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Preparation of cell-affinity adsorbents by immobilization of peptides to Sephadex G-10

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Abstract—In this study, affinity adsorbents for the binding of activated blood platelets and endothelial cells were prepared from Sephadex G-10 by immobilization of peptides, derived from the cell-adhesive amino acid sequence RGD (arginine-glycine-aspartic acid). Derivatization of Sephadex G-10 was accomplished by sequential coupling of specific dipeptides (RG and DV) (V = valine) and by coupling of the RGD-containing hexapeptide GRGDSP (S = serine, P = proline). Two types of gel were prepared by sequential coupling, designated as G10 (acetone) and G10 (dimethylformamide) (G10 (DMF)), containing peptides which had been coupled to the outer side of the beads and throughout the porous beads, respectively. The binding capacity of the prepared Sephadex-derivatives amounted up to 2×10^9 human blood platelets per millilitre GRGDV-Sephadex at immobilized peptide concentrations, that were in the nanomole range (per millilitre packed gel) and which differed a factor 10 between G10 (acetone) and G10 (DMF).

In a second series of experiments, different amounts of the hexapeptide GRGDSP were coupled to carboxylated Sephadex G-10 and carboxylated Sepharose CL 6B. The binding of human umbilical vein endothelial cells to the resulting materials was studied. Up to 10^6 endothelial cells attached per ml GRGDSP-derivatized hydrogel at peptide concentrations of 15 nmol GRGDSP/ml Sephadex and at ±300 nmol GRGDSP/ml Sepharose. Substitution of the arginine residue of the RGD-sequence by glutamine abolished the cell-binding activity of the immobilized peptide towards activated blood platelets but not towards endothelial cells.

From the results of this study it can be concluded that small peptides can be coupled to the outer side of the porous Sephadex beads, resulting in high effective ligand densities for cell-affinity applications. In this respect, Sephadex G-10, derivatized according to 'the acetone method', is a good alternative for polystyrene and other solid phase materials.

Key words: Peptides; immobilization; affinity-binding of cells; Sephadex.

INTRODUCTION

Cellular affinity chromatography is directed towards the specific isolation of intact cells, as well as cell organelles, cell membranes, and also of viruses [1]. One type of cellular affinity chromatography, biospecific affinity chromatography, is based on the interaction of immobilized ligands with specific receptors on the cell surface. Not only proteins but also low molecular weight substances like dinitrobenzene, pyridoxamine phosphate and *p*-aminophenylthiogalactopyranoside have been used as cell-specific ligands [1]. One important group of small cell-adhesive molecules are peptides containing the RGD-sequence (R = arginine, G = glycine and D = aspartic acid). The RGD-sequence is an important cell-binding site which is

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present in adhesive proteins like fibrinogen, fibronectin, vitronectin, and von Willebrand factor [2]. Plasma membrane proteins of blood platelets, endothelial cells, and several other cell types can bind RGD-containing peptides, whether the peptide is present in solution or immobilized on a surface [3,4].

The ideal chromatographic support contains pores which are large enough for proteins to penetrate, and have a relatively large surface area to which proteins can bind [5]. However, cells (even smaller ones like blood platelets (mean diameter 3 μm)) are very large compared to the pore diameters present in chromatographic supports. Sepharose 6B, for example, has a mean pore diameter of about 80 nm [6] and therefore the inner bead volume is not accessible for cells. For this reason, the cell-binding capacity of porous adsorbents is only related to the external particle surface area and not to the total pore surface area.

Coupling of proteins like leucine aminopeptidase (M_w 326 kDa) and Immunoglobulin M (M_w 900 kDa) to cyanogen bromide-activated Sepharose 4B takes place throughout the porous beads [7,8]. As a consequence, relatively large amounts of ligand must be employed in order to attain significant cell binding to the Sepharose beads. Scaling up of this technique is too costly when expensive ligands have to be used. A theoretical disadvantage of the use of these adsorbents in cellular affinity chromatography is that the effective ligand density, i.e. the amount of ligand on the outer side of the adsorbent that is accessible for cells, is unknown.

Because of their non-porosity, polystyrene beads form an alternative for the hydrophilic, porous media. Disadvantages of polystyrene, if it is used in combination with blood platelets, are the nonspecific binding of these cells and the induction of platelet activation. It was found that retention of platelets in columns with polymer-coated glass beads is high for a polystyrene coating and low for a polyethyleneoxide coating [9]. Upon contact of blood platelets with polystyrene latex beads, the cytoplasmic free calcium level in platelets increases to the same extent as observed after addition of thrombin or Ca^{2+} ionophore A23187 to platelets [10]. Moreover, Kasuya *et al.* reported platelet responses like platelet aggregation and platelet secretion of ADP when the platelets are mixed with copoly(styrene–acrylamide) microspheres [11].

Hydrophilic supports like Sepharose have advantageous properties in combination with blood platelets, as compared to hydrophobic materials such as polystyrene. For instance, gel filtration of blood platelets with Sepharose 2B does not result in platelet activation. Comparison of several morphological and metabolic parameters of gel-filtered platelets and control platelets reveals no differences [12]. Another type of hydrophilic adsorbent, Sephadex G-25, shows a relatively low non-specific adherence of cells like lymphocytes and red cells with no concomitant irreversible binding, in contrast to the high level of irreversible adhesion of these cells to glass beads and polymethylmethacrylate beads [13]. For the above mentioned reasons, the use of hydrogel-like solid phases like Sepharose and Sephadex is preferred over polystyrene when they are applied in combination with cells. However, as mentioned before, coupling of ligands to media like Sepharose takes place throughout the porous bead structure which is undesired.

In this study it is shown that a porous matrix like Sephadex can be derivatized with low molecular weight ligands, like diaminoethane or dipeptides, in such a way that only the outer layer of the Sephadex beads is involved in the coupling reaction. Furthermore, it is shown that sequential coupling of dipeptides is a useful method

for *in situ* synthesis of RGD-like peptides. The integrity of the immobilized peptides and the high ligand density on the outer surface of the derivatized adsorbents could be proven with a binding assay in which human blood platelets and human endothelial cells were used.

MATERIALS AND METHODS

Materials

Sepharose CL 6B and Sephadex G-10 were obtained from Pharmacia (Uppsala, Sweden). All the dipeptides, used in this study, were purchased from Bachem (Bubendorf, Switzerland). The hexapeptides, GRGESP and GRGDSP, were from Novabiochem (Laufelingen, Switzerland). Hirudin (grade V), $K_2B_4O_7.4H_2O$, Pipes, Hepes, and (human) thrombin were obtained from Sigma (St. Louis, MO, USA). $NaHCO_3$ and PGI_2 were obtained from Janssen Chimica (Geel, Belgium). Fungizone, Medium 199 and RPMI 1640 were from Gibco Biocult Co. (Paisley, UK). Heparin was purchased from Organon (Oss, The Netherlands). Human serum albumin (20% solution) and the murine anti-platelet GPIIb-IIIa monoclonal antibody CLB-C17 were obtained from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands), *N*-hydroxysuccinimide was obtained from Fluka AG (Buchs, Switzerland), ^{111}Indium oxine (1 mCi ml^{-1}) from Mallinckrodt Medical BV (Petten, The Netherlands), D(+)-glucose from BDH Chemicals Ltd (Poole, UK), and 2,4,6-trinitrobenzene sulfonic acid was obtained from ICN (Costa Mesa, CA, USA). Jeff Amine 600 was obtained from Texaco Chemical Company (Bellaire, TX, USA). All other chemicals were supplied by Merck (Darmstadt, Germany).

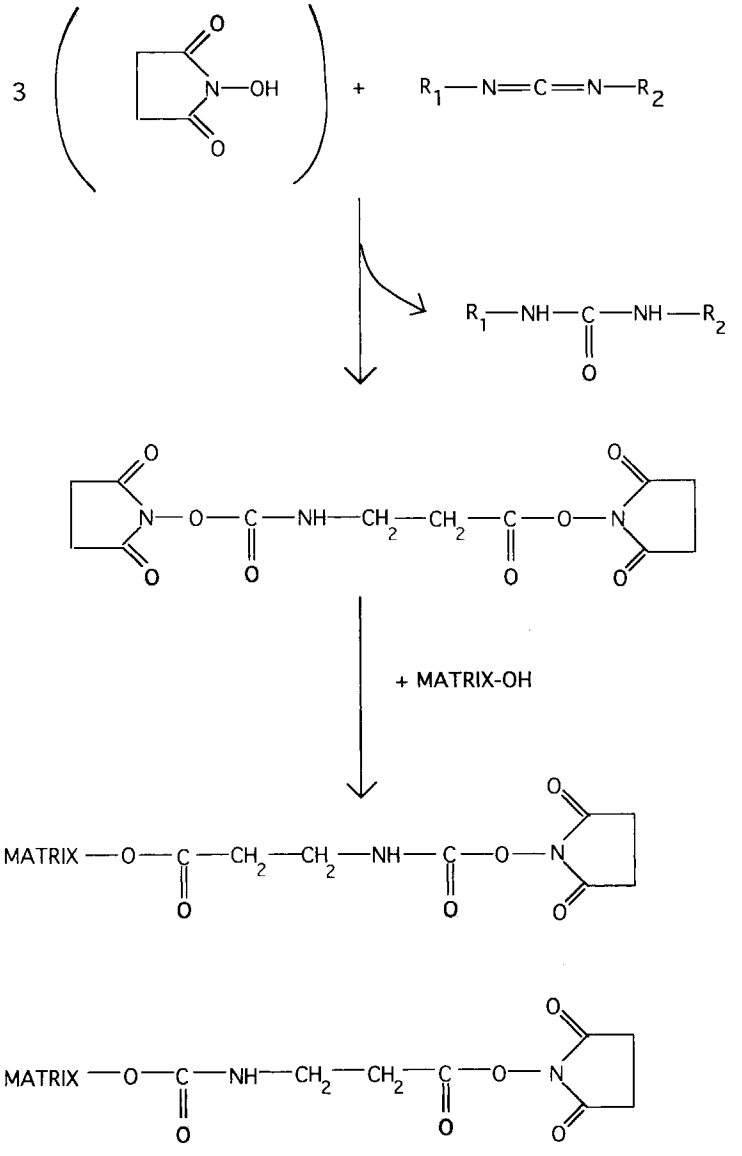
Methods

Solid substance content of Sephadex G-10 in different suspending media. One gram of Sephadex G-10 was suspended in 12 ml of one of the following solvents: water, *N,N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), diethyl ether, ethyl acetate, acetonitrile, acetone, and mixtures of acetone and water (90, 95, and 98% acetone). The suspensions were mixed by rotation for 16 h at room temperature. After standing for 2 h the packed gel volume was determined and from this volume the solid substance content (in g Sephadex per ml packed gel) was calculated.

Activation of Sephadex G-10 with 1,1'-carbonyldiimidazole (CDI). Sephadex G-10 and Sepharose CL 6B were transferred from water to acetone and DMF, respectively, by stepwise washing on a glass filter (Borosilikat, G3, Robuglas) with threefold volumes of 30 and 70% solutions of organic solvent in water, followed by extensive washing with pure solvent (five times with five-fold volumes). The gels were activated by addition of ten volumes of 0.25 M CDI in acetone or five volumes of 0.25 M CDI in DMF to one volume of packed gel, suspended in the same solvent. At different times after addition of CDI, the activated gels were washed (with acetone or DMF, respectively; the acetone-suspended gel was transferred to DMF) and the gels were subsequently treated with diaminoethane (DAE) (2 ml 1 M DAE in DMF per millilitre packed gel; reaction time 1 h). Reaction of DAE with the CDI-activated gels yielded gel-immobilized primary amino groups. The amounts of primary amino groups on the gels were determined by conductivity titration. Before

titration, the gels were washed extensively with deionized water, 2 M NaCl, and again deionized water, after which the pH of the gel suspensions was adjusted to a value of 10.7 with 0.1 N NaOH. For the assay, 0.100 N HCl was added with an autoburette while conductivity and pH of the gel suspension were measured continuously.

Immobilization of β-alanine during carbodiimide/NHS activation. Wilchek and Miron observed that treatment of Sepharose CL 4B with a combination of carbodiimide and NHS results in the immobilization of a β-alanine/NHS ester (Scheme 1)

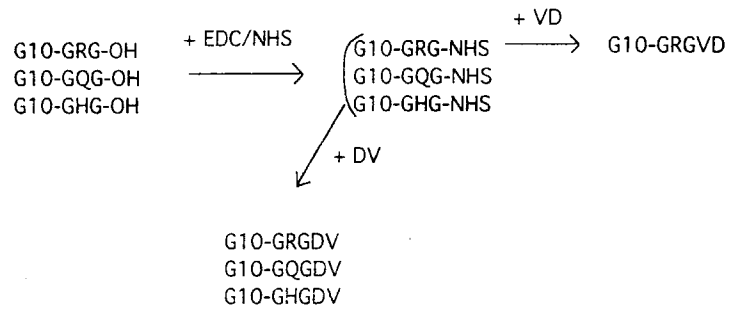


Scheme 1. Immobilization of NHS-activated β-alanine to hydroxyl-containing matrices, as a result of carbodiimide/NHS treatment.

[14]. To investigate the occurrence of this side reaction during the EDC/NHS activation of Sephadex, underivatized Sephadex G-10 was treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) (for each 0.5 mmol ml^{-1} gel, in DMSO, at room temperature), which is similar to the procedure as described by Wilchek and Miron [14]. After treatment with carbodiimide and NHS the gels were washed with organic solvent, transferred to water and suspended in 0.2 *N* NaOH for 30 min. Alkaline treatment of the β-alanine derivative, which is bound to the gel via an ester bond (Scheme 1), causes a rearrangement which results in the immobilization of β-alanine having a carboxylic end group [14]. After the alkaline treatment, the Sepharose suspension appeared to contain precipitate, which very likely consisted of *N,N'*-dicyclohexylurea. This precipitate was removed by extensive washing of the Sepharose with ethanol. Numbers of ionizable groups on the treated Sephadex and Sepharose were determined by conductivity titration.

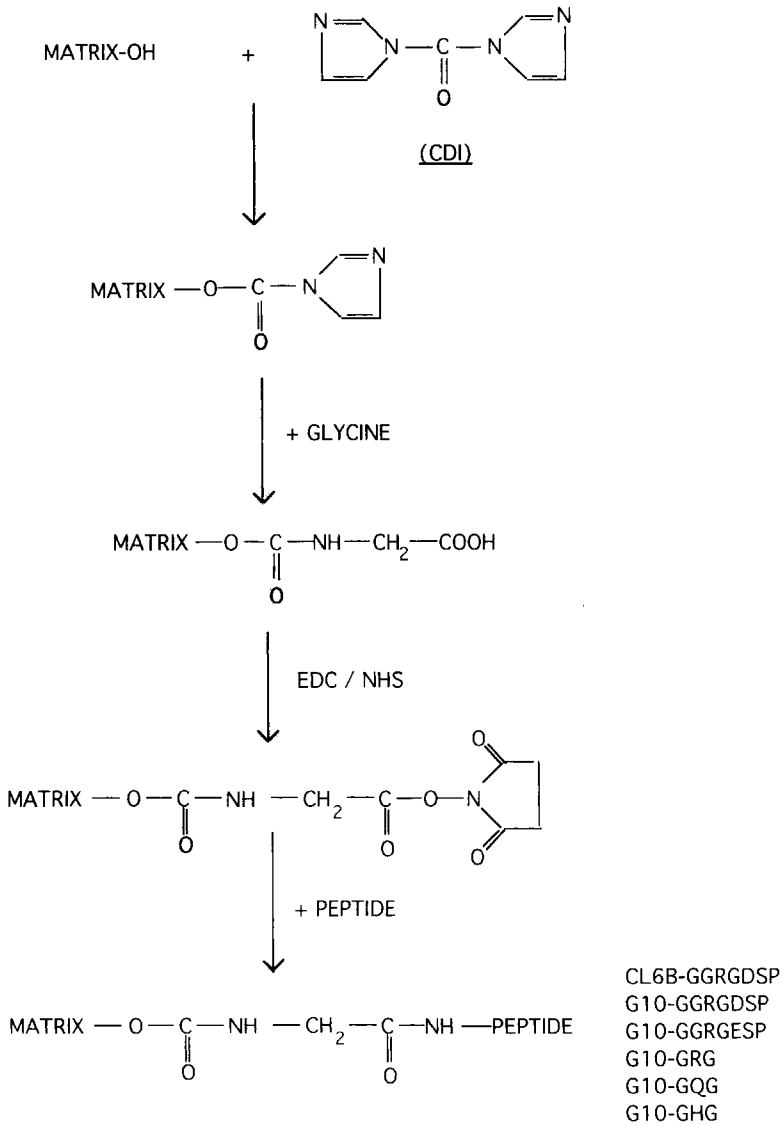
Preparation of GRGDV-derivatized Sephadex (Schemes 2 and 3). After swelling in water (for at least 3 h) Sephadex G-10 was transferred to acetone or DMF, and ten and five volumes of 0.25 M CDI in acetone and DMF, respectively, were added to one volume of packed gel suspended in the respective solvent. Activation time was 30–90 min. The gels which were derived from Sephadex, activated with CDI in acetone and CDI in DMF, will be designated in this paper as G10 (acetone) and G10 (DMF), respectively. The activated gels were washed with the appropriate organic solvent and transferred to a solution of glycine in water (pH 9.6–9.7). For the reaction with glycine, ratios of five volumes of 1 M glycine to one volume of packed G10 (acetone), and one volume of 0.5 M glycine to one volume of packed G10(DMF) were taken. The relative amounts of glycine which were added to the two types of gel were quite different in order to reach a near equal coupling yield of glycine, despite the unequal activation levels of gels (differing a factor 40). The reaction of CDI-activated gel with glycine was carried out for at least 16 h. The amounts of glycine-derived carboxylate groups, as measured with conductivity titration, averaged 10–12 μmol ml^{-1} G10 (acetone) and 45–60 μmol ml^{-1} G10 (DMF).

The carboxylated gels were transferred to DMSO and the carboxylate groups were activated by addition of a ten-fold molar excess (calculated with regard to the number of carboxylate groups) of EDC and NHS. This activation method is very



Scheme 2. Sequential derivatization of Sephadex, resulting in different immobilized peptides; starting material was obtained as illustrated in Scheme 3.

efficient for the coupling of amino compounds to carboxylated hydrogel, as demonstrated in a previous study with NHS-activated glycine-Sepharose [15]. Activation time was 2 h after which the activated gel was washed with DMSO. The NHS-activated glycine-Sephadex was allowed to react with the dipeptide Arg-Gly (2 μmol dipeptide/ml G10 (acetone) and 10 μmol dipeptide/ml G10 (DMF); this proportion between the added amounts of dipeptide is equal to the ratio of the numbers of activated groups on the two different gels). After 2 h reaction time, aminoethanol (0.2 ml 98% aminoethanol/ml packed gel) was added to block the remaining NHS-activated groups on the gel. The derivatized gel, G10-GRG-OH, was transferred



Scheme 3. Coupling of peptide to Sephadex G-10.

to water and extensively washed with deionized water (ten times with a four-fold volume) to remove the excess of aminoethanol. The washed gel was transferred to DMSO, activated with at least a ten-fold molar excess (as calculated with regard to the amount of the immobilized peptide RG) of EDC and NHS (activation time 2 h), washed with DMSO, transferred to 1,4-dioxane, and dried under high vacuum for at least 2 h. Portions of dry NHS-activated G10-GRG (300–600 mg) were suspended in DMSO and were allowed to react with different amounts of dipeptide Asp–Val (coupling time 16 h). Gel to which VD was coupled instead of DV, was used in the platelet binding experiments as the negative control gel. Depletion of dipeptide, as determined with the TNBS assay, from the supernatant of gels in which 1 μmol dipeptide/ml gel was added for coupling, revealed considerable differences between different batches of G10–GRG–NHS. Some batches showed a nearly complete depletion of dipeptide while in other batches depletion averaged only one third of the initial amount of dipeptide. Moreover, the coupling capacity seemed to decrease with increasing storage time (storage at 4°C). Addition of triethylamine (20–80 μmol ml^{-1} gel) restored the coupling capacity of all gell batches, indicating that the variation in coupling extent in the absence of triethylamine is probably caused by the presence of variable amounts of free acid in the batches. In this study, different amounts of triethylamine were added to the coupling mixture: (a) two molar equivalents as compared to the amount of the free dipeptide DV to neutralize protons, which could have been liberated from the dipeptide; and (b) 2 μmol triethylamine/ml G10 (acetone) and 10 μmol triethylamine/ml G10 (DMF) which corresponds to the maximal amount of NHS-activated groups on the gels. After coupling, the gels were transferred to water and incubated for 3 h in aqueous buffer (0.1 M $K_2B_4O_7$, pH 9.3) to hydrolyze the remaining activated groups on the gels. Part of the control gel, to which only triethylamine and no dipeptide was added, was washed with 0.1 M HCl/2 M NaCl in an attempt to remove non-covalently bound triethylammonium salt.

Preparation of GRGDSP-Sephadex and GRGDSP-Sepharose. Sephadex G-10 and Sepharose CL 6B were suspended in acetone and activated with CDI (addition of three volumes of a solution of 0.25 M CDI in acetone to one volume of packed gel). After 1.5 h activation time, the gels were washed with acetone, transferred to deionized water, and suspended in a solution of Jeff Amine 600 (JA600) (0.6 M JA600 in 0.25 M $NaHCO_3$, pH 9.5). After a coupling time of 16 h, the gels were washed extensively with deionized water, 2 M NaCl, and again deionized water. The amounts of JA600, immobilized to Sephadex and Sepharose, as determined with conductivity titration, averaged 9 and 8 μmol ml^{-1} gel, respectively.

The gels were suspended in 0.5 M $K_2B_4O_7$ after which the gel-bound amino groups were succinylated by addition of five portions succinic anhydride (SA) from a freshly prepared batch of 2 M SA in acetone. Each portion corresponded with 100 μmol SA/ml gel and the time between each two successive additions was about 1 min. Accomplishment of the conversion of the gel-bound primary amino groups into amide groups was qualitatively examined as follows. One ml of 50 mM TNBS and 2 ml of 0.5 M $K_2B_4O_7$ were added to 0.5 ml of packed gel and the reaction was proceeded for at least 30 min. JA600-derivatized gels coloured bright orange, indicating the presence of primary amino groups, while the SA-treated gels developed nearly no colour. From this striking colour difference it was concluded that the

SA-treatment, which was applied to the JA600-derivatized gels, resulted in succinylation of nearly all gel-bound amino groups. The succinylated gels were activated with EDC and NHS as described above.

To the NHS-activated gels different amounts of GRGDSP were added, corresponding with peptide densities ranging from a few nanomoles up to several micromoles per millilitre packed gel. Coupling of hexapeptide (in DMSO, at room temperature, coupling time 16 h) appeared to be complete, as checked with the TNBS assay, for the gel portions of both series to which the largest amount of peptide was added (1.4 μmol ml^{-1} Sephadex and 5.4 μmol ml^{-1} Sepharose).

Derivatization of Sephadex with substituted peptides. The activated gel G10-Gly-NHS was allowed to react with Arg-Gly, Gln-Gly, and His-Gly (for all dipeptides : 1 μmol dipeptide/ml gel) (Scheme 3), in DMSO, for 3 h at room temperature. Completion of the reaction was confirmed with the TNBS depletion assay. After reaction with the dipeptide, aminoethanol was added to the gel (0.25 ml 98% aminoethanol/ml gel) to block the remaining NHS-activated groups on the gel. Coupling of the second dipeptide Asp-Val (in a concentration of 0.5 μmol ml^{-1} gel) was performed as mentioned above (Preparation of GRGDV-derivatized Sephadex). In order to compare gels obtained by sequential coupling of dipeptides with gels obtained by direct coupling of hexapeptides in the endothelial cell binding experiments, the hexapeptides GRGESP and GRGDSP were coupled to G10-Gly-NHS (1 μmol hexapeptide/ml gel, coupling in DMSO, coupling time 16 h).

Trinitrobenzenesulfonic acid (TNBS) assay. The coupling between Arg-Gly and N-hydroxysuccinimide-activated glycine-Sephadex (G10-Gly-NHS) and the reaction of Asp-Val with G10-GRG-NHS, as well as the reactions of the hexapeptide Gly-Arg-Gly-Asp-Ser-Pro with NHS-activated Sephadex G-10 (carboxylated with glycine or with succinylated Jeff Amine 600 (JA/SA)) and NHS-activated glycine-Sepharose CL 6B, were studied as a function of the coupling time. The dipeptides were added in an amount of 5 μmol ml^{-1} gel. The amount of added hexapeptide was 2.5 μmol ml^{-1} Sephadex, and 10 μmol ml^{-1} Sepharose. The amounts of NHS-activated groups, initially present on the gels were 11 μmol ml^{-1} for G10-Gly-NHS, 5 μmol ml^{-1} for G10-GRG-NHS, 10 μmol ml^{-1} for G10-JA600/SA-NHS, and 110 μmol ml^{-1} for Sepharose-Gly-NHS. Reactions were performed with a 50% gel slurry in DMSO at room temperature. The depletion of the amino compounds from the supernatant of the gel suspensions could be measured photometrically after treatment of the supernatant with TNBS. Usually, 100–250 μl of supernatant was mixed with 1.00 ml of 0.1 M borate buffer ($K_2B_4O_7$) and 250 μl of 50 mM TNBS in deionized water. After a reaction time of at least 30 min, the TNBS-treated sample was diluted with 0.1 M borate buffer in order to get an extinction (at 420 nm) of less than 2.0 OD. The extinction at 420 nm was measured and taken as a relative measure for the concentration of the amino compound in solution.

The reaction of GRGDSP with the activated Sepharose appeared to be very rapid. Sampling of supernatant was accomplished by spinning down of the gel beads into a silicon oil layer by centrifugation in an Eppendorf centrifuge (the silicon oil layer consisted of a mixture of one part chloroform and 2.22 parts of silicon oil DC200 (Serva); the resulting mixture had a calculated density of 1.125 kg l^{-1}).

Isolation of blood platelets. Washed platelet suspensions were prepared by a modification of the procedure of Cazenave [16]. Platelet washing was performed in 50 ml polypropylene tubes (Greiner, Frickenhausen, Germany). Buffy coats from regular whole blood donation, which had been stored for not more than 24 h, were obtained from the Blood Bank Enschede (The Netherlands). After addition of heparin (30 Uml^{-1}), the buffy coats were diluted with a threefold volume of Tyrode Simple Buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 10 mM Pipes, 5.6 mM glucose, 0.20% human serum albumin; pH 7.0) and centrifuged at low speed (180 *g*, 15 min, no brake). The platelet-rich supernatant was transferred to clean tubes and an additional volume of Tyrode Simple Buffer was added (up to 1 v/v platelet-rich supernatant). After addition of prostaglandin I_2 (PGI_2) (final concentration 0.5 μM) the platelets were spun down (1600 *g*, 13 min, 37 °C). The platelet pellet was resuspended in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM Hepes, 5.6 mM glucose, 0.35% human serum albumin; pH 7.35) to which PGI_2 was added (final concentration 0.5 μM). Platelets were labeled after the first washing step by incubation with ^{111}Indium oxine (1–2 μCi/10^9 platelets), during 15 min at 37 °C. The resulting labeling efficiencies were found to be 70–95%. After addition of PGI_2 (final concentration 0.5 μM), the platelet suspension was kept at 37 °C for 10 min, before being centrifuged again (1600 *g*, 13 min, 37 °C). This washing step was repeated once more after which the platelet count was adjusted to 600×10^6 platelets/ml in Tyrode buffer. Apyrase was added to the platelet suspension (0.05 Um^{-1} l) and the platelet suspension was incubated at 37 °C for 30 min, whereafter platelets were kept at room temperature (22 ± 3°C).

Binding of blood platelets. The binding of washed blood platelets to the derivatized Sephadex gels was determined at room temperature (22 ± 3°C). To 2.0 ml 600×10^6 platelets/ml, 0.2 ml thrombin solution (1 Uml^{-1}) was added. After 20 s activation time, 0.2 ml hirudin solution (2 Uml^{-1}) was added in order to neutralize the thrombin. Twenty seconds after addition of hirudin, the activated platelets were combined with Sephadex-derivative (0.6 ml platelet suspension with 0.125 ml packed gel) and this suspension was manually mixed for 40 s. The beads were allowed to sediment (for 10–30 s), and the platelet suspension above the sedimented beads was removed. Unbound platelets and platelets which were loosely bound to the gel were removed by washing the gel with Tyrode buffer (2×3.5 ml). The radioactivity of the resulting gel fraction was determined with an LKB compugamma 1282 γ-counter (LKB, Stockholm, Sweden) from which the number of gel-bound platelets was calculated.

Binding of endothelial cells to peptide-derivatized gels. Human endothelial cells were isolated from umbilical cord veins according to the method of Willems *et al.* [17] and cultured as described by van Wachem *et al.* [18]. The human umbilical vein endothelial (HUVE) cells were harvested after the third passage by treatment with 0.05% trypsin/0.02% EDTA. After harvesting, the cells were washed twice with complete medium (a 1:1 mixture of Medium 199 and RPMI 1640, supplemented with 2 mML-glutamine, 100 Uml^{-1} penicillin, 100 μgml^{-1} streptomycin, and 2.5 mg ml^{-1} fungizone) and finally the cells were suspended in complete medium at a density of 525 000 cells ml^{-1}.

Cell binding to GRGDSP-Sephadex and GRGDSP-Sepharose. Sephadex G-10 and Sepharose CL 6B, both carboxylated with succinylated JA600 and derivatized with different amounts of GRGDSP, were combined with HUVE cells (0.2 ml packed gel with 1.0 ml cell suspension) in a sealed polystyrene tube. In negative control experiments, endothelial cells were preincubated for 30 min with the monoclonal (anti-platelet GPIIb-IIIa) antibody C17 (60 μgml^{-1}), and with free GRGDSP (1 mM), before mixing of the cells with the gel beads. The suspension was incubated at 37 °C with intermittent gentle shaking (cycles of 10 min shaking and 20 min resting). Total incubation time was 1.5 h after which the gel was washed with 2 × 3.5 ml of complete medium to remove unbound cells. After incubation of the washed gel in 0.1 M citric acid/0.1% crystal violet (for 30 min at 37 °C), and vigorous vortexing of the suspension, cell numbers were determined by counting the cell nuclei which were released in the supernatant, using a Burker chamber.

Cell binding to immobilized peptides obtained by sequential coupling of dipeptides to Sephadex. A volume of 0.4 ml cell suspension was added to 0.125 ml packed Sephadex, derivatized with different peptides, and the suspension was kept at 37 °C, in a sealed polystyrene tube, with intermittent gentle shaking for 3 h. The gel was washed with 3 × 3.5 ml complete medium to remove unbound cells. Counting of gel-bound cell numbers was carried out as mentioned above.

RESULTS

Swelling of Sephadex and Sepharose in different organic solvents

Sephadex G-10 showed various degrees of swelling depending on the solvents used. The swelling in water, DMSO, and DMF was very obvious while hardly any swelling occurred in acetone, acetonitrile, ethyl acetate, and diethyl ether. This different swelling behaviour is clearly reflected in the various solid substance contents of Sephadex in the different solvents (Fig. 1). Sepharose CL 6B showed a shrinking of about 5 and 20% after transfer from water to acetone and DMF, respectively.

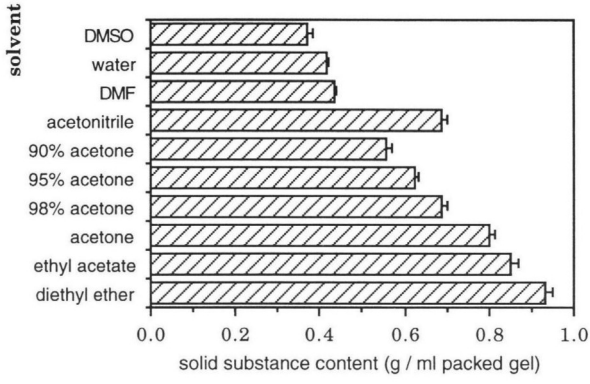


Figure 1. Solid substance content (gml^{-1}) of packed Sephadex G-10, suspended in different organic solvents and in water (mean ± S.D., n = 4). The electrostatic factor (EF) of the different solvents were: DMSO, EF = 209.2; water, EF = 144.4; acetonitrile, EF = 144; DMF, EF = 140.3; acetone, EF = 59.6; ethyl acetate, EF = 10.9; diethyl ether, EF = 5.9.

CDI-activation of Sephadex and Sepharose in acetone and DMF

The activation level of CDI-treated Sephadex G-10 and CDI-treated Sepharose CL 6B was studied by determination of the density of primary amino groups on the gel after reaction of the activated gel with diaminoethane. The increase of the activation of Sephadex with increasing activation time was very dependent on the solvent which was used as suspending medium. When DMF was used, the activation of Sephadex already increased to a high level within 10 min of activation time (Fig. 2a and b). In acetone, the increase of the activation as a function of time was slow and after 48 h of activation only a moderate degree of Sephadex activation was observed (Fig. 2a). The increase of the activation of Sephadex in the first 5 min was fast and was followed by a slower increase in time (Fig. 2b). The content of primary amino groups of the Sephadex after 5 min CDI-activation in acetone or DMF, followed by the reaction with diaminoethane, averaged 8 and 400 μmol ml^{-1} gel, respectively (Fig. 2b). In contrast to Sephadex, the activation degree of Sepharose CL 6B was not dependent on the type of suspending medium, and the change in activation of Sepharose with increasing activation time followed the same pattern whether the activation reaction was carried out in acetone or DMF (Fig. 2a).

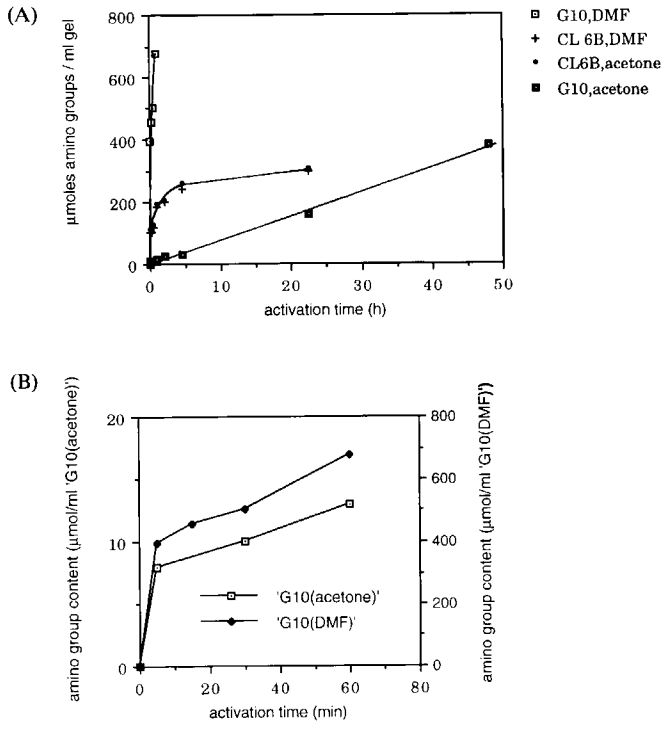


Figure 2. (A) The content of primary amino groups of Sephadex G-10 and Sepharose CL 6B, after CDI-activation and treatment with diaminoethane, as a function of activation time. (B) Increase of the relative degree of CDI-activation of Sephadex G-10, during the first 60 min of activation. Activation was performed in acetone and DMF, respectively.

Formation of β-alanine during carbodiimide/NHS-activation

Treatment of underivatized Sephadex G-10 and Sepharose CL 4B with carbodiimide and NHS led to the introduction of ionizable groups on the gel as demonstrated by conductivity titration. The number of ionizable groups on Sepharose CL 4B increased from 0.2 up to 1.6 μmol ml^{-1} gel and the number of ionizable groups on Sephadex G-10 increased from 3 up to 28 μmol ml^{-1} gel, after 2 days treatment of the gels with carbodiimide and NHS (Table 1). The residual number of ionizable groups that remained on the Sephadex when EDC/NHS treatment of Sephadex was followed by addition of aminoethanol, appeared to be equal to the number of ionizable groups on native, untreated Sephadex ($\pm$ 3 μmol ml^{-1} gel, results not shown).

Table 1.
Contents of ionizable groups, present on Sephadex G-10 and Sepharose CL 4B, after treatment of the native gel with carbodiimide and NHS (0.5 mmol EDC and NHS per ml Sephadex, in DMSO; 0.5 mmol DCC and NHS per ml Sepharose, in dioxane) (mean ± S.D., n = 4).

Time of treatment (h)	μmol ionizable groups/ml gel	
	Sephadex G-10	Sepharose CL 4B
0	3.11 ± 0.05	0.17 ± 0.022
2	5.39 ± 0.26	0.70 ± 0.055
24	22.6 ± 1.2	1.19 ± 0.081
48	28.4 ± 1.4	1.63 ± 0.087

Coupling of peptides to Sephadex and Sepharose

Coupling of the dipeptide Arg-Gly to the NHS-activated glycine-Sephadex was completed in 40 min (Fig. 3a). After blocking of the remaining NHS-activated groups with aminoethanol, the resulting G10–Gly–Arg–Gly was activated with EDC and NHS. The subsequent coupling of the dipeptide Asp-Val to G10–Gly–Arg–Gly–NHS was relatively slow; after coupling for 1 h, about 70% depletion from supernatant was found (Fig. 3a). Coupling of the hexapeptide GRGDSP to NHS-activated carboxylated Sephadex G-10 proceeded slow; completion of the coupling took about 10 h or more and the reaction seemed to be somewhat faster for G10–Gly–NHS as compared to G10-JA600/SA-NHS (Fig. 3a). The coupling between GRGDSP and NHS-activated glycine-Sepharose appeared to be fast; the reaction was completed in about 2 min (Fig. 3b).

Binding of blood platelets to G10-GRGDV, prepared from G10(acetone) and G10(DMF)

Nearly no platelets bound to the Sephadex derivatized with GRGVD (0.5 μmol peptide/ml gel) or with GRG (1 μmol peptide/ml gel) (Fig. 4) which were negative control gels. However, platelets did bind to the GRGDV-derivatized Sephadex at GRGDV-densities of $\geq$ 1 nmol GRGDV/ml G10(acetone) and at $\geq$ 10 nmol GRGDV/ml G10(DMF) (Fig. 4).

Addition of triethylamine alone and no dipeptide to the NHS-activated G10-GRG (2 μmol triethylamine/ml G10(acetone) and 10 μmol triethylamine/ml G10(DMF)) resulted in a material that showed a relatively high binding of platelets, especially by

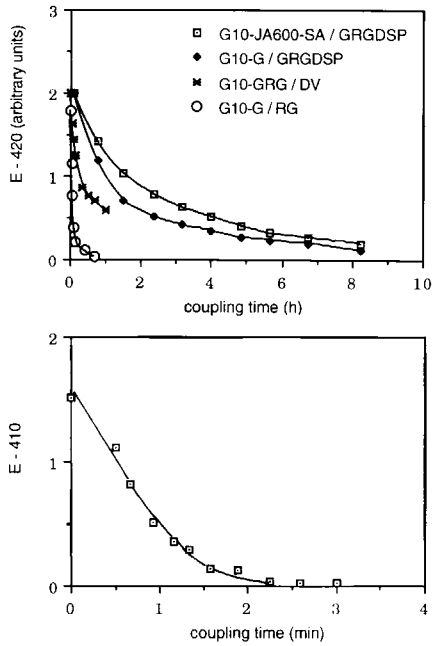


Figure 3. Depletion of peptide from the supernatant of suspensions of NHS-activated carboxylated Sephadex G-10 as a function of the coupling time. Depletion of peptide was determined by using the TNBS assay. (A) Arg-Gly was added to G10-Gly-NHS; Asp-Val was added to G10-Gly-Arg-Gly-NHS; Gly-Arg-Gly-Asp-Ser-Pro was added to G10-Gly-NHS; G10-JA600/SA-NHS. (B) Gly-Arg-Gly-Asp-Ser-Pro was added to Sepharose-Gly-NHS (see for more details Materials and Methods; the TNBS assay).

G10(acetone) (Fig. 5, DV = 0). This aspecific platelet binding was not affected by washing of the gel with 0.1 M HCl/2 M NaCl before using the material in the platelet binding assay (platelet binding to washed and unwashed gel: 210×10^6 and 236×10^6 bound platelets/ml gel, respectively). The use of triethylamine led to an increased specific platelet binding at lower GRGDV-concentrations as compared to the series to which no triethylamine was added, especially in the case of G10(DMF) (Figs 4 and 5). Platelet binding was measurable at ≤ 0.2 nmol GRGDV/ml G10(acetone) and ≤ 2 nmol GRGDV/ml G10(DMF) (Fig. 5). Platelet binding became maximal within the concentration ranges of 1–4 nmol GRGDV/ml G10(acetone) and 10–20 nmol GRGDV/ml G10(DMF) (Fig. 5). An optimum in the platelet binding to the gels was observed as the platelet binding to the gels with the highest peptide densities was less compared to the binding in the case of intermediate peptide densities (Figs 4 and 5).

Binding of human endothelial cells to peptide-derivatized Sephadex and Sepharose

Human umbilical vein endothelial cells bound to GRGDSP-derivatized Sephadex and Sepharose, which was accompanied by a coarse clumping of the beads during the first 30 min of incubation. As observed with phase contrast microscopy, many beads had bound one to three endothelial cells while part of the beads were clustered

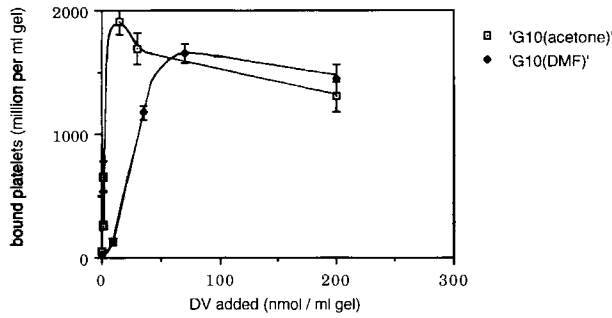


Figure 4. Binding of activated blood platelets to GRGDV-Sephadex as a function of the amount of DV, added to G10-Gly-Arg-Gly-NHS in the second coupling step. The number of bound platelets ($\times 10^6$ ml^{-1} gel) for the negative control gel GRGVD-Sephadex was found to be 89 ± 13 and 65 ± 5 for G10(acetone) and G10(DMF), respectively (mean ± S.D., n = 4).

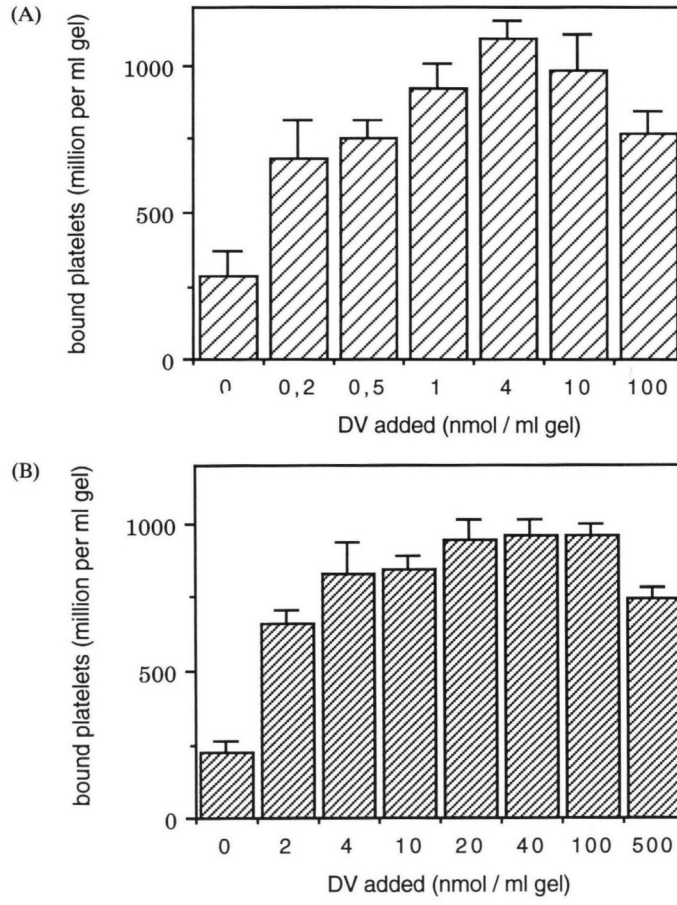


Figure 5. Binding of activated blood platelets to GRGDV-Sephadex as a function of the amount of DV, added to G10-Gly-Arg-Gly-NHS in the second coupling step. Coupling of DV was performed in the presence of triethylamine. (A) G10(acetone); 2 μmol triethylamine/ml gel. (B) G10(DMF); 10 μmol triethylamine/ml gel. (mean ± S.D., n = 4). The number of bound platelets ($\times 10^6$ml^{-1} gel) for the negative control gel GRGVD-Sephadex was found to be 287 ± 58 and 465 ± 94 for G10(acetone) and G10(DMF), respectively.

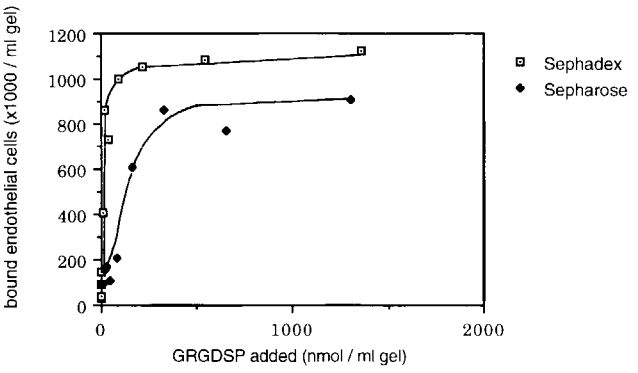


Figure 6. Binding of human endothelial cells to GRGDSP-derivatized Sephadex G-10 or Sepharose CL6B as a function of the amount of hexapeptide which was used for coupling.

by interconnecting cellular structures. Binding of the cells to immobilized RGD-peptide could be completely inhibited by preincubation of the cells with the monoclonal antibody C17 or by preincubation of the cells with free, i.e. soluble, GRGDSP (not illustrated). The extent of the cell binding to GRGDSP-Sephadex and to GRGDSP-Sepharose was dependent on the peptide-content of the gel beads. Cell binding occurred at ≥ 2 nmol GRGDSP/ml Sephadex and at >75 nmol GRGDSP/ml Sepharose (Fig. 6) and maximal cell binding was seen at ≥ 10 nmol GRGDSP/ml Sephadex and at ≥ 150 nmol GRGDSP/ml Sepharose (Fig. 6).

Binding of human endothelial cells and blood platelets to immobilized, substituted peptides

The extent of the binding of endothelial cells to GRGDV-Sephadex, prepared by sequential coupling of dipeptides, was comparable to the cell binding to GGRGDSP-Sephadex, obtained by one-step coupling of hexapeptide (Fig. 7). A relatively small number of HUVE cells bound to the immobilized negative control peptide GGRGESP (Fig. 7). Binding of endothelial cells to GQGDV-Sephadex

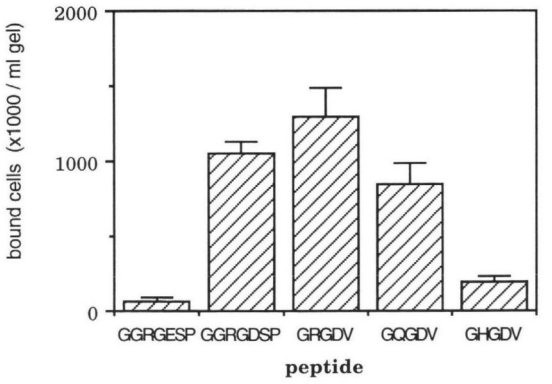


Figure 7. Binding of human endothelial cells to Sephadex derivatized with different peptides. The first two bars refer to materials which resulted from 'one step coupling' of hexapeptide, the other bars refer to materials derivatized by sequential coupling of dipeptides (mean ± S.D., $n = 6$).

was nearly as good as the binding to GGRGDSP-Sephadex while the number of cells which bound to GHGDV-Sephadex was not much larger as compared to the number found for the negative control (Fig. 7). In contrast with the endothelial cells, activated platelets did not bind to immobilized GQGDV ($6.4 \pm 0.7 \times 10^6$ bound platelets/ml G10-GQGDV ($n = 4$), to be compared with a positive control: $1110 \pm 24 \times 10^6$ bound platelets/ml G10-GGRGDSP ($n = 4$)).

DISCUSSION

The varying degree of swelling of Sephadex G-10 in the different organic solvents (Fig. 1) reflects a different degree of interaction of the Sephadex with solvent. Sephadex contains many hydroxylic groups and besides their involvement in intra- and interchain hydrogen bonding, the hydroxylic groups can also form hydrogen bonds with a solvent such as water. In polar solvents, which cannot form hydrogen bonds, dipole–dipole interactions with molecules of the solvent are formed. In apolar solvents, intra- and interchain hydrogen bonds prevail and consequently the swelling of the cross-linked polymer will be low. There seems to be some correlation between the electrostatic factor of the solvent (defined as the product of the dielectric constant and the dipole moment of the solvent) and the degree of swelling of Sephadex as observed in this study: a higher electrostatic factor of the solvent is attended with a higher extent of swelling, and consequently with a lower solid substance content (Fig. 1). Because of the good solubility of CDI in acetone, acetone was preferred to solvents like ethylacetate and diethyl ether in this study.

The different activation kinetics observed with Sephadex which was treated with CDI in either acetone or DMF, is most probably related to the difference in swelling of Sephadex in both solvents. The shrinkage of Sephadex G-10 in acetone was high (47% (v/v) as compared to the water-swollen gel), indicating that the porous network of the polymer beads had collapsed and therefore diffusion of substances inwards the beads had become restricted. For this reason, treatment of the shrunken Sephadex with CDI will mainly result in activation of the outer side of the Sephadex beads. As a consequence, coupling of low molecular weight compounds like diaminoethane and glycine to Sephadex, activated with CDI in acetone, will be restricted to the outer surface of the Sephadex beads and will not take place throughout the beads. The steady increase of the activation degree of CDI-treated Sephadex in acetone, which was still evident after 2 days of activation (Fig. 2a), may be explained by the slow diffusion of CDI through residual microporous cavities of the gel matrix.

In contrast to Sephadex G-10, Sepharose CL 6B showed only minimal shrinking after transfer from water to acetone or DMF (5% shrinkage in acetone and 20% shrinkage in DMF) and because of the high exclusion limit of Sepharose CL 6B (about 4×10^6 Da) it can be assumed that diffusion of CDI into the Sepharose beads is not influenced by the type of suspending medium. As shown in Fig. 2a, Sepharose CL 6B showed exactly the same increase of activation as a function of time whether CDI-activation was performed in acetone or in DMF. Therefore, it can be concluded that the kinetic differences, observed for the activation of Sephadex in the two solvents, was not caused by a solvent effect.

Treatment of native Sepharose CL 4B with DCC and NHS resulted in the immobilization of up to 1.6 μmol ionizable groups per ml gel, as measured with

conductivity titration (Table 1). Wilchek and Miron found about 100 μmol of β-alanine per gram dry Sepharose CL 4B after DCC/NHS treatment and alkaline washing, as determined by means of amino acid analysis [14]. Our value of 1.6 μmol ml^{-1} gel corresponds to 57 μmol g^{-1} dry gel (the dry substance content of Sepharose CL 4B is about 28 mg ml^{-1}) which is roughly comparable to the value reported by Wilchek and Miron.

After EDC/NHS treatment of Sephadex G-10, the amount of immobilized ionizable groups increased from 3 up to 28 μmol ml^{-1} gel (Table 1). This high value for Sephadex as compared to Sepharose can be explained by the high dry substance content of Sephadex (417 mg ml^{-1} gel). Untreated Sephadex has a significant number of gel-bound ionizable groups (3.11 μmol ml^{-1} gel, Table 1). After treatment of native Sephadex with EDC/NHS and subsequent addition of aminoethanol residual ionizable groups remained on the gel ($\pm$3 μmol ml^{-1} gel). This means that the ionizable groups initially present on the untreated gel cannot be blocked and therefore are not reactive and do not participate in the activation and coupling reactions which were carried out. The increase of the number of ionizable groups on Sephadex after 2 h EDC/NHS treatment may have consequences for the sequential coupling of dipeptides to the EDC/NHS-activated glycine-Sephadex. Part of the dipeptide DV may react with the immobilized β-alanine-NHS derivative, instead of reacting with the immobilized GRG-NHS, resulting in lower yields of immobilized GRGDV.

The slow coupling of hexapeptide to the NHS-activated Sephadex (Fig. 3a) is most likely caused by a restricted diffusion of the peptide into the activated outer layer of the Sephadex beads as well as the unfavourable high-density packing of the peptide, having multiple large side chains. In contrast to Sephadex, Sepharose has a very open pore structure, corresponding with a relatively large surface area to which peptide can be coupled. Therefore, the packing of the immobilized peptide will be diffuse and the restrictions as imposed by Sephadex will not be relevant for Sepharose, which explains the relatively high immobilization rate (Fig. 3b).

The platelet binding to GRGDV-Sephadex is specific with respect to the RGD-sequence as no platelet binding occurred with GRGVD-Sephadex (Fig. 4, legend). Platelet binding to the immobilized RGD-peptide is based on a specific interaction with the platelet GPIIb–IIIa complex as platelet binding is specifically inhibited in the presence of monoclonal antibody which is directed against GPIIB-IIIa (the corresponding results will be published in a forthcoming paper). The binding of activated platelets to GRGDV-Sephadex, prepared from Sephadex-Gly by sequential coupling of dipeptides, occurred at low peptide concentrations: G10(acetone) and G10(DMF) already bound platelets at 1 nmol GRGDV/ml gel and 10 nmol GRGDV/ml gel, respectively (Fig. 4). Platelet binding increased when the amounts of DV, added in the second coupling step of the preparation of GRGDV-Sephadex, were enlarged; maximal platelet binding occurred at DV-amounts of $\leq$ 15 nmol ml^{-1} G10(acetone) and at $\leq$ 70 nmol ml^{-1} G10(DMF) (Fig. 4). The difference in the effective GRGDV-density range between G10(acetone) and G10(DMF) can be explained by the lower peptide concentration which is necessary to react with the activated outer surface of the shrunken G10(acetone) particles compared to the relatively large activated surface area throughout the porous G10(DMF) gel beads.

Addition of triethylamine to the coupling mixture increased the nonspecific platelet binding whereas the DV-concentrations at which additional (specific)

platelet binding to the resulting gels is seen, were lower (Fig. 5). The increased specific platelet binding to GRGDV-Sephadex gel, which was prepared by coupling of peptide in the presence of the proton scavenger triethylamine, can be explained by the neutralization of acid, present in the coupling mixture. This acid, which was most likely present in the form of gel-bound carboxylic acid, was probably formed by hydrolysis of the immobilized NHS-active ester. Neutralization of free acid favours the coupling reaction with the dipeptide DV and results in a higher GRGDV-density as compared to the reaction without triethylamine. The specific binding of platelets to Sephadex, which was treated with triethylamine, is probably due to the immobilized triethylammonium salt. However, washing of the triethylamine-treated Sephadex with acid, in the presence of NaCl (2 M) did not diminish the aspecific platelet binding. Therefore, if the triethylammonium salt is responsible for the aspecific platelet binding, it must be concluded that the sustaining of this salt complex is not merely based on ionic interaction.

The decrease of platelet binding, observed for Sephadex with higher GRGDV-peptide densities as compared with intermediate peptide densities, may be explained by the corresponding higher negative charge density on the beads. This negative charge will impede the approach of negatively charged platelets and beads, because of electrostatic repulsion.

The binding of human umbilical vein endothelial (HUVE) cells to the GRGDSP-Sephadex was inhibited in the presence of the anti-platelet GPIIb-IIIa monoclonal antibody C17. HUVE cells possess an RGD-dependent (GPIIb-IIIa like) integrin which can bind adhesive proteins such as fibrinogen, von Willebrand factor, and vitronectin [19] and it is probably this receptor protein that is responsible for the HUVE cell binding to the immobilized RGD-peptide as described in the present study. For both GRGDSP–Sephadex and GRGDSP–Sepharose, peptide threshold levels can be distinguished above which endothelial cell binding became significant: $\pm$ 2 nmol GRGDSP/ml Sephadex and >75 nmol GRGDSP/ml Sepharose (Fig. 6). The occurrence of a peptide threshold level for the binding of cells to the RGD-peptide ligand has also been reported in the literature [20,21]. Our results with respect to cell binding give indirect support for the hypothesis that the coupling sites on G10(acetone) are mainly located in the outer layer of the porous beads while these sites have a more diffuse distribution on G10(DMF) and on Sepharose.

Human endothelial cells adhered to RGD–Sephadex but also to immobilized peptides in which arginine was substituted by glutamine or histidine (Fig. 7). In contrast with endothelial cells, activated platelets did not bind to the immobilized QGD-peptide. The choice of these substitutions in our study was partly based on the work of Tranqui *et al.* [22] who tested different synthetic peptides like RGDF, HGDF, and QGDF in solution for their ability to inhibit fibrinogen binding to blood platelets and endothelial cells. They found that substitution of arginine by glutamine or histidine had no effect on the binding affinity of the resulting peptide towards endothelial cells. However, the affinity of the respective peptide towards activated platelets was abolished as a result of the substitution of arginine by glutamine or histidine [22].

The different affinity of immobilized QGD-peptide for endothelial cells and blood platelets may be interesting with respect to blood vessel prostheses. Vascular prosthetic materials, derivatized with QGD-peptide, may facilitate endothelial cell seeding without promoting platelet adhesion, lowering the risk of occlusion of small diameter vessels due to thrombus formation.

The results presented in Fig. 7 illustrate that peptide-derivatized Sephadex, obtained by sequential coupling of dipeptides, is a very suitable material for the study of the binding of cells to immobilized peptides. Carboxylated Sephadex can be used for *in situ* synthesis of peptides by application of the procedure for coupling of dipeptides described in this paper. Coupling of dipeptides to G10-Gly-Arg-Gly-NHS is very easy and does not necessitate the use of protecting groups. However, in many other cases protecting groups must be introduced to prevent reactions with functional groups in the amino acid side chains, and racemization. Then, it is more appropriate to use peptides which are conventionally prepared and to immobilize these peptides in one step to the carboxylated Sephadex.

It can be concluded that (CDI-) activation of the porous Sephadex G-10 in acetone and subsequent coupling of glycine results in polymer beads which are mainly carboxylated in the outer layer. Subsequent activation with carbodiimide and *N*-hydroxysuccinimide gives an activated Sephadex, which can be used for the coupling of (small) ligands like oligopeptides, at the outer side of the beads.

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