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Removal of β_2-microglobulin using grafted affinity adsorbents as therapeutic approach for the treatment of hemodialysis patients

D. Müller-Schulte[a,*], H. Melzer[b], H. Mann[b]

[a]*Institut für Anorganische Chemie, RWTH Aachen, Professor-Pirletstr. 1, D-52074 Aachen, Germany*
[b]*Abteilung Innere Medizin II, RWTH Klinikum, Pauwelstr., D-52074 Aachen, Germany*

Abstract

Patients undergoing long-term hemodialysis are increasingly faced with the problem of developing amyloidosis. This is particularly manifested in a deposition of amyloid in diverse tissues and joints. For the treatment and prevention of amyloidosis, novel affinity adsorbents, which were prepared on the basis of radiation grafted polyamide, have been developed to remove β_2-microglobulin (β2-MG) which is the main constituent of the amyloid. Various affinity ligands, such as alkyl residues with different chain lengths, as well as collagen and gelatin were tested for removing β2-MG from human serum and hemofiltrates (HF). Collagen and gelatin carriers show the best adsorption performances. They remove $>95\%$ and $>50\%$ of the β2-MG from HF and serum, respectively. The results show that the present approach can be used as the basis for future development of β2-MG adsorbents as an additional means in the treatment of dialysis patients.

1. Introduction

In addition to their use in general separation technology, affinity media have increasingly been applied in the medical field over the recent years [1]. Some prominent examples are: the use of Protein A carriers for the removal of IgG from blood of hemophilia patients [2], removal of low density lipoprotein in the treatment of patients suffering from hypercholesterolemia [3], and the removal of blood-group antibodies from incompatible bone marrow transplants using blood-group oligosaccharide carrying adsorbents [4].

The recently introduced radiation grafted carrier media based on polyamide-6 for affinity chromatography and enzyme immobilization [5,6] are used for the adsorption of β_2-microglobulin (β2-MG) from human serum and hemofiltrates (HF).

Patients undergoing long-term hemodialysis are increasingly confronted with dialysis-related amyloidosis. Although the mechanism of the amyloid formation still remains to be clarified, it is known that β2-MG, a component of the major histocompatibility antigen (HLA) with a molecular mass of 11 800 Da, is one of the main constituents of the amyloid. It is deposited mainly in joints, diverse tissues, kidney, heart and other organs and may often lead to a fatal course of the disease [7]. As there is no estab-

* Corresponding author.

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lished treatment for this progressive disease, the removal of β2-MG from blood and other biological fluids as a means to decrease amyloid deposition has gained great interest in current nephrology.

Here we describe polyamide carrier media that were modified by a recently developed synergistic radiation grafting technique [3] by which alkyl chains with different chain lengths (C8, C12, C16, C18) as well as collagen and gelatin affinity ligands were covalently coupled. The aim is to determine the elimination rates for β2-MG in HF and human serum as a function of the adsorbent type and the ligands bound, as well as to assess the applicability of the present adsorbents in the treatment of dialysis patients.

Performance and specificity of the adsorbents were evaluated by analyzing the chromatographic fractions by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins separated by SDS-PAGE were detected with a modified silver staining method that allowed analysis of the proteins with very low background staining.

2. Experimental

2.1. Materials

CNBr-activated Sepharose 4B and Sepharose CL-6B were obtained from Pharmacia (Freiburg, Germany). Collagen (M_n: 5000–25 000) was supplied by the Deutsche Gelatine-Fabriken (Eberbach, Germany). All other chemicals were from Fluka (Neu-Ulm, Germany) and were of analytical grade. Pooled HF from dialysis patients and pooled blood sera were obtained from the Nephrology Department and Department of Clinical Chemistry both of the University Hospital, Aachen, Germany.

2.2. Preparation of affinity adsorbents

Grafting onto polyamide-6 micro carriers (BioGRAFT), bead size 80–150 μm, with 2-hydroxyethyl methacrylate as monomer and N-vinylpyrrolidone and acrylamide as co-monomers was performed as previously described [6,8]. The total dose applied amounted to 0.18 Mrad; this resulted in grafting yields of 80% (w/w) (BioGraft A), 85% (BioGRAFT B) and 102% (BioGRAFT C), respectively. Activation with epichlorohydrin and 2-fluoro-1-methylpyridinium toluene sulphonate (FMP) was carried out following a recently described method [5,9]. Coupling of octyl-, dodecyl-, hexadecyl- and octadecylamin to 1 ml epoxy-activated BioGRAFT A media was carried out by incubating 3 ml of a 20% (w/v) amine solution in methanol for 24 h at 35°C. To couple collagen, gelatin and glycine to epoxy-activated Biograft 3 ml of 1 *M* phosphate buffer (pH 7.5) containing 1% (w/v) ligand was incubated for 24 h at room temperature. After intensive washing with phosphate-buffered saline (PBS) (pH 7.2) containing 0.05% (v/v) Triton X-100, residual oxirane groups were blocked by incubating with 0.2 *M* of β-mercaptoethanol for 6 h at room temperature. Collagen and gelatin were coupled to the FMP-activated carriers by incubating with PBS, containing 1% (w/v) ligands. Coupling of collagen to CNBr-Sepharose 4B was carried out according to manufacturer's standard protocol [10]. The amounts of collagen, gelatin and glycine coupled to the carrier were determined by analysing the ligand concentrations in the coupling solutions before and after the coupling procedure using the bicinchoninic acid (37°, 30 min) standard method described by Smith *et al.* [11].

2.3. Affinity adsorption procedure

All adsorption tests were done at room temperature.

A 1-ml volume of coupled affinity gel was packed into a disposable plastic column (6.5 × 0.5 cm I.D.) and equilibrated with PBS (pH 7.2). A 20-ml volume of pooled HF and 20 ml of pooled human serum, respectively were passed through the column with a flow-rate of 0.25 ml/min. After washing with the loading buffer, bound proteins were successively eluted with 0.1 *M* Gly-HCl (pH 2.7), and 0.1 *M* Gly-HCl containing 1 *M* NaCl and 50% (v/v) ethylene glycol, pH 3.1. Effluents were continuously

monitored at 280 nm using a LKB 2510 Uvicord SD.

2.4. SDS-PAGE

SDS-PAGE was carried out using the PhastSystem of Pharmacia (Uppsala, Sweden). Ready made 8–25% SDS gradient gels, SDS agarose buffer strips and marker proteins were also supplied by Pharmacia. The SDS-PAGE patterns were analyzed by a laser densitometer, Ultrascan XL (Pharmacia). SDS-PAGE and silver staining are performed using the methods previously described [12].

2.5. Enzyme-immunosorbent assay

For the quantitative determination of β2-MG the standard protocol of the Microparticle Enzyme Immunoassay (MEIA) Technology by Abbott Laboratories was used [13]. The assays were performed in the Abbott IMx β2-Microglobulin System.

3. Results and discussion

The results of β2-MG adsorption on epoxy-activated BioGRAFT A carrying different affinity ligands are shown in Table 1. The amount of β2-MG adsorbed was calculated by measuring the concentration before and after passage through the column with a MEIA test. When one compares the different ligands, it is obvious that gelatin and collagen have distinct advantages over the hydrophobic alkyl residues C_8, C_{12}, C_{16} and C_{18}. The gelatin and the collagen matrices adsorb 12 and 18 μg β2-MG per ml adsorbent, respectively, whereas the adsorption capacities of the alkyl-carrying gels range from 1.2 (C_8) to 9.6 μg/ml (C_{12}). Glycine was applied as ligand because it is the most abundant amino acid in collagen and gelatin. Furthermore, as it is a simple and inexpensive chemical, it could serve as an alternative affinity ligand. However, its adsorption capacity of 10.8 μg/ml gel is well below that of the collagen matrix which attains a β2-MG clearance rate of over 50%.

Structural studies have revealed that β2-MG contains hydrophobic domains. Based on this result a hexadecyl alkyl chain was used as specific affinity ligand for β2-MG adsorption by Gejyo *et al.* [14]. The adsorption capacities stated, using a cellulose matrix of 1 mg β2-MG per ml gel cannot be corroborated by the present tests; the values presented here show distinctly lower adsorption values particularly when a hexadecyl ligand is used (Table 1). Considering

Table 1
Amount of β2-MG adsorbed onto grafted polyamide-6 (BioGRAFT A) from human serum as a function of different affinity ligands

Ligands	Ligand bound (mg/ml gel)	Adsorbed β2-MG	
		(μg/ml gel)	(%)
Octyl alkyl-	–[a]	1.2	3.6
Dodecyl alkyl-	–	9.6	28.8
Hexadecyl alkyl-	–	7.2	21.6
Octadecyl alkyl-	–	7.6	22.8
Glycine-	6.2	10.8	32.4
Gelatin-	2.0	12.0	36.0
Collagen-	2.1	18.0	54.0

[a] Not determined

A 20-ml volume of pooled human serum from healthy patients was loaded onto 1 ml BioGRAFT A affinity adsorbent pre-equilibrated with PBS (pH 7.2); flow-rate: 0.25 ml/min. Amount of adsorbed β2-MG was determined by a β2-MG specific MEIA test by calculating the difference of the amount in the serum before and after column passage (see Experimental for details).

the fact that grafted carriers generally show superior protein binding capacities when compared to polysaccharide matrices [4,6] (see also Table 2), the above discrepancies cannot be explained. Apart from their specific binding capacity, hydrophobic supports tend to adsorb unspecifically substantial amounts of proteins such as IgG, human serum albumin (HSA) and fibronectin. Hence, the hydrophobic interactions between proteins and the matrix in affinity chromatography have to be minimized as much as possible. Solely from this aspect and the fact that corresponding data concerning unspecific adsorption are not available from the above reference, the use of adsorbents carrying hydrophobic groups as medical adsorbents should be critically considered.

The extent of hydrophobic interactions leading to unspecific protein adsorption was assessed by recording chromatographic profiles of human serum on collagen affinity matrices using different solvents for elution. Fig. 1 shows elution profiles using collagen–Sepharose CL-6B and collagen–BioGRAFT A. All columns were loaded with 2 ml of serum; after washing with PBS, pH 7.2, elution was successively performed with two buffers: 0.1 *M* Gly-HCl, pH 2.7 (elution buffer 1), and 0.1 *M* Gly-HCl containing 1 *M* NaCl and 50% (v/v) ethylene glycol, pH 3.1 (elution buffer 2). The two desorption peaks resulting from the Sepharose (Fig. 1a, elution peaks 1 and 2) show clearly higher adsorption values than those of the BioGRAFT desorption peaks (Fig. 1b). In ligand–protein binding, pre-

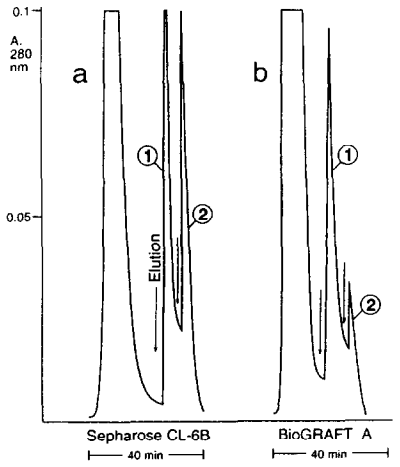

Fig. 1. Adsorption and elution profiles of human serum on (a) collagen–Sepharose CL-6B, and (b) collagen–BioGRAFT A. A 2-ml volume of pooled human serum was loaded at a flow-rate of 0.25 ml/min onto 1 ml affinity support at 20°C. Elutions were performed at a flow-rate of 0.25 ml/min with (1): 0.1 *M* Gly-HCl buffer (pH 2.7), and (2): 0.1 *M* Gly-HCL buffer (pH 3.1) containing 1 *M* NaCl and 50% (v/v) ethylene glycol.

Table 2
Amount of β2-MG from HF adsorbed on different affinity media

	Ligand bound (mg/ml gel)		Adsorbed β2-MG (μg/ml)	(μg/ml)	(%)	(%)
Ligand	Collagen	Gelatin	Collagen	Gelatin	Collagen	Gelatin
Adsorbent						
BioGRAFT A	4.2	3.8	409	402	98.3	96.6
BioGRAFT B	3.9	3.7	411	403	98.8	96.8
BioGRAFT C	3.3	2.9	402	312	96.6	75.0
CNBr-Sepharose 4B	2.7	2.4	65	–[a]	15.6	–[a]
FMP-Sepharose CL-6B	3.6	3.2	365	348	87.7	83.6

[a]Not determined.
A 20-ml volume of pooled HF from dialysis patients was loaded onto 1 ml affinity adsorbent pre-equilibrated with PBS (pH 7.2) using a flow-rate of 0.25 ml/min. Determination of adsorbed β2-MG was done as described in Table 1.

dominantly electrostatic and hydrophobic interaction forces are involved; hence, owing to the fact that ethylene glycol suppresses mainly hydrophobic interactions [15], one can conclude that the second elution peak represents mainly unspecific protein adsorption resulting from hydrophobic interactions. The second elution peak from the BioGRAFT sample (Fig. 1b), the extinction value of which is distinctly smaller compared to that of the Sepharose elution peak (Fig. 1a), indicates that hydrophobic properties are more pronounced in the collagen–Sepharose gel than in the grafted carrier. This appears to contradict the fact that agarose is a far more hydrophillic material than the polyamide that we used as matrix for grafting. This phenomenon may arise from (a) grafting with the monomers 2-hydroxyethyl methacrylate and the co-monomers acrylamide and N-vinylpyrrolidone, which are all very hydrophillic, (b) the high ligand binding density that results from the high accessibility of the grafted matrix compared to polysaccharide derivatives [6], and (c) the fact that serum may contain specific agarose binding proteins.

A further important aspect of biomedical adsorbents is their biocompatibility. It has been reported [16] that the use of cellulose as dialysis membrane results in the release of the enzyme elastase. This enzyme attacks the connective tissue whereas polyamide and polysulfone membranes only cause very little release of this enzyme. Therefore, if one envisages medical application, the polyamide material used here appears to be a more favourable adsorbent.

In order to elucidate the influence of the carrier type and the coupling technique, different affinity supports were tested using collagen and gelatin as ligands. For these tests, HF from dialysis patients was used; this generally contains 10- to 20-times more β2-MG than normal serum. Table 2 presents the adsorption data of three grafted carriers (BioGRAFT A, B and C) and two differently activated Sepharose gels. The influence of the coupling technique can be deduced from the distinctly different adsorption behaviour of CNBr-Sepharose and the FMP activated derivative. FMP activation leads to a *ca.* 6-fold increase of β2-MG adsorption (365 μg compared to 65 μg). This result as well as the increase in the amount of bound collagen/gelatin ligands (2.7/2.4 mg/ml CNBr-Sepharose compared to 3.6/3.2 mg/ml FMP-Sepharose) underlines the superior properties of FMP as activation and coupling agent compared to CNBr. As for most of the common coupling agents, the disadvantage of FMP is that absolutely anhydrous conditions are a prerequisite to achieve good coupling yields. This necessitates drying of both carrier and coupling solvent thus causing an overall increase of manufacturing costs, an important aspect that has to be considered when applying this technique for medical use. The results for the adsorption performances of BioGRAFT carriers and FMP-Sepharose (Table 2), show that the grafted media exhibit a *ca.* 10% higher adsorption yield. When comparing the adsorption properties as a function of the ligands, collagen tends to produce somewhat better results. This is in accordance with the adsorption test in serum (Table 1) and also seems to correspond with the higher coupling yields obtained with collagen (0.2–0.6 mg/ml gel). However, on the basis of the present data, no general conclusion regarding the degree of adsorption of collagen or gelatin matrices for β2-MG can be made. The reason for this is that the manufacturing conditions and in particular the conditions used for hydrolysis, may have a marked influence on the properties of collagen/gelatin as affinity ligands. These parameters will have to be elucidated in future studies. Only collagen–BioGRAFT C reveals a markedly higher adsorption compared to the gelatin derivative (402 μg *versus* 312 μg/ml). The corresponding β2-MG clearance rates are between 83% and 87% for FMP-Sepharose CL-6B and generally above 95% for the BioGRAFT carriers. These differences seem to be quite marginal. If, however, one considers the highly different unspecific protein adsorption of collagen–Sepharose (Fig. 1a, elution peak 2), the BioGRAFT media are doubtlessly to be preferred for medical applications.

The specificity of β2-MG adsorption by collagen–BioGRAFT is shown in the electropho-

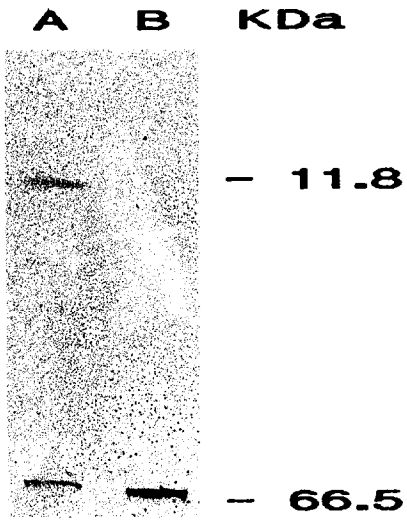

Fig. 2. SDS-PAGE of HF under reducing conditions using silver staining. Lane A: pattern of initial HF. Lane B: HF in effluent after passing collagen–BioGRAFT A adsorbent (for chromatographic condition see legend Table 2). Position and mass (in kDa) of molecular mass markers are indicated on the right.

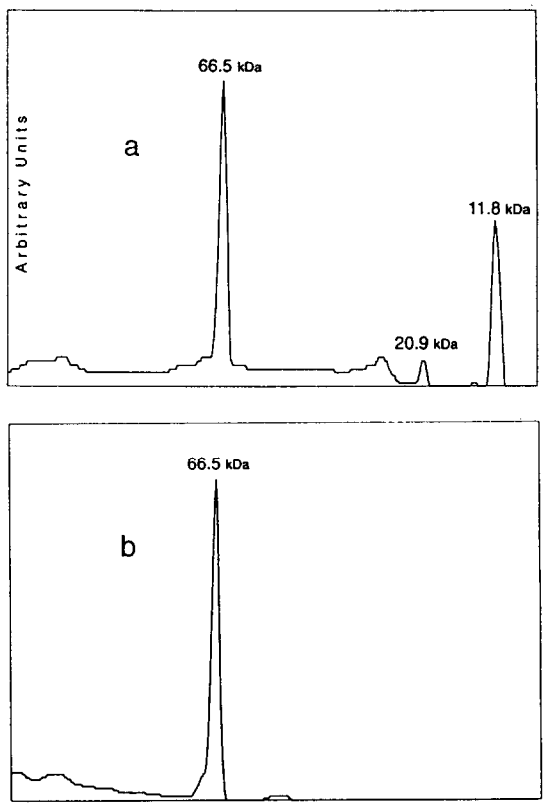

Fig. 3. Laser densitograms of SDS-PAGE analysis according to Fig. 2. (a): Initial HF; (b): HF in effluent. Albumin peak (66.5 kDa), retinol binding protein (21 kDa), and β2-MG (11.8 kDa) are marked.

retic pattern of the HF fractions before and after passing through the adsorbent column (Fig. 2). HSA and β2-MG are clearly separated as two distinct bands at 11.8 kDa and 66.5 kDa (lane A) in the original HF. In the column eluates, however, the β2-MG band is completely absent (lane B), whereas the HSA band remains almost unchanged. These results are confirmed by the corresponding densitograms (Fig. 3) which reveal almost no background staining.

A further approach for the development of affinity supports for β2-MG adsorption is the use of immunoaffinity adsorbents onto which anti-β2-MG antibodies are attached. These matrices have recently been described by Mogi *et al.* [17]. Due to their specificity, antibodies as affinity ligands have certain advantages over other ligands. Most of the immunoaffinity carriers, however, lack sufficient binding capacity [4,5]; they are prone to microbial degradation and necessitate high cost investments. Insofar, the carrier media described here using simple and inexpensive ligands that enable high β2-MG adsorption can be considered as interesting alternatives to the adsorbents presently being used.

4. References

[1] K. Mosbach, *Methods Enzymol.*, 137 (1988) 441.
[2] I.M. Nilsson, S. Jonsson, S.B. Sundqvist, A. Ahlberg and S.E. Bergentz, *Blood*, 58 (1981) 38.
[3] D. Müller-Schulte, *Radiat. Phys. Chem.*, 42 (1993) 891.
[4] D. Müller-Schulte, S. Manjini, M. Vijayalaksmi, *J. Chromatogr.*, 539 (1991) 307.
[5] D. Müller-Schulte and H.G. Thomas, *Radiat. Phys. Chem.*, 35 (1990) 93.
[6] D. Müller-Schulte, *J. Chromatogr.*, 510 (1990) 115.
[7] H. Hampl, H. Lobeck, S. Bartel, H. Stein, M. Eulitz and R.P. Linke, *Trans. Am. Soc. Artif. Organs*, 10 (1987) 250.

[8] D. Müller-Schulte, Ger. Offen. 4129901 A1 (1993).
[9] T.T. Ngo, *Makromol. Chem. Macromol. Symp.*, 17 (1988) 229.
[10] CNBr-activated Sepharose 4B, Standard Protocoll, Pharmacia, Uppsala, Sweden.
[11] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk, *Anal. Biochem.*, 150 (1985) 76.
[12] H. Kierdorf, H. Melzer, H. Mann and H. Sieberth, *Electrophoresis*, 14 (1993) 820.
[13] β2-Microglobulin IMx MEIA Test, Abbott Laboratories, Abbott Park, IL 60064, USA, 1992.
[14] F. Gejyo, N. Homma, S. Hasegawa and M. Arakawa, *Artif. Organs*, 17 (1993) 240.
[15] J. Li and S. Hjerten, *J. Biochem. Biophys. Methods*, 22 (1991) 311.
[16] D. Falkenhagen, *Dialyse J.*, 39 (1992) 17.
[17] M. Mogi, M. Harada, K. Kojima, T. Adachi and S. Nakamura, *Clin. Chem.*, 39 (1993) 277.