Temperature-Responsive, Polymer-Modified Surfaces for Green Chromatography

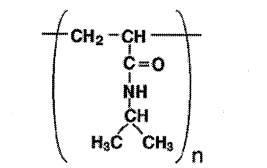
*Akihiko Kikuchi, Teruo Okano**

Institute of Advanced Biomedical Engineering & Science, Tokyo Women's Medical University, 8-1 Kawadacho, Shinjuku, Tokyo 162-8666, Japan

Summary: Thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm) and its derivatives were utilized as chromatography column matrix modifiers to develop novel supports for thermoresponsive hydrophobic chromatography with aqueous mobile phase. In the column, matrix surfaces show thermoresponsive hydrophilic/hydrophobic property alterations, which alter interaction with and retention of solutes to be separated. We have also demonstrated that the electrostatic interaction of ionic solutes and charged, thermoresponsive polymer-modified surfaces can be modulated temperature changes in the aqueous mobile phase alone.

Introduction

We have been investigating the preparation and characterization of a series of thermoresponsive polymer-modified surfaces. Poly(*N*-isopropylacrylamide) (PIPAAm) shows temperature-responsive soluble-insoluble changes across its lower critical solution temperature (LCST) at 32°C in aqueous solution[1,2] (Fig. 1). The thermoresponsive solubility change can be explained by the hydration/dehydration changes of polymer sidechain isopropyl groups. When PIPAAm is covalently incorporated onto solid surfaces to form densely polymer-grafted gel-like surfaces, these surfaces show temperature responsive hydrophilic/hydrophobic surface property alterations.[3-5] Utilizing these thermoresponsive surface property alterations, PIPAAm-modified surfaces have been utilized to recover cultured cells noninvasively for possible application in tissue engineering[6-11]. Furthermore, PIPAAm-modified surfaces are



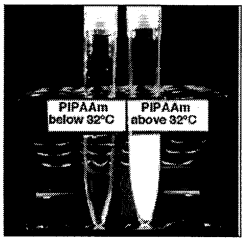


Fig. 1. Structure and solution appearance of PIPAAm.

 DOI: 10.1002/masy.200450319

also applied to develop a series of new column matrixes for chromatography using water as a sole mobile phase.[5,12-15] Several of the thermoresponsive surface types drawn in Fig. 2 are currently being investigated in our research groups. In the present paper, brief discussion will be made on these newly developed aqueous temperature responsive chromatography systems.

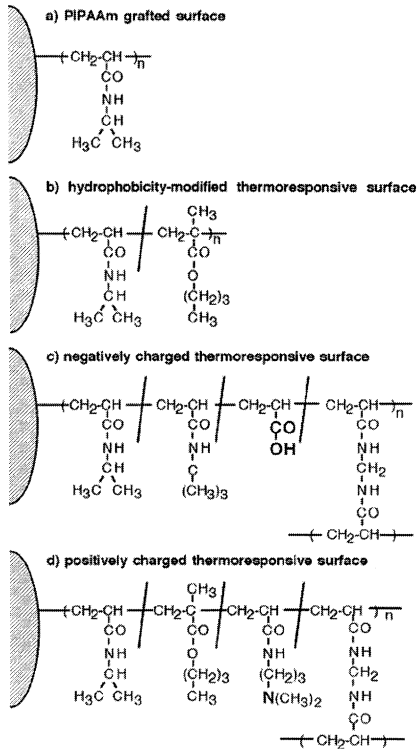


Fig. 2. Several surface types used in our studies.

Preparation and Wettability Changes of PIPAAm-Modified Surfaces

We have prepared four-types of PIPAAm-modified surfaces.[4,5] Those are, A) PIPAAm-grafted surface at one chain end, B) multipoint grafted PIPAAm surfaces, C) combination of the surfaces A) and B), and D) PIPAAm thin hydrogel layer grafted surfaces. PIPAAm-terminally grafted surfaces (type A) were prepared using end-carboxylated PIPAAm to graft onto aminated surfaces. Poly(IPAAm-*co*-*N*-acryloxysuccinimide) was used to prepare the model surface b). In this case, solvent composition was revealed to be an important factor affecting the polymer graft conformation on the surfaces. When 1,4-dioxane, a good solvent of PIPAAm, was used as the reaction solvent, PIPAAm was grafted on the surface with a relatively extended conformation, resulting in the disappearance of the transition in water contact angle changes. By contrast, addition of a small amount of non-solvent (toluene) for PIPAAm in dioxane resulted in a large, discontinuous water contact angle changes at a definite temperature, as described in a latter section. To prepare the surface type C), the latter preparation procedure was followed to obtain a large contact angle change at the polymer transition temperature. Thin PIPAAm hydrogel layer on glass surfaces was prepared using azo initiator-immobilized glass plates. Polymerization was initiated by

the surface-modified radical initiators, and a thin hydrogel layer was formed on the surfaces. Those four surface types are illustrated schematically in Fig. 3.

All four types of PIPAAm-modified surfaces showed thermoresponsive hydrophilic/hydrophobic surface property alterations, being hydrophilic at low temperature below transition temperature, and became hydrophobic above the transition temperature, as shown in Fig. 3. Thermoresponsive wettability changes arose from the characteristic of PIPAAm, which shows hydration changes with temperature. At low temperatures below the LCST, hydrophobic hydration around the side isopropyl groups has considerable influence on solubility of PIPAAm in water. Hydrophobic hydration is destroyed at higher temperature due to the increased entropy of water molecules, and thus, the hydrophobic nature of PIPAAm side chains determines the solubility of PIPAAm molecules in water above the LCST. The transition temperatures were slightly shifted according to the PIPAAm-graft configuration. For surfaces with freely mobile PIPAAm graft chains (surface type A) and C)), the transition temperature was in good accordance with the LCST observed for soluble PIPAAm molecules. On the other hand, transition temperatures observed for surface types B) and D) were slightly lower temperature than for surfaces types A) and C). Surface grafted PIPAAm molecules might have restricted molecular motion arising from the multi-point attachments, which reduces the transition temperature. These differences affected the molecular interaction as shown in the next section.

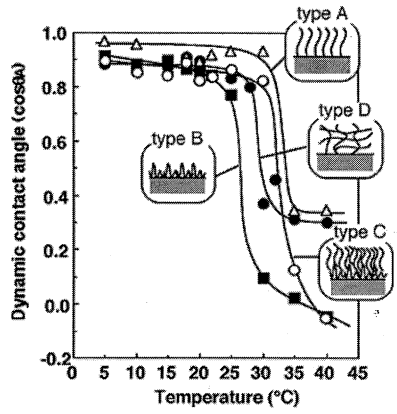


Fig. 3. Thermoresponsive contact angle (cosθ) changes for four types of PIPAAm-modified surfaces.

Hydrophobic Chromatography Using Sole Aqueous Mobile Phases

We then utilized these PIPAAm-modified surfaces for chromatography column matrices. Commercially available aminated silica beads (average diameter 5μm) were modified according to the above described methods to obtain several types of PIPAAm-modified beads with different PIPAAm conformation on the surfaces. These beads were then

packed into a stainless steel column (i.d. 4.6mm, 150mm length). The polymer-modified bead-packed column was connected to a high performance liquid chromatography system. Column temperature was controlled within ± 0.1°C with circulating water connected to a thermostated water bath. UV absorbance was used for detection. We applied thermoresponsive hydrophilic/hydrophobic surface property alteration to the recognition of hydrophobicities of substances by liquid chromatography with an aqueous mobile phase. Fig. 4 shows the effects of temperature on hydrophobic steroid elution from a terminally PIPAAm-modified, silica-packed column.[12]. At lower temperatures where the PIPAAm-modified surfaces are hydrophilic, steroids were eluted in one broad peak near void volume. With increasing temperature each peak can be recognized, but incomplete peak resolution was obtained at 25°C, below the polymer transition temperature. By sharp contrast, steroids were separated nicely above the polymer transition temperature. As a substance's hydrophobicity is usually defined as the logarithm of its octanol/water partition coefficient, log *P*, retention changes of steroids on PIPAAm-modified surfaces at various temperatures were analyzed using the log *P* values. With increasing the log *P* value, retention time became longer regardless of

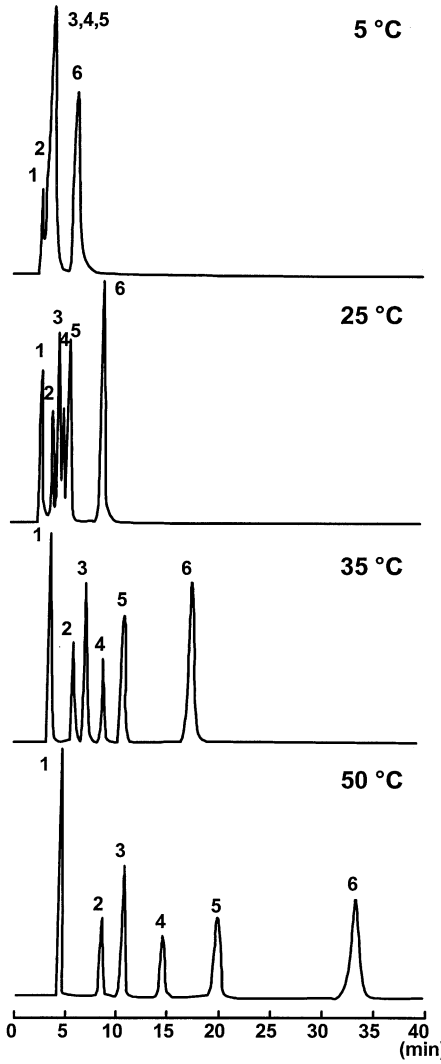


Fig. 4. Thermoresponsive chromatograms of steroids from PIPAAm-end grafted surfaces (type a) with water as a sole mobile phase. 1; benzene, 2; hydrocortisone, 3; prednisolone, 4; dexamethasone, 5; hydrocortisone acetate, 6; testosterone.

temperature, meaning that more hydrophobic substances have stronger interaction with PIPAAm-modified surfaces. Interestingly, when the natural logarithm of the capacity factor, *k*, values (ln *k*) was plotted against inverse temperature (1/T), inflection points were observed in the plots for all steroid types at a definite temperature. The temperature corresponds to the PIPAAm's LCST. This means that steroid interaction toward PIPAAm-modified surfaces is influenced by the polymer property alteration responding to temperature; i.e., the higher the temperature, the stronger the hydrophobic interaction is observed. The elution profiles of steroids from PIPAAm-modified columns are totally different from those observed using conventional reserved-phase chromatography column, octadesylsilane-column, in which elution time was shortened with temperature.

As described in the previous section, graft conformation of PIPAAm on the surface greatly influences the temperature-responsive wettability changes. Thus, it is possible to consider that the graft conformation of the PIPAAm on the column matrix surfaces should affect the elution behavior of steroids. The temperature-dependent elution behavior of steroids was examined on PIPAAm looped chain grafted surfaces, freely PIPAAm grafted onto PIPAAm loops, and PIPAAm thin hydrogel grafted surfaces ([5] for hydrogel surface). On those three PIPAAm-modified surfaces, low separation efficiency was observed in the chromatograms at 5 °C. At 45 °C under surface graft conditions, PIPAAm chains are in dehydrated state, and elution of hydrophobic steroids is retarded. As surface grafted polymer layers have higher grafted polymer densities than the surfaces with freely mobile linear PIPAAm-grafted chains, sample partitioning within densely grafted PIPAAm layers should have an influence on the extension of steroids retention times and peak broadening. When PIPAAm with chains with free ends were grafted onto PIPAAm loop chains, the thickness of the PIPAAm layer increased simultaneously with the graft reaction process. Thus, longer retention was seen on the surface with free end PIPAAm grafted onto PIPAAm loops than with PIPAAm loops alone. Significant peak broadening was seen in the case of PIPAAm thin hydrogel grafted surfaces. Partitioning of steroid molecules should be large for thin PIPAAm hydrogel grafted surfaces, which have a 3-dimensional cross-linked structure. Furthermore, the mobility of the PIPAAm chains is restricted, influencing peak broadening and the retardation of elution of steroids.

Steroid interactions were modulated by incorporating as hydrophobic comonomer, butyl

methacrylate (BMA) into PIPAAm sequences, which were utilized as column matrix modifiers (Fig. 2b).[13] Steroids interacted with the matrix, and separated even below the transition temperature of the polymer modifiers, probably due to the hydrophobic interaction with hydrophobic comonomer side chains below the polymer transition temperature. Above the LCST, more pronounced interaction took place, and baseline separation was achieved. Interestingly, steroid elution was easily modulated with stepwise temperature changes, similar to the gradient elution in the reversed-phase chromatography method. These results strongly support the feasibility of temperature-responsive chromatography as an alternative method for separation of bioactive compounds in aqueous systems.

The poly(IPAAm-*co*-BMA) modified column was further utilized to evaluate the interaction with peptides, elution profiles were observed for three peptides; insulin chain A, insulin chain B, and β-endorphin fragment (amino acid residues 1-27) at two different temperatures.[13] All three peptides have approximately the same molecular weight of 3,000 but with different amounts of hydrophobic amino acid residues. At low temperature, where polymer modifiers on the surface of column matrix are in the hydrophilic state, minimum interaction took place with all types of peptide samples, however, these peptides were separated nicely above the polymer transition temperature. Elution became retarded as the amount of hydrophobic amino acid residues in peptide sequences increased. The results indicate that thermoresponsive chromatography using an aqueous mobile phase can be utilized to separate not only low molecular weight substances, but also polypeptides, at least with molecular weights of around 3,000-4,000.

Modulation of Electrostatic Interaction with Ionic, and Thermoresponsive Polymer-modified Columns

Many biologically active substances have both hydrophobic and ionic nature in their molecular structures, such as peptides, DNA, and RNA. As the electrostatic nature can be altered by temperature in many ionic substances as is seen in synthetic polyelectrolytes.[16-18] Therefore, we hypothesized that the electrostatic interaction of ionic biological substances with thermoresponsive IPAAm copolymers bearing charged side groups might be modulated by only changing temperature. Poly(IPAAm-*co*-acrylic acid (AAc),[15] poly(IPAAm-*co*-t-butylacrylamide (tBAAm)-*co*-AAc),[19] or poly(IPAAm-*co*-BMA-*co*-*N,N*-dimethylaminopropylacrylamide (DMAPAA))[20] were

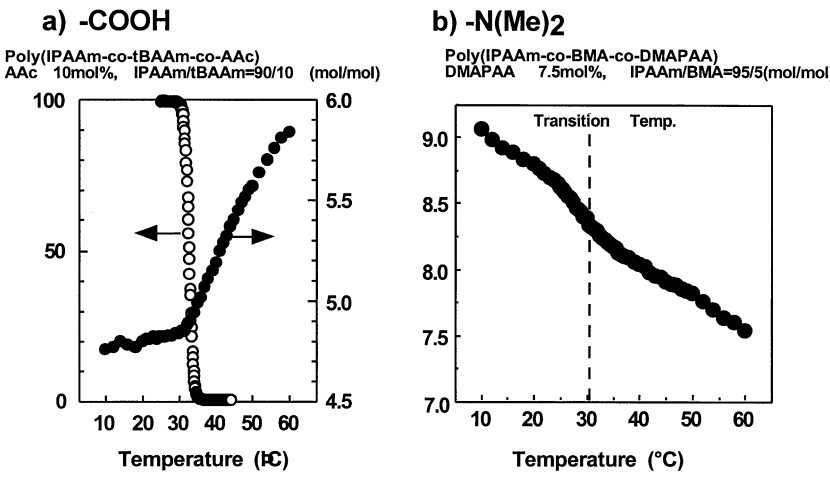


Fig. 5. Temperature dependent pKa' shift for a) carboxyls and b) amines in IPAAm copolymers.

prepared, and p*Ka'* changes were evaluated with temperature change. Both ionic polymer systems showed relatively large p*Ka'* changes around the polymer transition temperature; increasing p*Ka'* values for negatively charged polymer systems, and decreasing p*Ka'* for positively charged polymers (Fig. 5). In the case of positively charged poly(IPAAm-*co*-BMA-*co*-DMAPAA), with increasing surrounding temperature, dehydration of IPAAm isopropyl side chains took place, locally increasing the hydrophobic microenvironment around polymer amino groups. Furthermore, surface potentials of polymer-modified beads decreased and became negative above the polymer transition temperature. Previous reports document the weakening of tertiary amine basicity in IPAAm copolymers at elevated temperature, especially above the LCST, while there is nearly no observable change in amine basicity in water-soluble polyacrylamide derivatives over a wide temperature range.[17] The decrease in amino group basicity in PIPAAm derivatives is due to the decreased dielectric constant around the amino groups at higher temperature where the polymer loses hydration and becomes hydrophobic. Urry[16] also reported a carboxyl p*K*a shift in synthetic polypeptides by modulating peptide hydrophobicity. These observations indicated that the charge density distributions of polymer molecules, or polymer-modified surfaces become lower as polymer is in a hydrophobic, aggregated state above the respective transition temperature even at neutral pH. Therefore, the interaction of charged bioactive compounds with

thermoresponsive and ionic polymers can be modulated by temperature alteration alone. Fig. 6 shows the typical result of elution for adenosine nucleotides, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), all these share the same adenosine chemistry but different numbers of anionic phosphate units. All of these molecules are important for energy metabolism in living systems. As is shown in this figure, poor separation of adenosine nucleotides was observed in nonionic PIPAAm-modified columns. By sharp contrast, retention time increased with increasing number of anionic phosphate units in the molecules, regardless of temperature, on the amino-containing, terpolymer-modified column. This means that adenosine nucleotides interact with poly(IPAAm-*co*-BMA-*co*-DMAPAA)-modified surfaces through primarily an electrostatic interaction. Interestingly, stronger interaction took place at lower temperatures, and all substrates show longer retention times. By increasing temperature, retention times of adenosine nucleotides dramatically decreased, especially above the polymer transition temperature. These results strongly suggest that polymer matrix charge density alteration with temperature affects to the order of electrostatic interaction with oppositely charged biological molecules. Without charged groups, i.e., PIPAAm-modified surfaces, such retention time changes were not observed. Therefore, the temperature-modulated charge density alteration could be utilized to regulate the interaction with charged, hydrophobic biological compounds. Although the thermoresponsive ionic interaction chromatographic systems need longer analyses times,

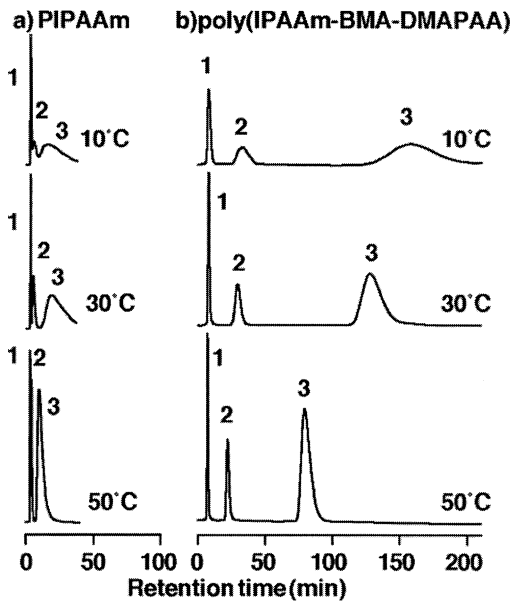


Fig. 6. Temperature dependent adenosine nucleotides elution on a) PIPAAm, and b) cationic thermoresponsive polymer surfaces at pH7.0. 1; AMP, 2; ADP, 3; ATP. flow rate; 0.5mL/min.

column conditions could be optimized through chemical composition of copolymer modifiers, size of column, and silica bead diameter, and so on. Adenosine nucleotides are currently analyzed with reversed-phase columns in the absence of[21,22] or in the presence of additive alkylamine (ion-pair RPC)[23-25] to modulate analyte sample properties, interactions with stationary phase surfaces, and of course, elution time.

To regulate the interaction of positively charged bioactive compounds with negatively charged thermoresponsive polymer modified columns, we introduced the poly(IPAAm-*co*-*N*-*t*-butylacrylamide (*t*BAAm))-*co*-AAc) thin hydrogels onto the silica bead surfaces.[19] A hydrophobic monomer, tBAAm, was incorporated to maintain thermoresponsive property of the polymer molecules, which may be disrupted by the increase in the hydrophilic, negatively charged carboxylate anions. The prepared bead-packed column was then utilized to investigate the elution behavior of positively charged substances, including catecholamines and angiotensin subtypes, as low molecular weight substances and peptides, respectively. Catecholamine interaction with poly(IPAAm-*co*-*t*BAAm-*co*-AAc) with 10mol% of AAc was evaluated. At pH 4.0, where all carboxyl side chains in the surface-grafted polymer were in protonated state (no charge) while amino containing sample analytes were all protonated, negligible interaction took place with catecholamines regardless of temperature change. By sharp contrast, catecholamines, excepting zwitterionic DOPA, are retained on the poly(IPAAm-*co*-*t*BAAm-*co*-AAc) modified column. As catecholamines did not interact with the non-ionic PIPAAm column, these interacted were primarily through electrostatic interaction with poly(IPAAm-*co*-*t*BAAm-*co*-AAc) surfaces at pH 7.0. Elution times were increased in the order DOPA<adrenaline<dopamine<tyramine. This order is in agreement with the molecule hydrophobicity parameter, i.e. the logarithm of the partition coefficient.

The data indicate that the analyte interaction with negatively charged surfaces is affected by the two parameters: electrostatic, or coulombic interaction, and hydrophobic interaction. When positively charged sample molecules interact with negatively charged surfaces, coulombic interaction neutralizes apparent charges. Then, microenvironmental hydrophobicity around the analyte molecules increases due to repulsion of the polarized and hydrated water molecules around charged groups. As analyte hydrophobicity increases, the microenvironmental hydrophobicity also increases. Thus, more hydrophobic substances interact strongly with negatively charged polymer surfaces. Temperature alteration affected peak resolution, and around the surface-modified

polymer LCST at pH 7.0 the resolution of tyramine and dopamine was maximal. Above the transition temperature, the resolution become worse due to decreased electrostatic interaction with hydrophobized polymer surfaces.

Three angiotensin subtypes; angiotensin I, II, and III, are peptides differing in sequence and biological affinity. Due to their structural similarity, separation of these subtypes are often difficult with conventional chromatography methods, and combination of reversed-phase chromatography and ion exchange chromatography is applied for separation of these substances. In our newly developed column of poly(IPAAm-*co*-*t*BAAm-*co*-AAc) modified silica, it is possible to separate these three angiotensin subtypes with only one chromatographic run at constant pH and ionic strength, but changing temperature with a sole aqueous mobile phase.

The use of intelligent materials should prove valuable in the design of novel aqueous 'green' chromatography systems.

Conclusions

Poly(*N*-isopropylacrylamide) (PIPAAm) and its derivatives were utilized as chromatographic matrix modifiers to develop novel hydrophobic chromatography using a sole aqueous mobile phase. Thermoresponsive soluble-insoluble changes of the PIPAAm derivatives were used to modulate surface hydrophilic-hydrophobic property alterations. The surface property alteration then influenced the interaction with biological entities, such as peptides, proteins, and also cells. The results briefly demonstrated here indicate that formation of very thin, hydrogel-like, dense thermoresponsive polymer layers on the solid surfaces is a feasible approach for surface property alteration. This technique can be utilized for novel aqueous chromatography matrices and/or cell culture and detachment control substrates without using cell-lytic enzymes. These technologies are promising for future environmentally friendly chromatography developments as well as for tissue engineering.

Acknowledgments

This work was financially supported in part by the Japan Chemical Innovation Institute (JCII) operated under the New Energy and Industrial Technology Development Organization (NEDO), and the Grant-in-Aid for Scientific Research (Grant No. 13680955) by the Japan Society for Promotion of Science. The authors would be

grateful to the collaborators, Professor Kiyotaka Sakai of Waseda University, and Professor Hideko Kanazawa of Kyoritsu College of Pharmacy for their collaboration as well as valuable comments, and suggestions throughout the work.

[1] M. Heskins, J. E. Guillet, E. James, *J. Macromol. Sci., Chem. A* **1968**, *2*, 1441.
[2] Y. H. Bae, T. Okano, S. W. Kim, *J. Polym. Sci.: Part B: Polym. Phys.* **1990**, *28*, 923.
[3] Y. G. Takei, T. Aoki, K. Sanui, N. Ogata, Y. Sakurai, T. Okano, *Macromolecules* **1994**, *27*(21), 6163.
[4] T. Yakushiji, K. Sakai, A. Kikuchi, T. Aoyagi, Y. Sakurai, T. Okano, *Langmuir* **1998**, *14*(16), 4657.
[5] T. Yakushiji, K. Sakai, A. Kikuchi, T. Aoyagi, Y. Sakurai, T. Okano, *Anal. Chem.* **1999**, *71*(6), 1125.
[6] T. Okano, N. Yamada, H. Sakai, Y. Sakurai, *J. Biomed. Mater. Res.* **1993**, *27*, 1243.
[7] T. Okano, N. Yamada, M. Okuhara, H. Sakai, Y. Sakurai, *Biomaterials* **1995**, *16*, 297.
[8] A. Kikuchi, M. Okuhara, F. Karikusa, Y. Sakurai, T. Okano, *J. Biomater. Sci., Polym. Ed.* **1998**, *9*(12), 1331.
[9] M. Yamato, A. Kikuchi, S. Kohsaka, T. Terasaki , H. A. von Recum, S. W. Kim, Y. Sakurai, in: „*Tissue Engineering for Therapeutic Use 3*“, Y. Ikada, T. Okano, Eds., Elsevier; Amsterdam 1999, p. 99.
[10] M. Yamato, M. Utsumi, A. Kushida, C. Konno, A. Kikuchi, T. Okano, *Tissue Eng.* **2001**, *7*(4), 473.
[11] T. Shimizu, M. Yamato, A. Kikuchi, T. Okano, *Tissue Eng.* **2001**, *7*(2):141.
[12] H. Kanazawa, K. Yamamoto, Y. Matsushima, N. Takai, A. Kikuchi, Y. Sakurai, T. Okano, *Anal. Chem.* **1996**, *68*(1), 100.
[13] H. Kanazawa, Y. Kashiwase, K. Yamamoto, Y. Matsushima, A. Kikuchi, Y. Sakurai, T. Okano, *Anal. Chem.* **1997**, *69*(5), 823.
[14] H. Kanazawa, T. Sunamoto, Y. Matsushima, A. Kikuchi, T. Okano, Anal. Chem. **2000**, *72*(24), 5961.
[15] J. Kobayashi, K. Sakai, A. Kikuchi, T. Okano, *Anal. Chem.* **2001**, *73*(9), 2027.
[16] D. W. Urry, *Prog. Biophys. Molec. Biol.* **1992**, *57*, 23.
[17] H. Feil, Y. H. Bae, J. Feijen, S. W. Kim, *Macromolecules* **1993**, *26*(10), 2496.
[18] A. Kikuchi, T. Tsuruta, K. Kataoka, J. Biomater. Sci., Polym. Ed. **1994**, *5*(6), 569.
[19] J. Kobayashi, A. Kikuchi, K. Sakai, T. Okano, *J. Chromatogr.* **2002**, *958,* 109.
[20] A. Kikuchi, T. Iwasa, J. Kobayashi, K. Sakai, T. Okano, submitted.
[21] G. Leoncini, E. Buzzi, M. Maresca, M. Mazzei, A. Balbi, *Anal. Biochem.* **1987**, *165*, 379.
[22] T. Teerlink, M. Hennekes, J. Bussemaker, J. Groeneveld, *Anal. Biochem.* **1993**, *214*, 278.
[23] K. K. Tekkanat, I. H. Fox, *Clin. Chem.* **1988**, *34*(5), 925.
[24] B. Schobert, *Anal. Biochem.* **1995**, *226*, 288.
[25] J. Blanco, E. I. Canela, J. Sayos, H. Mallol, C. Lluis, R. Franco, *J. Cell. Physiol.* **1993**, *157*, 77.