anaphylactic reactions. Therefore, with respect to the properties of immobilized heparin and the permeability of the membrane we recommend gamma irradiation for sterilization of heparinized Cuprophan for clinical applications.

## Acknowledgements

This study was financially supported by the Dutch Kidney Foundation (Grant C 91.1077).

## References

1. M. AMIJI and K. PARK, *J. Biomater. Sci. Polymer Edn.* **4** (1993) 217.
2. G. H. M. ENGBERS and J. FEIJEN, *Int. J. Artif. Org.* **14** (1991) 199.
3. O. LARM, R. LARSSON and P. OLSSON, in "Heparin. Chemical and biological properties. Clinical applications, edited by D. A. Lane, and U. Lindahl (Edward Arnold, London, 1989), p 597.
4. G. H. M. ENGBERS, Thesis, University of Twente, Enschede, The Netherlands, 1990.
5. R. BLACKBURN, B. IDDON, J. S. MOORE, G. O. PHILLIPS, D. M. POWER and T. W. WOODWARD, in "Radiosterilization of medical products" (International Atomic Energy Authority, Vienna, 1975) p. 351.

6. H. E. EDWARDS, A. R. MENZIES, G. O. PHILLIPS, Y. NAKAMURA, M. SHIMADA and S. TAKIGAMI, *Carbohydrate Polym.* **5** (1985) 473.
7. A. R. MENZIES, D. M. BENOLIEL and H. E. EDWARDS, *J. Pharm. Pharmacol.* **41** (1989) 512.
8. A. ALBANESE, R. BARBUCCI, J. BELLEVILE, S. BOWRY, R. ELROY, H. D. LEMKE and L. SABATINI, *Biomaterials* **15** ( 1994) 129.
9. Y. IDEZUKI, H. WATANABE, M. HAGIWARA, K. KANASUGI, Y. MORI, S. NAGAOKA, M. HAGIO, K. YAMAMOTO and H. TANZAWA, *Trans. Amer. Soc. Artif. Int. Organs.* **21** (1975) 436.
10. A. P. BROEK, H. A. TEUNIS, D. BARGEMAN, E. D. SPRENGERS and C. A. SMOLDERS, *J. Membr. Sci.* **73** (1992) 143.
11. A. P. BROEK, D. BARGEMAN, E. D. SPRENGERS and C. A. SMOLDERS, *Int. J. Artif. Org.* **15** (1992) 25.
12. E. KLEIN, F. F. HOLLAND, A. DONNAUD, A. LEBEOUF and K. EBERLE, *J. Membr. Sci.* **2** (1977) 349.
13. S. TAKESAWA, S. SATOH, H. HIDAI, M. SEKIGUCHI and K. SAKAI, *Trans. Amer. Soc. Artif. Intern. Organs* **33** (1987) 584.
14. S. TAKESAWA, S. OHMI, Y. KONNO, M. SEKIGUCHI, S. SHITAOKOSHI, T. TAKAHASHI, H. HIDIA and K. SAKAI, *Nephrol. Dial. Transplant* **1** (1987) 254.
15. F. H. VERHOEK, *J. Amer. Chem. Soc.* **58** (1936) 2577.
16. M. MILLER-ANDERSSON, H. BORG and L.-O. ANDERSSON, *Throm. Res.* **5** (1974) 439.
17. P. K. SMITH, A. K. MALLIA and G. T. HERMANSON, *Anal. Biochem.* **109** (1980) 466.
18. K. A. SMITH, C. K. COLTON, E. W. MERRILL and L. B. EVANS, *Chem. Eng. Prog. Symp. Series 84* **64** (1968) 45.
19. A. ROSOWSKY, in "Heterocyclic compounds with three- and four-membered rings. Part 1", edited by A. Weissberger (Interscience, New York, 1964) p. 1.
20. B. CASU, *Adv. Carbohydr. Chem. Biochem.* **43** (1985) 51.
21. B. CASU, in "Heparin. Chemical and biological properties. Clinical applications", edited by D. A. Lane, and U. Lindahl (Edward Arnold, London, 1989), p. 26.
22. W. D. COMPER, "Heparin (and related polysaccharides)" (Gordon and Breach, New York, 1981).
23. P. OLSSON and O. LARM, *Int. J. Artif. Organs* **1** ( 1991) 453.
24. A. v. BERLO and D. J. ELLENS, *Adv. Exp. Med. Biol.* **238** (1988) 341.
25. C. K. COLTON, *Blood Purification* **5** (1987) 202.
26. L. W. HENDERSON and D. CHENOWETH, *ibid.* **5** (1987) 100.
27. F. MAILLET, D. LABARRE and M. D. KAZATCHKINE, *Transfus. Sci.* **11** (1990) 33.

*Received 7 July*
*and accepted 7 September 1995*