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Concanavalin A Binding on PHEMA Beads and Their Interactions with Myeloma Cells

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The aim of this study is to prepare concanavalin A (Con A) bound poly(2-hydroxy ethyl methacrylate) (PHEMA) beads for cell affinity chromatography. In the first step, PHEMA beads were produced by suspension polymerization, and activated by cyanogen bromide (CNBr) in an alkaline medium (pH 11.5), and then, the bio-ligand "Con A" was attached by covalent binding onto the CNBr activated beads. PHEMA beads were characterized by scanning electron microscopy (SEM), surface area and pore size measurements. The PHEMA beads have a spherical shape and porous structure. The specific surface area of the PHEMA beads was found to be 39.7 m^2/g with a size range of 150–200 μm in diameter and the swelling ratio was 55%. The amount of bound Con A was controlled by changing pH and the initial concentrations of CNBr and Con A. The non-specific adsorption of Con A on the plain PHEMA beads was 0.1 mg/g. The maximum Con A binding was 4.8 mg/g at pH 7.25. Both plain and Con A bound PHEMA beads were interacted first with the myeloma cell suspension in phosphate buffer. Myeloma cell attachment was very low for the plain PHEMA beads, while the number of myeloma cells attached increased almost 20 fold when the Con A bound beads were used. In order to look at whether or not the interaction of the Con A bound PHEMA beads and myeloma cells are affected from the biological molecules and other cells in the medium. We selected sheep blood itself as the medium, and mixed with the myeloma cell suspension and changed the environment. Cell adhesion decreased but not very significantly by changing the medium from simple buffer to sheep blood.

Keywords: Myeloma cell adsorption, Concanavalin A, Lectin affinity chromatography, PHEMA beads

1 Introduction

The technology for cell isolation and identification is rapidly increasing in parallel to the advances in novel cell based therapies (1). In affinity separation, glycoaffinity chromatography allows the purification and the study of two interacting components: a lectin binding protein and a glyco-ligand. The interaction of these two components is due to the aptitude of a particular protein domain (carbohydrate recognition domain) to bind specifically to a certain sugar sequence in oligosaccharides and glycoconjugates (2). Numerous applications of lectins as affinity adsorbents are reported where their biochemical and biological properties are used not only for preparative chromatography but also for other applications such as detection of sugar chains on cell surface (3–5), separation of proteins, enzymes, antibodies and cells (6–16) on a preparative scale by bioaffinity chromatography. Another attractive application of lectins is their use in cell targeting for drug delivery systems because of their specificity for the sugar chains on different cell surfaces (17).

Glycans of cell membrane glycoproteins have gained increasing attention in recent years. Owing to their markedly different properties, their functions e.g., as adhesion mediators, or immunological markers are involved in complex processes such as development, differentiation and proliferation (18). Alterations in activities or concentrations of these proteins, associated with diseases, in body fluids are widely used as diagnostic tools and indices of therapeutic responses (19–24). Recently, cancer associated changes in the sugar chains of various enzymes or other proteins have been reported, suggesting that studies of alterations in sugar chains of these proteins may become a possible diagnostic tool for human diseases (18–21, 25).

Concanavalin A (Con A), a 104 kDa tetrameric protein, is one of the most widely studied lectin-ligands which interacts rather specifically and strongly with the mannosyl chain containing glycoconjugates on cell surface (18). It exhibits a series of remarkable properties. It has been used as haemaglutinin for studies of cell surface and cell division, to examine the nature of the carbohydrate residues responsible

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for blood group specificity and to distinguish between malignant or abnormal and normal cells (26). Therefore, Con A can be used for histochemical detection of sugar chains on the cell surface. Con A can also be used in different kinds of chemical and biological assays for testing the cellular mitogenic activities, studying the carbohydrate moieties in glycoproteins, purifying glycoproteins and treatment of tumourogenic cells (27–30).

The aim of this work is to develop a cell affinity system which enables distinguishing normal and malignant cells by means of their affinity of sugar chains on the cell membrane structures. This work focused on three major aspects; preparation of poly(2-hydroxyethyl methacrylate) (PHEMA) beads as an affinity matrix, immobilization of Con A onto the beads activated by cyanogen bromide (CNBr), and interaction of myeloma cells with the Con A carrying PHEMA beads in buffer suspensions and in blood.

2 Experimental

2.1 Materials

Concanavalin A (Jack Bean from Canavalia Ensiformis) and cyanogen bromide (CNBr) were all obtained from the Sigma-Aldrich Chemical (St. Louis, MO) and used as received. HEMA and EGDMA were obtained from Fluka AG. Commercial 2-hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) contain residual impurities due to production method. HEMA and EGDMA monomers were distilled under reduced pressure (0.01 mBar, 70°C). The initiator, azobisisobutyro-nitrile (AIBN, BDH Chem. Ltd., Poole, England) was recrystallized from methanol. Magnesium oxide as a stabilizing agent (MgO) was obtained from Sigma and used as received. All other chemicals were the guaranteed or analytical grade reagents commecially available and used without further purification. All water used in the binding experiments was purified using a Barnstead (Dubuque, IA, USA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANO pure® organic/colloid removal and ion exchange packed-bed system.

2.2 Preparation of PHEMA Beads

Preparation of PHEMA beads had been reported in our previous reports (31–33). Briefly, polymerization experiments were carried out in an aqueous dispersion medium containing magnesium oxide which was used to decrease the solubility of the monomer, HEMA in the medium. The monomer phase containing HEMA, EGDMA and AIBN was added to the dispersion medium within a laboratory type reactor (i.e., a two-neck flask with a volume of 500 ml) provided with a blade type stirrer. In order to produce polymeric beads in the range of 150–200 μm in diameter and with a narrow size distribution, the HEMA/EGDMA ratio, the monomer phase/dispersion phase ratio, the amounts of EGDMA and AIBN, and the agitation speed were 1/3 (v/v), 1/10 (v/v), 0.33 (mol EGDMA/mol HEMA), 0.0015 (mol AIBN/mol HEMA), 600 rpm, respectively. Polymerization was carried out at 70°C for 3 h and then at 90°C for 1 h. After cooling, the polymeric beads were separated from polymerization medium and residual MgO was removed by washing with a dilute HCI solution. The beads were also washed with water and ethanol, and then dried at vacuum dessiccator at room temperature.

2.3 Characterization of PHEMA Beads

2.3.1. *Density measurement*

The hydrated density of the PHEMA beads was measured with a 25 ml pycnometer by dispersing the beads in ethanol.

2.3.2. *SEM studies*

The surface morphology and internal structure of the PHEMA beads were observed by scanning electron microscope (JEOL, JEM 1200EX, Tokyo, Japan). PHEMA beads were dried at room temperature and coated with a thin layer of gold (about 100 Å) in vacuum and photographed in the electron microscope with ×1000 magnification. The bead size was determined by measuring at least 100 beads on photographs taken on a SEM.

2.3.3. *Surface area and pore size measurements*

The PHEMA beads were dried in a vacuum oven at 50°C for 24 h. Pore diameter greater than 20 Å were determined by mercury porosimeter up to 2000 kg/cm^2 using a Carlo Erba model 200 (Milano, Italy). The specific surface area was determined from the BET plot ($p/p_0 = 0.05$–0.95). Prior to the measurement, the samples were degassed at room temperature for 12–16 h in nitrogen flow. The average size and size distribution of the beads were determined by screen analysis performed by using Tyler Standard Sieves.

2.3.4. *Elemental analysis*

In order to evaluate the degree of imidocarbonate residues, the PHEMA beads were subjected to elemental analysis using a Leco Elemental Analyzer (Model CHNS-932, USA).

2.4 CNBr Activation

Prior to activation process, the PHEMA beads were kept in distilled water for about 24 h and washed on a glass filter with 0.5 M NaCl solution and water in order to remove impurities. Cyanogen bromide (CNBr, Sigma) aqueous solutions (100 ml) with different initial concentrations (5–50 mg CNBr/ml distilled water) were prepared. The pH of this solution was quickly adjusted to 11.5 with 2 M NaOH while it was magnetically stirred. One gram of the PHEMA

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beads was then added to this solution and activation procedure was continued for 60 min at a constant pH of 11.5. After the activation reaction, in order to remove the excess activation agent, the PHEMA beads were washed with 0.1 M $NaHCO_3$ and any remaining active groups (e.g., isourea) on the surfaces were blocked by the treatment with ethanol amine (pH: 9.1 M) and $FeCl_3$ solution for 1 h. Then, the activated PHEMA beads were washed four times with distilled water containing 0.5 M NaCl.

2.5 Concanavalin A Binding

CNBr activated PHEMA beads were magnetically stirred (at 50 rpm) at a constant temperature of 25°C for about 4 h (i.e., equilibrium time) with 50 ml of a Con A solution containing 100 μM $CaCl_2$, 100 μM $MnCl_2$ and 2% (w/v) glucose. Binding of Con A was carried out by reaction between the amino groups of Con A and the imidocarbonate residue of CNBr-activated PHEMA beads (34). Since the binding reaction occured at random between the amino group and the imidocarbonate residue, a haptenic sugar was added to the reaction mixture to protect the carbohydrate binding site of Con A. In order to observe the effects of the CNBr initial concentration and pH on covalent binding of Con A to the CNBr activated PHEMA beads, the CNBr initial concentration and the medium pH were varied between 5–50 mg/ml and 5.0–8.5, respectively. The initial concentration of Con A was 15.6 μg/ml. To obtain the effect of the Con A concentration on binding, the initial concentration of Con A was varied between 3.9 and 62.4 μg/ml in which pH of the solution (containing 0.1 M $NaHCO_3$ + 0.5 M NaCl) was 7.25.

After binding, the Con A bound PHEMA beads were washed with 0.1 M borate buffer + 0.15 M NaCl (pH 8.8), with 2 M urea + 0.15 M NaCl, and finally with 0.1 M $NaHCO_3$ + 0.5 M NaCl (pH 9.5). The amount of Con A bound on the CNBr activated PHEMA beads was determined by measuring the decrease of Con A concentration and also by considering the Con A molecules adsorbed non-specifically (the amount of Con A adsorbed on the plain PHEMA beads), by using Coomassie Brilliant Blue as described by Bradford with BSA as a standard (35). The experiments were performed in replicates of three and the samples were analyzed in replicates of three as well. For each set of data present, standard statistical methods were used to determine the mean values and standard deviations. Confidence intervals of 95% were calculated for each set of samples in order to determine the margin of error. The amount of bound lectin was calculated as

$$q = [(C_i - C_f)V]/m \tag{1}$$

where q is the amount of bound lectin onto PHEMA beads (mg/g), C_i and C_f are the initial and final concentrations of Con A in the adsorption medium (mg/ml), respectively, V is the volume of the reaction medium (ml) and m is the mass of the beads (g).

2.6 Interaction of Myeloma Cells with Polymeric Beads

Myeloma cells were obtained from Hammersmith Hospital (NSO, Immunology Department, London) and cultured in RPMI 1640 medium containing 10% bovine serum under CO_2 atmosphere at 37°C to reach enough cell population. Then, they were first suspended in 10 ml of phosphate-buffered saline (PBS, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.25) and incubated with Con A bound PHEMA beads (100 mg) in a batch system. Incubation was continued for 30 min in order to ensure complete equilibrium which was reached in about 10–15 min as determined in the preliminary studies. To obtain the number of cells attached to the polymeric beads, the cell counts in the initial suspension and in the supernatant after equilibrium were measured as follows: A few drops of cell suspension was spread out on a microscope slide and dried at room temperature for prefixation. Then, slides were interacted with May Grun Wald dye for 3 min in order to dye the cell cytoplasm and Giemsa dye for 15 min for the nucleus. Then cells were count with an optic microscope (Nikon, Alphapot YS, Japan). In order to calculate standard deviations, each measurement was repeated at least three times.

In the last part of the study, 10 ml of myeloma cells suspension in PBS was taken and mixed with 10 ml sheep blood and normal human blood. This mixture was incubated with Con A bound PHEMA beads in a batch system and cell counts were obtained with the same procedure given above.

3 Results and Discussion

3.1 PHEMA Beads

Con A bound PHEMA beads were prepared as an affinity adsorbents for myeloma cell adhesion from the cell suspension. PHEMA beads (150–200 μm in diameter) prepared in this study are well-known hydrophilic and crosslinked structures, i.e., hydrogels. They were selected as the basic carrier matrix due to their high inertness against proteins and cells (means low protein adsorption and cell attachment), and biocompatibility in addition to their high mechanical, chemical and biological stabilities (36). The biocompatibility of PHEMA is due to its ability to mimic tissues in relation to its high hydration and to its special surface properties. PHEMA hydrogels are hydrophilic polymer networks capable of imbibing large amounts of water yet remain insoluble and preserve their three-dimensional shape. The equilibrium water uptake of the PHEMA beads produced in this study with the formulation and condition given in the previos section was 55% (w/w), which

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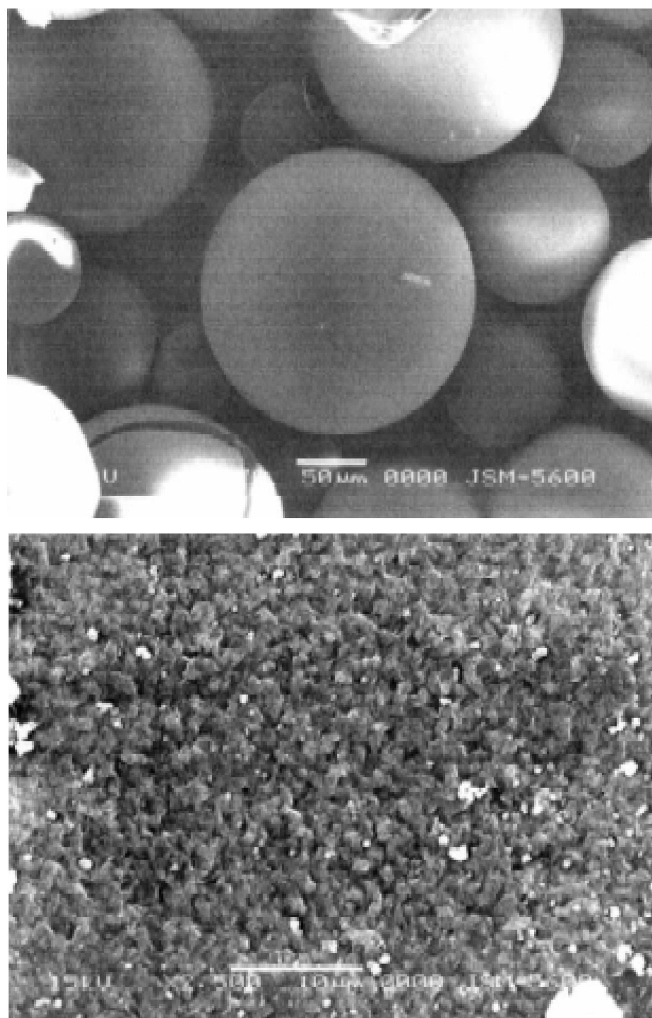

Fig. 1. SEM photographs of PHEMA beads.

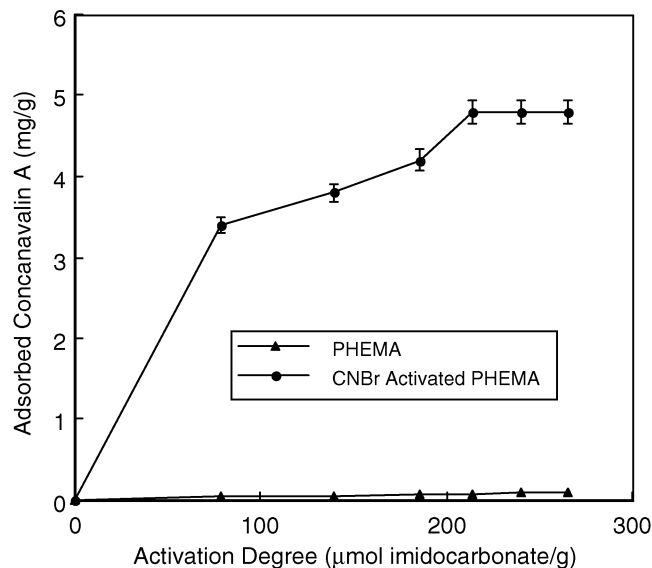

Fig. 2. Effect of CNBr activation degree on Con A binding. Con A concentration: 15.6 μg/ml; pH: 7.25; T: 25°C.

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was high enough to reach the required hydrophilicity for high inertness. Note that the equilibrium swellings did not change after CNBr activation and Con A binding steps.

The surface morphology and internal structure of PHEMA beads are exemplified by the scanning electron pictures in Figure 1. As seen in Figure 1A, PHEMA beads have a spherical form and a rough surface containing macropores due to the formation during the polymerization procedure. The picture in Figure 1B was taken with broken beads to observe the internal parts of PHEMA beads. The presence of pores within the bulk structure was clearly seen in the photograph. It can be concluded that the PHEMA beads have a porous structure. According to mercury porosimetry data, the average pore size of the beads was 600 nm. The specific surface area of the PHEMA beads was found to be 39.7 m^2/g. This indicated that the beads contained mainly macropores. The hydrated density of the PHEMA beads measured at 25°C was 1.05 g/ml.

3.2 Con A Binding

3.2.1. *Effect of CNBr activation degree*

The CNBr activation degree was changed between 78.7 and 265.3 μmol imidocarbonate/g polymer in order to change the number of the activated binding sites on the PHEMA surfaces. The PHEMA beads with different activation degrees were then incubated with Con A aqueous solutions at pH 7.25. Figure 2 shows that the amount of Con A bound on the beads increased by increasing the activation degree of CNBr, up to 214.1 μmol/g and then almost reached a plateau. Note that an increase in the CNBr activation degree corresponds to a larger number of activated binding sites on the surface of the carrier. Therefore, as expected, higher amounts of Con A are coupled on the CNBr activated PHEMA beads with higher number of activated sites. It should be noted also that, there is always a saturation capacity which depends on the number of functional groups on the matrix and the size of the bioligand molecules. However, the key factor in the performance of ligands bound on an adsorbent surface is the ligand mobility after binding rather than the total number of ligands available for binding. By binding Con A molecule tightly to the surface with multiple interactions would certainly reduce effective utilization of active sites on the Con A molecule. Considering these data we assumed that 214.1 μmol/g activation degree is optimal, and used this value in further studies.

3.2.2. *Effect of pH*

Con A was used as the bioligand for selective interaction with myeloma cells. In order to optimize the binding of Con A onto the CNBr activated PHEMA beads, the effects of medium pH, initial concentrations of CNBr and Con A were investigated in batch adsorption-equilibrium studies.

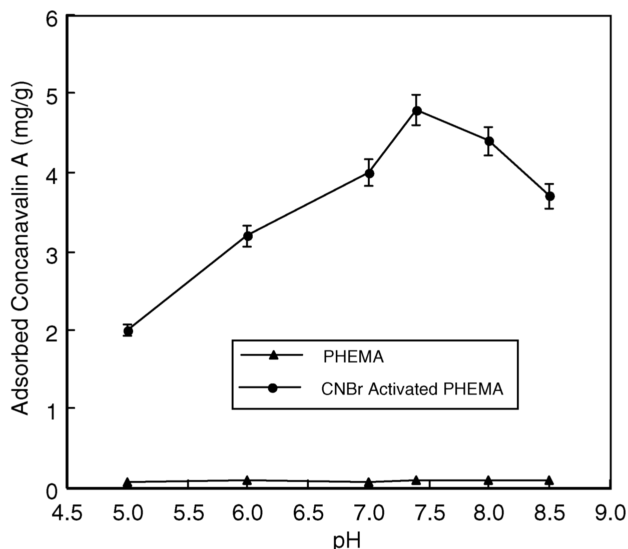

Fig. 3. Effect of pH on Con A binding: CNBr activation degree: 214.1 μmol/g; T: 25°C.

In the first group, the pH of the medium was changed in the range of 5.0–8.5 and the amount of Con A bound onto the beads was obtained. It was found that the amount of Con A adsorbed was significantly dependent on pH and the maximum adsorption capacity appeared around pH 7.25. We have assumed this pH as optimal and used in the later part of this study. As seen in Figure 3, the non-specific Con A adsorption (adsorption on the plain PHEMA beads) was pH undependent and quite low (0.1 mg Con A/g). However, covalently bound Con A onto the CNBr activated beads was 4.8 mg/g.

3.2.3. *Effect of Con A concentration*

Figure 4 shows both non-specific adsorption of the bioligand on the plain PHEMA beads and binding of Con A

Table 1. Normal human cell and myeloma cell adhesion studies

Polymer	*Myeloma cell adhesion (Cell/g beads)*	*Normal cell adhesion (Cells/g beads)*
PHEMA	4500 ± 1100	2300 ± 597
PHEMA/ Con A	89900 ± 2008	4500 ± 985

onto the CNBr-PHEMA beads. Note that we have changed only the Con A concentration in this group of experiments between 3.9–62.4 μg/ml. The non-specific adsorption was low, about 0.1 mg Con A/g PHEMA, while the amount of Con A molecules bound onto the bead surfaces first increased and then reached a saturation value (i.e., 4.8 mg/g PHEMA) at around 15.6 μg/ml Con A concentration, which is accepted as the optimal value and applied in the rest of the studies.

Yields of Con A binding on affinity matrices reported in the related literature changes in a wide range from about 5 mg Con A per gram of CNBr activated Sepharose (37) up to 49 mg/g for oxirane carrying poly(butadiene-hydroxyethyl methacrylate) particles (4). Recently, very high Con A binding yields up to 80 mg/g of aldehyde and tresylate functionalized silica beads were also reported (6). The maximum ligand incorporation achieved in the present study was 4.8 mg/g which we believe to be enough for the interaction with the relatively large size cells.

Ligand leakage may be a serious problem in affinity chromatography applications (38). Therefore we have looked at Con A leakage in PBS, and observed almost no leakage, most probably, due to very low nonspecific lectin adsorption and/or effective washing if there is any lectin non-specifically adsorbed on the beads, and also successful covalent bonding of the bioligand in the procedure described and applied in this study.

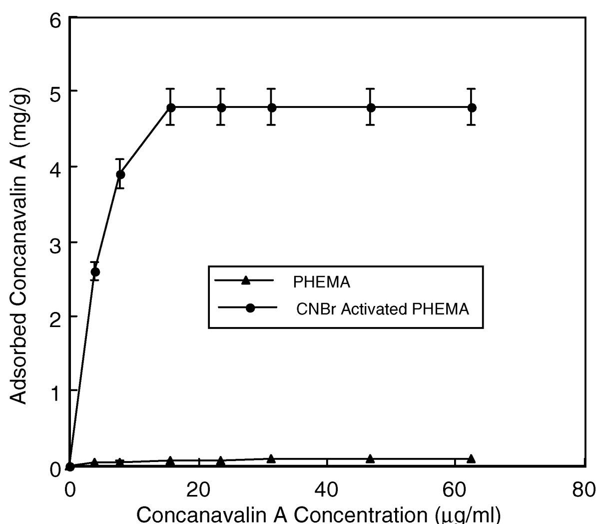

Fig. 4. Effect of Con A concentration on Con A binding: CNBr activation degree: 214.1 μmol/g; pH: 7.25; T: 25°C.

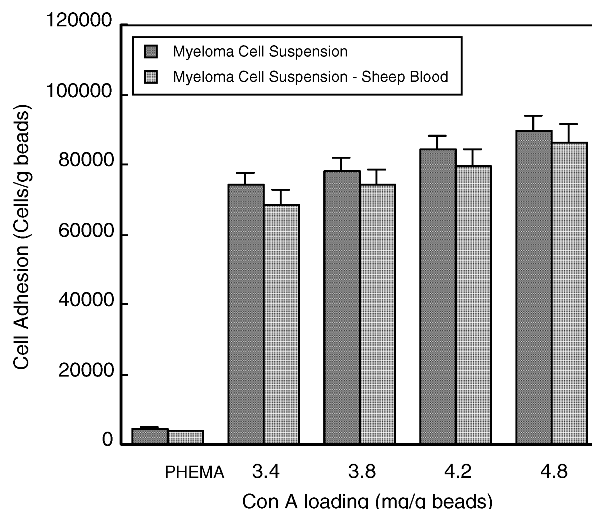

Fig. 5. Myeloma cell adhesion onto the plain and Con A bound PHEMA beads.

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3.3 Myeloma Cell Adhesion

Myeloma cell suspensions were prepared in phosphate-buffered saline, and interacted with both the plain and Con A bound PHEMA beads in a batchwise mode (four different Con A content 3.4 mg/g, 3.8 mg/g, 4.2 mg/g and 4.8 mg/g). Figure 5 summarized the data obtained in this first group of cell-material interaction studies. As seen here, cell adhesion onto the plain PHEMA beads is very low, as expected, due to the hydrophilic nature of PHEMA bead surface. There is a clear and important contribution of the Con A molecules bound onto the PHEMA beads. Con A binding increased the cell adhesion up to 20 fold. Increasing the surface concentration, in other terms Con A content onto the beads causes in an increase the cell attachment noticeably.

In the last part of the study, in order to look at whether or not the interaction of the Con A bound PHEMA beads and myeloma cells are affected from the biological molecules and other cells in the medium, we selected sheep blood itself as the medium, and mixed with the myeloma cell suspension and changed the environment. As seen in Figure 5, cell adhesion decreased but not very significantly by changing the medium from simple buffer to sheep blood, which is the one of the important results of this study. As seen here, the number of normal human cell adhesion is much lower than the number of myeloma cells (Table 1). Our efforts are continued in this direction to exhibit the specific affinity of the Con A carrying PHEMA on myeloma cells in the existance of other cell types including normal cells.

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4 Conclusions

Lectins have numerous applications as affinity adsorbents, where their biochemical and biological properties are used not only for preparative chromatography but also for other applications such as detection of sugar chains on cell surface (3–5). Cancer associated changes in the sugar chains of various enzymes and proteins have been reported, suggesting that studies of alterations in sugar chains of these proteins may become a possible diagnostic tool for human diseases (18–21). Concanavalin A (Con A), a 104 kDa tetrameric protein, is one of the most widely studied lectin-ligands which interacts rather specifically and strongly with the mannosyl chain containing glycoconjugates on cell surface (18). It exhibits a series of remarkable properties. It has been used as haemaglutinin for studies of cell surface and cell division, to examine the nature of the carbohydrate residues responsible for blood group specificity and to distinguish between malignant or abnormal and normal cells (26). In this study, the PHEMA beads produced by suspension polymerization were bound by Con A after CNBr activation. The adsorbents containing 4.8 mg Con A/g PHEMA beads were used for the Myeloma cell attachment. Myeloma cell attachment was very low for the plain PHEMA beads, while the number of myeloma cells attached increased almost 20 fold when the Con A bound beads were used. When the sheep blood was used as the adsorption medium, Myeloma cell adhesion decreased, but not very significantly.

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