abrupt decreases in their I_1/I_3 values when temperature increased across its LCST of 32 °C, indicating the hydrophobic changes of polymer chains [19,20]. P(IPAAm-*co*-CIPAAm) solutions also showed similar abrupt decreases in this ratio when temperature was increased across 32 °C. Introduction of 4.8 mol% CIPAAm to PIPAAm did not alter the original LCST (see Fig. 2). This is asserted to result from strong monomer structural similarities and the resulting uninterrupted sequences of isopropyl side groups in both homo- and co-polymers. In contrast, P(IPAAm-*co*-CNPAAm) copolymers showed higher polarity ratios than PIPAAm solutions at any temperature, suggesting a weaker hydrophobic interaction among copolymer side chains, weaker aggregated collapse and elevated LCST above 32 °C. This could be produced by the different carboxylate side chain group position that cannot permit isopropyl side chain alignment and cooperative collapse with IPAAm-based copolymer after LCST dehydration as in the CIPAAm case.

The p*K*'a value for the P(IPAAm-*co*-CIPAAm) copolymer was considerably higher than that for P(IPAAm-*co*-CNPAAm), while the CIPAAm monomer's p*K*'a was very similar to that of CNPAAm (Table 1). Specifically, carboxyl groups in the copolymer exhibit suppressed dissociation at pH 7.4 at 10 °C below the LCST. This could result from cooperative hydrophobic interactions among isopropyl groups that either lower the local dielectric environment of the side chains, promote known carboxylate hydrogen bonded dimerization in this environment, or restrain repulsive forces between dissociated carboxyl groups. Fig. 3 shows p*K*'a values observed for IPAAm copolymers plotted against solution temperature. At any temperature, P(IPAAm-*co*-CNPAAm) exhibits higher

Table 1
Apparent p*K*'a values measured by titration at 10 °C

Samples	p*K*'a
CIPAAm monomer	4.58
CNPAAm monomer	4.70
P(IPAAm-*co*-CIPAAm) (CIPAAm 4.8 mol%)	6.17
P(IPAAm-*co*-CNPAAm) (CNPAAm 4.0 mol%)	4.90

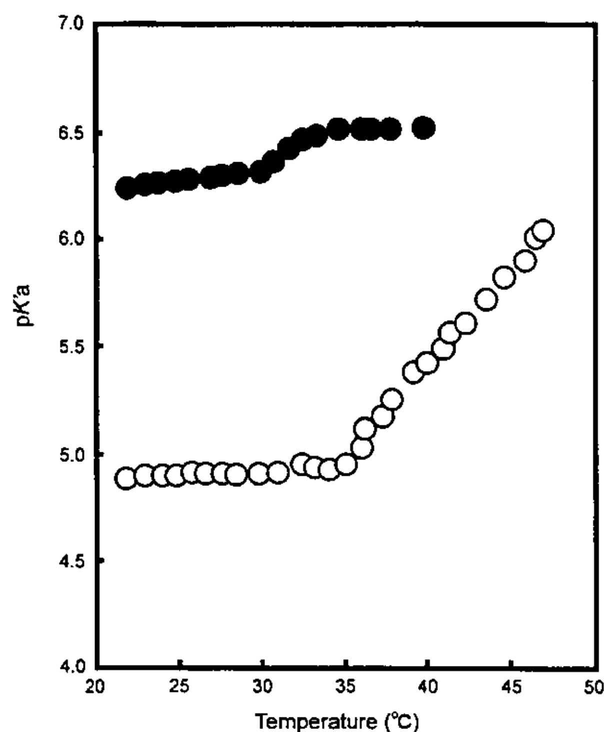


Fig. 3. Temperature dependence for apparent p*K*'a values for IPAAm-based copolymers. P(IPAAm-*co*-CIPAAm) (CIPAAm content 4.8 mol%): closed circles, P(IPAAm-*co*-CNPAAm) (CNPAAm content 4.0 mol%): open circles.

acidity than P(IPAAm-*co*-CIPAAm). Each copolymer has constant acidity below its LCSTs. But, solution acidity increases with temperature to 47 °C for the P(IPAAm-*co*-CNPAAm) copolymer. These observations are consistent with the copolymer hydrophobicity increasing with increasing temperature above their respective LCSTs [14, 23,24]. These would result from protonation of carboxyl groups and subsequent hydrophobic aggregation for P(IPAAm-*co*-CNPAAm), distinct from the other polymers tested. The solution p*K*'a increase for P(IPAAm-*co*-CIPAAm) over this temperature increase was small, reflecting reduced dissociation of carboxyl groups in P(IPAAm-*co*-CIPAAm) solutions. These results clearly indicate the physicochemical affects of the slight structural difference between these two monomers (*iso*-propyl versus *normal*-propyl), and the resulting significant differences in their phase transition behaviors when introduced into copolymers. The p*K*'a for IPAAm-AAc copolymers is around 5 [14,23,24],

which is close to the p*K*'a for P(IPAAm-*co*-CNPAAm).

3.2. Cell spreading and detachment on polymer-grafted surfaces in culture

PIPAAm, P(IPAAm-*co*-CIPAAm), and P(IPAAm-*co*-CNPAAm) were polymerized in situ and covalently grafted onto TCPS surfaces by electron beam irradiation. The graft amounts estimated by ATR-FTIR were approximately 1.8–1.9 $\mu g/cm^2$. We have reported attachment, spreading and detachment results for BAECs on pure PIPAAm-grafted surfaces. These results indicate that BAECs attach and spread normally on the PIPAAm homopolymer-grafted culture surface above the polymer LCST, and detach readily once below the LCST [5]. Fig. 4 shows BAEC spreading after 2-day culture at 37 °C on these surfaces. On copolymer-grafted surfaces, cell spreading was continually and increasingly hindered with increasing CNPAAm content in copolymer grafts. No BAEC attachment or spreading was ever obtained on copolymer-grafted surfaces containing 5 mol% feed-ratio of CNPAAm. Interestingly, BAEC spreading was not influenced by the introduction of analogous CIPAAm up to 5 mol% feed ratio. These observations are consistent with the observed phase transition data for these copolymers. P(IPAAm-*co*-CNPAAm) exhibits significant hydrophilicity even at 37 °C similar to P(IPAAm-*co*-CIPAAm) below its LCST shown already to repel cell attachment even in serum-supplemented culture media (see Fig. 2 and Ref. [17]).

Cell detachment upon reducing culture temperatures to 20 °C was examined with both PIPAAm- and copolymer-grafted surfaces with co-monomer composition controlled to 1 mol% feed ratios. Spread cell densities at 37 °C were nearly identical (see Fig. 4). Fig. 5 shows the time course of wettability changes of each grafted surface in response to decreasing temperature changes from 37 to 20 °C. All polymer-grafted surfaces showed similar wettability at 37 °C and became more hydrophilic (more wettable) in response to the temperature reduction. This is due to the common polymer chain hydration that occurs below the LCST. Both P(IPAAm-*co*-CIPAAm) and P(IPAAm-*co*-CNPAAm)-grafted surfaces demonstrated more rapid and larger hydrophobic to hydrophilic

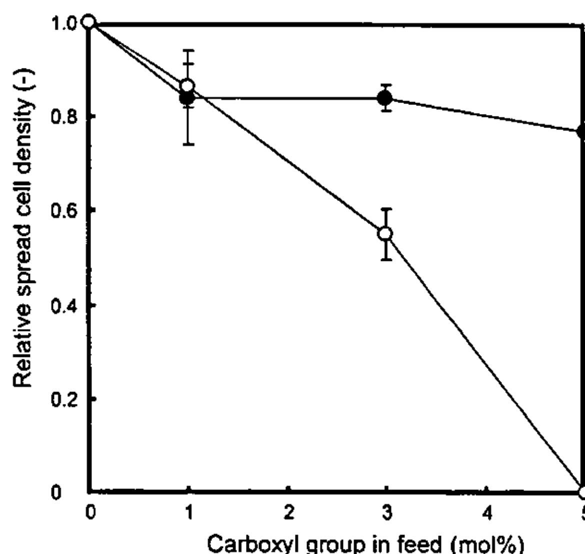


Fig. 4. Relative spread cell densities in culture. BAECs were seeded on IPAAm-based copolymer-grafted TCPS dishes and cultured for 2 days at 37 °C in serum-containing media (see text for details). P(IPAAm-*co*-CIPAAm) grafted surfaces: closed circles, P(IPAAm-*co*-CNPAAm) grafted surfaces: open circles.

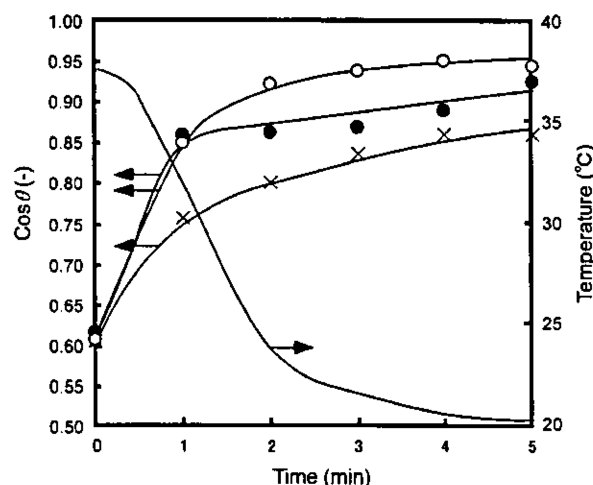


Fig. 5. Surface wettability changes for IPAAm-based grafted surfaces versus time and temperature. Advancing contact angles θ of all samples were measured 5 times with the sessile aqueous droplet technique and plotted as mean values±S.D. Pure PIPAAm: crosses, P(IPAAm-*co*-CIPAAm) grafted surfaces (CIPAAm 1 mol% in feed): closed circles, P(IPAAm-*co*-CNPAAm) grafted surfaces (CNPAAm 1 mol% in feed): open circles.

changes in their surface properties than PIPAAm-grafted surfaces, indicating carboxyl groups in the copolymer cause a rapid and excessive hydration of both types of polymer chains below their LCSTs. P(IPAAm-*co*-CNPAAm)-grafted surfaces were more hydrophilic (lower contact angle with water) than P(IPAAm-*co*-CIPAAm)-grafted surfaces at 20 °C. This could reflect the observed p*K*′a for P(IPAAm-*co*-CNPAAm) at 20 °C being lower than that for P(IPAAm-*co*-CIPAAm) (see Fig. 3) and is also consistent with the assertion that ionized carboxyl groups likely produce rapid and excess hydration of the grafted surface since the PIPAAm homopolymer grafted surface does not behave this way. Fig. 6 shows the detached cell to total cell ratios after lowering culture temperatures to 20 °C. Rapid cell detachment is observed on the surfaces grafted with P(IPAAm-*co*-CIPAAm) and P(IPAAm-*co*-CNPAAm). This result corresponds to the observed hydrophilicity on these surfaces at 20 °C and the rapid swelling kinetics for these charged polymer surfaces predicted from these data below the LCST.

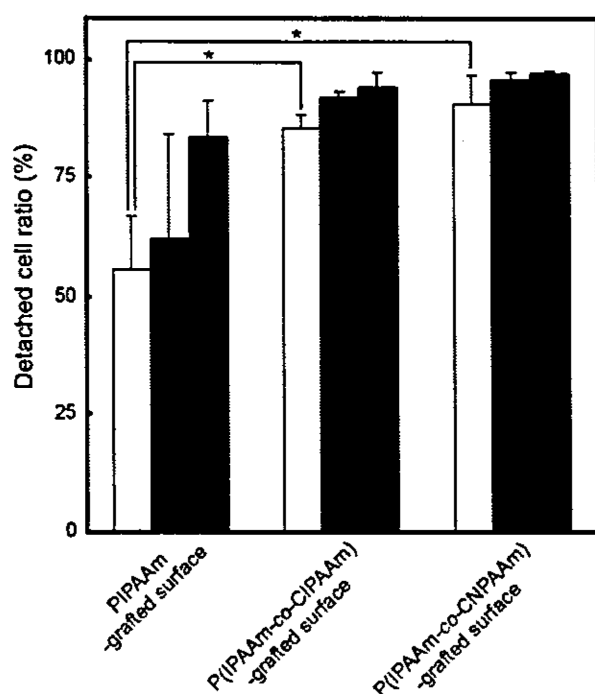


Fig. 6. Cell detachment upon reducing culture temperature to 20 °C. Percentage of detached cells to total cells prior to lowering temperature is plotted for grafted TCPS surfaces containing pure PIPAAm, P(IPAAm-*co*-CIPAAm) (1 mol% CIPAAm in feed), and P(IPAAm-*co*-CNPAAm) (1 mol% CNPAAm in feed). Remaining cell numbers counted after 30- (white), 60- (gray), and 90-min (black) incubations at 20 °C are shown. Statistically significant differences are indicated using Bonferroni test (*, $P<0.01$).

4. Conclusions

A newly synthesized and novel carboxylate-containing acrylamide monomer, CNPAAm, was characterized in thermally sensitive co-polymer grafted surfaces. Temperature-responsive behavior for both P(IPAAm-*co*-CNPAAm) and P(IPAAm-*co*-CIPAAm) were compared. The detected p*K*′a for P(IPAAm-*co*-CIPAAm) was considerably higher than that for P(IPAAm-*co*-CNPAAm) and other previously reported carboxylate co-polymers [14,23,24]. This reflects reduced or hindered carboxylate group dissociation in the P(IPAAm-*co*-CIPAAm) copolymer. Furthermore, temperature-dependent association/dissociation changes in copolymer carboxylate groups in P(IPAAm-*co*-CIPAAm) were smaller than those observed in P(IPAAm-*co*-CNPAAm). Slight structural differences between the two analogous monomers (*iso* or *normal* side group substitutions) are asserted to produce these significant differences in thermally induced phase transition behaviors of their IPAAm copolymers and are correlated with observed apparent p*K*′a values and contact angle results. Spread cell density on P(IPAAm-*co*-CNPAAm)-grafted surface above its LCST decreased with increasing CNPAAm content because of its LCST shift to higher temperature above 37 °C correlated with its lower apparent p*K*′a, higher hydrophilicity and resulting higher content of ionized carboxyl groups below the LCST. Because the LCST value for P(IPAAm-*co*-CIPAAm) is not altered significantly by CIPAAm content [23], spread cell density above 37 °C is not significantly affected by increasing temperature and is comparable to the PIPAAm-grafted surface for cell attachment and detachment performance. By contrast, incorporation of the carboxylate co-monomers into PIPAAm-grafted surfaces similarly accelerates cultured cell detachment below the respective polymer LCST.

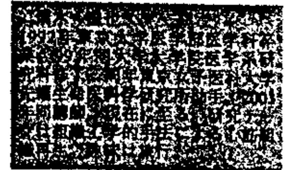
Acknowledgments

We appreciate the continued useful comments and technical criticism from Prof. D.W. Grainger (Colorado State University, USA). This work is supported in part by Grants-in-Aid for Scientific Research by Ministry of Education, Culture, Sports, Science and Technology of Japan. The present study is supported in part by Japan Ministry for Culture, Sports, Education, Science and Technology, Core Research for Evolutional Science and Technology (CREST).

References

[1] Y.H. Bae, T. Okano, S.W. Kim, J. Polym. Sci. Part B: Polym. Phys. 28 (1990) 923.
[2] M. Heskins, J.E. Guillet, J. Macromol. Sci. Chem. A2 (1968) 1441.
[3] H. Kanazawa, K. Yamamoto, Y. Matsushima, N. Takai, A. Kikuchi, Y. Sakurai, T. Okano, Anal. Chem. 68 (1996) 100.
[4] H. Feil, Y.H. Bae, J. Feijen, S.W. Kim, J. Membrane Sci. 64 (1991) 283.
[5] T. Okano, N. Yamada, H. Sakai, Y. Sakurai, J. Biomed. Mater. Res. 27 (1993) 1243.
[6] T. Shimizu, M. Yamato, Y. Isoi, T. Akutsu, T. Setomaru, K. Abe, A. Kikuchi, M. Umezu, T. Okano, Circ. Res. 90 (2002) e40.
[7] M. Yamato, M. Utsumi, A. Kushida, C. Konno, A. Kikuchi, T. Okano, Tissue Engng. 7 (2001) 473.
[8] M. Yamato, C. Konno, A. Kushida, M. Hirose, M. Utsumi, A. Kikuchi, T. Okano, Biomaterials 21 (2000) 981.
[9] K. Yoshizako, K. Akiyama, H. Yamanaka, Y. Shinohara, Y. Hasegawa, A. Kikuchi, T. Okano, Anal. Chem. 74 (2002) 4160.
[10] T. Yakushiji, K. Sakai, A. Kikuchi, T. Aoyagi, Y. Sakurai, T. Okano, Anal. Chem. 71 (1999) 1125.
[11] G. Chen, A.S. Hoffman, Nature 373 (1995) 49.
[12] G. Chen, A.S. Hoffman, Macromol. Chem. Phys. 196 (1995) 1251.
[13] H. Yu, D.W. Grainger, J. Appl. Polym. Sci. 49 (1993) 1553.
[14] H. Feil, Y.H. Bae, J. Feijan, S.W. Kim, Macromolecules 26 (1993) 2496.
[15] Y. Kaneko, S. Nakamura, K. Sakai, T. Aoyagi, A. Kikuchi, Y. Sakurai, T. Okano, Macromolecules 31 (1998) 6099.
[16] T. Aoyagi, M. Ebara, K. Sakai, Y. Sakurai, T. Okano, J. Biomater. Sci. Polymer Edn. 11 (2000) 101.
[17] M. Ebara, M. Yamato, M. Hirose, T. Aoyagi, A. Kikuchi, K. Sakai, T. Okano, Biomacromolecules 4 (2003) 344.
[18] A. Katchalsky, P. Spitnik, J. Polym. Sci. 2 (1947) 432.
[19] J.E. Chung, M. Yokoyama, K. Suzuki, T. Aoyagi, Y. Sakurai, T. Okano, J. Colloid. Surf. B: Biointerfaces 9 (1997) 37.
[20] F. Kohori, K. Sakai, T. Aoyagi, M. Yokoyama, Y. Sakurai, T. Okano, J. Control. Rel. 55 (1998) 87.
[21] K. Kalyanasundaram, J.K. Thomas, J. Am. Chem. Soc. 99 (1977) 2039.
[22] D.C. Dong, M.A. Winnik, Can. J. Chem. 62 (1984) 2560.
[23] H. Feil, Y.H. Bae, J. Feijan, S.W. Kim, Macromolecules 25 (1992) 5528.
[24] J. Kobayashi, A. Kikuchi, K. Sakai, T. Okano, J. Chromatogr. A 958 (2002) 109.

細胞シート工学を利用した組織再構築



■清水 達也・岡野 光夫
東京女子医科大学 先端生命医科学研究所

Key words：再生医療，組織工学，細胞シート

はじめに

近年，欠損部あるいは機能不全に陥った組織・臓器に対する新たな治療法として再生医療が注目を集め，下肢虚血に対する骨髄単核球細胞移植など細胞を使ったいくつかの治療法が臨床応用されるに至っている。再生医療には細胞を注射針などで不全部に注入する細胞移植療法と組織工学 (tissue engineering) 的手法により細胞から組織を再構築したうえで移植する方法がある。後者はこれまでの医学だけでは実現が困難であった研究領域であり工学的な技術との融合により急速に進歩しつつある。ここでは，組織工学を基盤とした再生医療の現状について概説し，当研究所が開発した独自の組織工学的手法『細胞シート工学』による種々の組織再構築に関する研究を紹介する。

1．組織工学 (tissue engineering)

組織工学は1993年，工学者であるLangerおよび外科医であるVacantiが提唱した学際的な学問である。細胞から組織を再構築するという研究は皮膚などに関してそれ以前より行われていたが，彼らはマウスの背中で耳を再生させたことにより組織工学を世界に知らしめた。組織の再生には細胞，細胞の足場となる細胞外マトリックス (ECM)，細胞の分化・増殖のためのサイトカインが必要であるとし，その足場として3次元の生体吸収性材料を用いた。細胞を3次元の支持体に播種・培養後，生体内に移植すると，生体吸収性の支持体が徐々に分解，細胞が産生するECMと置換され生体に類似した組織が再構築されるという手法である。組織工学において組織再生の足場として用いられる生体材料の多くは生体吸収性の高分子である。これには天然高分子と合成高分子がある。いずれも酵素分解あるいは加水分解によって高分子主鎖が切断され吸収される。天然高分子の中でよく利用されているのは生体のECMの主成分であるコラーゲンである。一方，合成高分子としては，ポリ乳

Cell sheet engineering for tissue reconstruction : Tatsuya Shimizu, Teruo Okano, Institute of Advanced Biomedical Engineering and science, Tokyo Women's Medical University

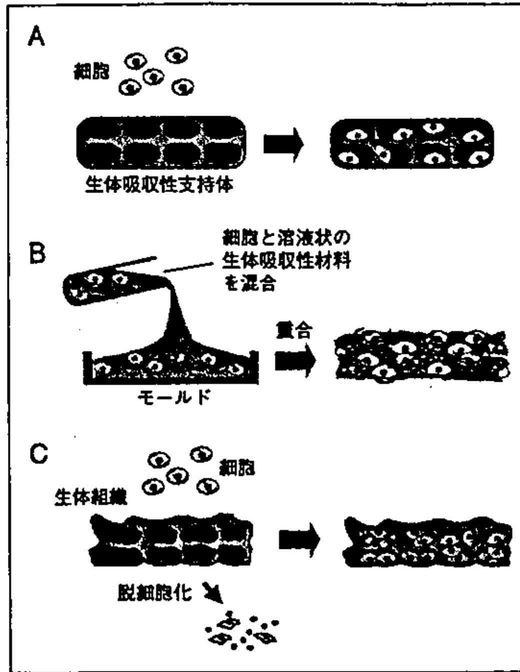


図1　生体吸収性支持体を用いた組織工学的手法
A. 生体吸収性高分子からなる支持体を作成し，それを足場として細胞を播種する手法。
B. 溶液状の支持材料と細胞を混合したのち重合する手法。
C. 生体由来の組織を脱細胞化したのちにそれを支持体として細胞を播種する方法。

酸（PLA），ポリグリコール酸（PGA），およびそれらの共重合体が最も盛んに使用されている。また，生体由来の組織が支持体として用いられる場合もある。具体的な方法としては①生体吸収性高分子からなる多孔性の3次元支持体を作製，それを足場として細胞を播種する方法（図1 A），②溶液状の生体吸収性材料と細胞を混合したのちモールドに流し込み，重合させることで細胞を3次元化する方法（図1 B），③生体組織を界面活性剤やマイクロウェーブを用いて脱細胞化した結合組織を支持体として細胞を播種し培養する方法（図1 C）がある。それぞれ支持体は生体が産生するECMと置換されて組織が再生される。

これら生体吸収性の3次元生体材料を細胞の足場として用いた組織工学の研究は殆どすべての組織に対して行われている。既に組織工学的に製造された軟骨・皮膚は商品化されており，また血管に関しても臨床応用されている。

2．細胞シート工学による組織再構築

生体吸収性の支持体を用いる組織工学的手法においては支持体内へ十分な細胞を播種することが困難なことやまた移植後支持体の分解に伴い炎症反応が生じることが問題となっている。また骨，軟骨，血管，弁など細胞が疎な組織の作製には適しているが心筋，肝臓など細胞の密な組織を作製するには新たな技術開発が必要となっている。そこで我々は最初から3次元的な足場を整えることにより組織を再生するという既存のコンセプトに対し，温度変化のみで細胞の接着・脱着を制御できる培養基材を用い，細胞をシート状に回収，この細胞シートを一つの組織として移植したり，積層化により3次元組織を再構築したうえで移植するという独自のコンセプト「細胞シート工学」を提唱し新たな治療法の確立に取り組んでいる。

この培養基材は通常のポリスチレン培養皿上に温度応答性高分子であるポリ（*N*-イソプロピルアクリルアミド）を電子線を用いて薄く（数十μm）共有結合させたもので，通常の培養温度である37℃では疎水性表面となり細胞接着性であるが，32℃以下では親水性表面に変化し細胞非接着性となる。この培養皿の使用により，接着した細胞をトリプシンやディスパーゼなどの蛋白分解酵素を用いること

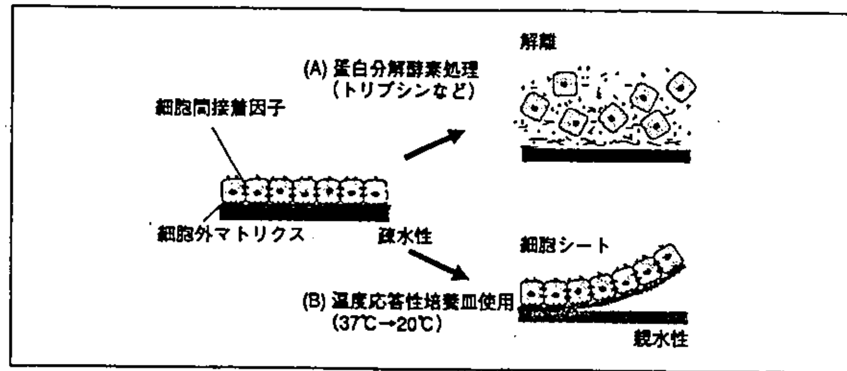


図2　温度応答性培養皿からの細胞シートの脱着

細胞を密に培養した場合は細胞と細胞が細胞間接着因子により互いに接着する。(A) 蛋白分解酵素を用いた場合は細胞と培養皿の接着が解離するとともに，細胞間接着も破壊されるため，それぞれの細胞は解離して浮遊することになる。(B) これに対し，温度応答性培養皿を使った低温処理においてはこの細胞間接着は全く影響を受けずに，シート下面のECMと培養皿表面の接着のみ解離するため細胞がシート状に脱着する。

なく脱着させることが可能である。さらに，細胞を密に培養し細胞が互いに接着した状態では，温度を降下させることにより細胞がその下面の接着因子・ECMとともに培養皿から脱着するものの，細胞間の結合は全く解離せず維持されるため蛋白分解酵素を用いたときのように細胞をばらばらにすることなく，シート状に回収できる（図2）。また細胞シート下面の接着因子が新たな培養基材や別の細胞シート上への移動時に糊の役目を果たすため速やかな接着・積層化が可能である。既にこの培養皿を用い，種々の細胞シートの回収が可能となっている。

細胞シートの再生医療への応用としては①単層シートの移植 ②同一細胞シートの積層化により構築した均一な組織の移植 ③数種の細胞シートの積層化により構築した層状構造を呈する組織の移植がある（図3）。それぞれ対象組織・臓器として ①角膜，膀胱（上皮），歯周組織，②心筋，③肝臓などが挙げられる。以下に，それぞれについてのこれまでの研究成果を示す。

3．単層シート

(1) 角　膜

角膜移植に関しては，ドナー角膜の不足が問題となっており，組織工学的手法による角膜再生が追求されてきた。角膜は組織構造が比較的単純な層構造を呈し，細胞シートによる再生医療のもっとも良い応用例の一つである。我々は，自己の健常側角膜輪部より採取した角膜上皮の幹細胞を温度応答性培養表面上で培養して細胞シートを作製，これを損傷した病変角膜部に移植する治療法を開発した。さらに，両眼性疾患に対しては，自己の口腔粘膜から採取した細胞を用いて細胞シートを作製し移植する方法も確立した。既に本法のヒトへの臨床応用を開始，視力の回復を確認している（大阪大学眼科との共同研究）。

(2) 膀　胱

現在，膀胱機能不全や悪性腫瘍などによる膀胱の欠損に対しては自己の消化管を使用した再建が行われているが，消化管粘膜の残存

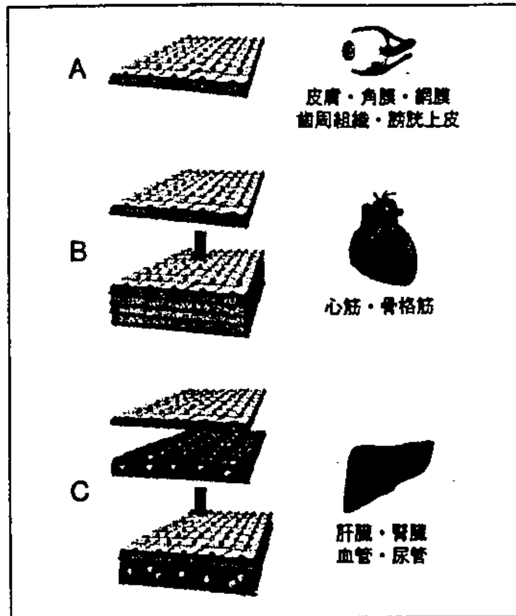


図3 細胞シートの再生医療への応用
温度応答性培養皿から回収した細胞シートの再生医療への応用として3つのコンセプトがある。単層シートの移植(A)。同一の細胞シートを重層化した均一な組織の移植(B)。異なる細胞シートの重層化による層状組織の移植(C)。

に起因する電解質異常，結石の形成などの合併症が大きな問題となっている。これらの問題を解決するために尿路上皮細胞シートを作製し，消化管粘膜を切除した消化管平滑筋層上に移植した。尿路上皮シートは消化管平滑筋層上に生着し，膀胱組織と同様の尿路上皮組織層が再生された。この粘膜置換型フラップは従来の手術方法に細胞シート技術を融合した新規の手法であり，早期の臨床応用が期待されている。

(3) 歯周組織

歯周病は，成人の8割以上が思っているといわれており，歯根膜を中心とする歯周組織の炎症により，放置すれば歯の脱落を招く。従来の治療法では失われた歯周組織は再生することなく，高齢化社会におけるQOL (Quality of Life) の低下の大きな要因といえる。そこで，我々は歯根膜組織由来細胞シートを歯周組織欠損部位へ移植することで，きわめて効果的に歯根組織膜が再生することを明らかとした(東京医科歯科大学歯学部との共同研究)。この技術は歯周組織の再生治療や歯根膜を持った次世代の人工歯根の開発に大きく貢献するものと考えている。

4．同一細胞シートの積層化

心 筋

心筋組織に対する再生医療としては，心筋細胞の代替として自己の筋芽細胞や骨髄由来の細胞を用いた浮遊液注入による細胞移植治療が既に臨床応用されている。一方，組織工学的なアプローチとしてコラーゲンゲル，ポリ乳酸，アルギン酸あるいはゼラチンからなる3次元の支持体を用いた心筋細胞の3次元培養の研究が報告されている。しかし，3次元支持体を用い心筋細胞培養を行うことは細胞の密な接着や自由な収縮弛緩の妨げになると考えられる。そこで我々は心筋細胞シートを重層化することで支持体を用いない心筋組織の再生を追求してきた。重層化した心筋細胞シート間には電気的にも形態的にも結合が生じ，組織全体が同期して拍動することが確認され，*in vitro*で4枚まで積層化したところ肉眼レベルで拍動する心筋組織が構築された。さらにヌードラット背部皮下組織への移植実験を行ったところホストの心電図とは異なるグラフト由来の電位が確認された。また心筋グラフトの肉眼レベルでの拍動が確認さ

れるとともに組織切片上，多数の新生血管を認め，心筋様組織が再生されていた。既に移植後1年まで，心筋グラフトが拍動を維持したまま生着しうることを確認している。さらに重層化心筋細胞シートの心筋梗塞モデルへの移植実験により心機能が改善することが確認されている（大阪大学臓器機能制御外科との共同研究）。将来，幹細胞生物学の発展によりヒトに移植可能な心筋細胞の分化誘導法が確立されれば体外で再生した心筋組織を不全心に移植することも可能となることが予測される。

5．異なる細胞シートの積層化

肝　臓

肝臓は生体内である程度の再生能をもつものの，その形態・機能の複雑さ故に組織工学的手法での再生が困難な組織のひとつである。肝組織は肝実質細胞と血管内皮細胞が層状に配列し，生体内における合成，代謝を効率的に行っている。肝実質細胞は，通常の培養環境ではその分化機能を著しく低下させ，長期培養も困難であることが知られている。そこで肝細胞シートと内皮細胞シートの重層化共培養を行ったところ，アルブミン合成能を維持した2ヶ月以上にわたる肝細胞の長期培養が可能となっている。これは，肝細胞と内皮細胞との層状の接着により生体に似た環境を再現し，細胞相互間のコミュニケーションを可能にしたことによるものと考えられる。この手法を基盤として，3次元的な肝組織を再生・移植するための技術開発を行っている。

以上に挙げた組織・臓器の他，皮膚・網膜・尿管・血管・腎臓などに関しても細胞シートの技術を使った研究を進めている。

おわりに

再生医療は今まで治療が困難であった疾患に対する新たな治療法として期待され，種々のアプローチで開発が行われ，研究者人口も増大している。その中で温度応答性培養皿およびそれを使った細胞シート工学は既存の培養技術では不可能であったシート状の細胞の回収・移動・重層化を実現し世界的にも注目されており，再生医療分野をさらに発展させる新技術として貢献するものと考える。

<BIO Information>

第18回 日本臨床内科医学会 in 岡山のお知らせ

会　期：2004年9月19日（日）～20日（月・祝）
会　場：岡山衛生会館・おかやま三光荘・岡山プラザホテル
テーマ：21世紀の理想の医療を目指して
～良質な医療提供とかかりつけ医の役割～
会　長：亀山一郎（亀山内科院長）

主なプログラム：基調講演　医療保険制度と診療報酬体系（講師　遠藤久夫・学習院大学経済学部教授）
インフルエンザの展望（講師　柏木征三郎・福岡日赤血液センター所長）
良質な医療提供とかかりつけ医の役割（講師　伴信太郎・名古屋大学医学部教授）

教育講演／ワークショップ／シンポジウム／市民公開講座　他

Regeneration of Tissues and Organs
— New Technique Opens Up New Possibilities for Regenerative Medicine through Control of Interaction of Polymers with Water —

Institute of Advanced Biomedical Engineering and Science,
COE Program for 21st Century, Tokyo Women's Medical University
Associate Professor
KIKUCHI Akihiko

Institute of Advanced Biomedical Engineering and Science,
COE Program for 21st Century, Tokyo Women's Medical University
Professor
OKANO Teruo

1 Introduction

Human bodies consist of approximately 60 trillion cells. Such a huge number of cells plays an important role in the formation of the body's tissues and organs. These tissues and organs have a close relationship with each other and express cooperatively various essential functions within the body. When this equilibrium is disturbed, the body becomes sick and treatment is necessary. Although it depends on the particular disease concerned, in some cases treatment can be accomplished by taking medicine alone. In other cases, however, disease can lead to organ dysfunction, which can afflict a person throughout their entire life. In such cases, artificial organs are used to replace damaged ones.

Up until now, much research has been carried out both within Japan and overseas and although artificial organs have been developed that are now in practical use, at present they can only replace some of the functions of the body's organs. Since the 1990s, the concept of tissue engineering has been presented as a development technique for artificial organs as it is more similar to biological organs.[1] Now, tissue engineering research is being actively carried out in numerous places throughout the world with regard to various tissues and organs.[2] In some of the early work in tissue engineering, materials such as poly(lactic acid) (PLA) and poly(lactic-*co*-glycolic acid) (PLGA), which are biodegradable materials, with porous structure, were used to grown target cells. While the cells on these materials divide and proliferate to form tissue, the material itself gradually biodegrades within the living organism to eventually leave only the regenerated tissue. This method is being used to regenerate tissue in the ears and nose, bone and cartilage in the digits, and for the regeneration of other tissue.

In our laboratory we have not been using the technique of transplanting grown cells on biodegradable materials into living organisms, but rather have been taking the cells and tissues that have been grown in newly developed culture dishes and transplanting them directly in single or multiple layers for use in regenerative medicine-related research and clinical applications. In this article we will offer an explanation of the nature and applications of the thermoresponsive surface that we have developed.

2 Surfaces that Change Properties in Response to Changes in Temperature

Since the 1980s, we have been interested in poly(*N*-isopropylacrylamide), hereafter referred to as, "PIPAAm" (**Fig. 1**) and have been investigating its application as matrices for the drug delivery systems (DDS)[3),4)] and the development of new polymer-enzyme bioconjugates to control enzymatic reactions with thermal stimuli.[5),6)] Surfaces modified with PIPAAm exhibit temperature responsive changes in water: that is hydrophilic behavior at low temperatures and become hydrophobic above 32°C in water,

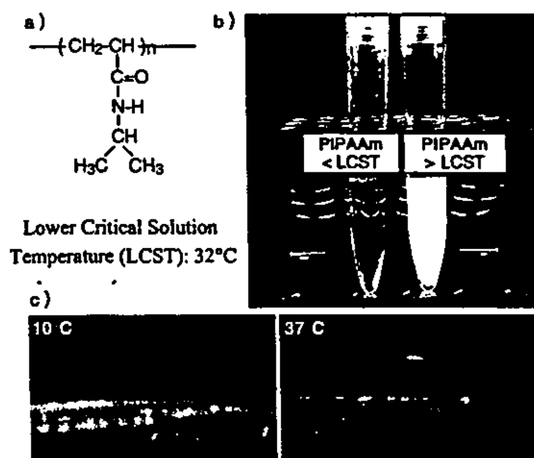


Fig. 1 Characteristics of thermoresponsive poly (*N*-isopropylacrylamide) (PIPAAm)
The chemical structure (a), changes in water solubility in response to temperature changes (b) of PIPAAm, and thermoresponsive wettability changes of PIPAAm-grafted surfaces

showing that it has thermoresponsive characteristics (Fig. 1c).[7), 8)]

In order to prepare PIPAAm-modified surfaces you can either use amide bonding between PIPAAm containing carboxyl groups at one chain end and amino groups generated on the surfaces or expose to an electron beam immediately after casting them with IPAAm monomer solution. During the electron beam irradiation, polymerization of the IPAAm monomer and fixation to the surfaces occur simultaneously.[9)] With either method, an ultra-thin layer of PIPAAm grafts is formed on the surface of the material. We ablated the PIPAAm modified surfaces with a low-energy UV excimer laser, peeled back the PIPAAm layer until the original surface of the material was exposed by the absorption of a hydrophobic fluorescent dye. These surfaces were then analyzed with time-of-flight secondary ion mass spectrometry (ToF-SIMS) so that we verified the exposure of the surface of the material. By scanning the ablated area using an atomic force microscope (AFM) we confirmed that the thickness of the surface grafted layer was approximately 20nm (article in press). The amount of the surface grafted polymers was determined using attenuated total reflectance Fourier transform infrared spectroscopy (ATR/FTIR) and found to be approximately 2μg/cm^2, which is very similar to the film thickness as calculated when the specific gravity of PIPAAm is 1. In either case, this would be like stretching a thin membrane with the thickness of a single human hair over the entire width of a baseball stadium and having an ultra thin layer formed on the surfaces of the polymeric base materials.

The PIPAAm modified surfaces respond to changes in environmental temperature due to the thermoresponsive property changes in the PIPAAm grafted molecules, exhibiting hydrophilic behavior at low temperatures while at 32°C the water contact angle changes discontinuously and at high temperatures dehydration of the PIPAAm chains occurs and the surface exhibits hydrophobic behavior (see Fig.1c).

3 Separating Molecules

We have been carrying out joint research together with Prof. Hideko KANAZAWA and her colleagues of Kyoritsu College of Pharmacy, covalent grafting thermoresponsive polymer layers on silica beads in the same way as described above and then placing them in a stainless steel column to create a new chromatography system using only a water as mobile phase.[10)~12)] Using this column we were able to control surface hydrophilic/hydrophobic characteristics with temperature, and separate hydrophobic compounds in a mixture of various steroid hormones through the modulation of hydrophobic interaction between column matrix surfaces and steroid hormones with different hydrophobicities (Fig.2). More hydrophobic steroids showed longer retention times, thus a mixture of steroids were successfully separated. Furthermore, we discovered that it is possible to dynamically control column temperature during elution to achieve gradient elution. In other words, after separating solutes with small degree of

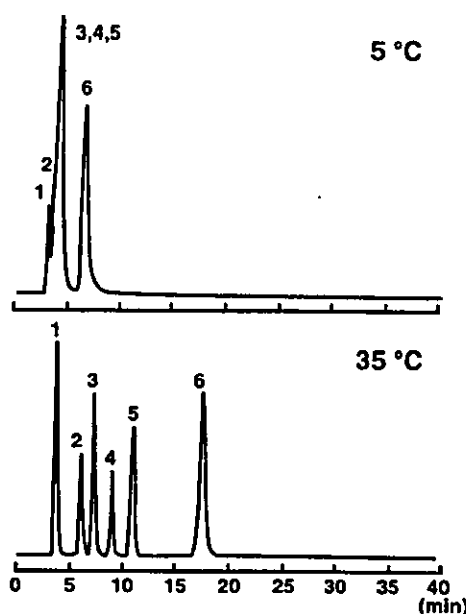


Fig. 2 Changes in the retention behavior of steroids and benzene at various temperatures on a thermoresponsive column. (Eluent: water)

Peaks: 1. Benzene; 2. Hydrocortisone; 3. Prednisolone; 4. Dexamethasone; 5. Hydrocortisone Acetate; 6. Testosterone.

hydrophobicities at high temperature, we lowered the temperature of the column and accelerated the elution by weakening the interaction between the solutes with the high degree of hydrophobicity and the column matrices to achieve a high efficiency separation within a short period of time and realize separation based on the stepwise temperature gradient. It became clear that for polypeptides with molecular weights of approximately 3,000, by simply controlling the hydrophobic interaction we were able to realize separation in an aqueous environment.[11)] Considering how important it is to be able to maintain biological activity and a high survival rate after separation — especially when separating protein and cells — as with this method separation can be carried out only in a water-based eluent it would be highly suitable as it satisfies those conditions. The column packed with octadecyl silica beads are widely used for separation in a mixture eluent of water and organic solvents and recognized as one of the powerful tool for analyses. However, the reversed-phase chromatography cannot be utilized for separation of proteins and cells. Therefore, it became clear that the newly developed thermoresponsive columns could be used in place of the reversed-phase chromatography for carrying out separation.

By introducing weak acid or base groups to the thermoresponsive polymers as the column matrix modifiers, bioactive molecules with charged, and hydrophobic characteristics, can be separated.[13), 14)] Angiotensin peptides consist of three subtypes having similar molecular weights but distinct biological activities, and are conventionally separated by combining a number of stages of

reversed-phase chromatography and ion exchange chromatography. With newly developed charged,thermoresponsive column, those angiotensin peptides were effectively separated.

We have considered that the thermoresponsive polymer chain extension and aggregation through temperature dependent hydration/dehydration changes of PIPAAm molecules attached on the silica matrixes should be utilized to modulate ligand-receptor affinity control. Thus at high temperature, target molecules with receptor sequences can easily be interacted with surface immobilized ligand molecules because the co-grafted PIPAAm molecules are in dehydrated, and aggregated conformation. By lowering temperature, then hydration and expansion of PIPAAm molecules interfere the affinity binding of target molecules and surface immobilized ligand molecules, and eventually forced-releasing the substances without the addition of binding inhibitor molecules.[15), 16)]

We have thus been continuing the researches on the development of novel chromatography matrices with thermoresponsive PIPAAm and its derivatives as modifiers to realize new systems for effective separation of bioactive substances while maintaining their structure and functions using an aqueous mobile phase.

4 Creating Tissues from Cells

Let us now return to the subject of regenerative medical researches utilizing the thermoresponsive culture dishes for tissue formation. Variety types of the cells that have been seeded on thermoresponsive culture dishes are adhered, divided, and proliferated all over the surfaces of the culture dishes in the same way as with commercially available tissue culture polystyrene dishes (TCPS).[9)] With the cells on TCPS, digestive trypsin treatment is carried out to both dissolve the extracellular matrix (ECM) proteins and to chelate and remove the calcium ions. Without trypsin treatment, one cannot recover the cells from the culture dishes. Alternatively, a scraper can be used to physically peel them off the dish surfaces. With trypsin treatment, each of the individual cells is recovered as in suspension. When a scraper is used the cells can only be recovered as irregularly shaped and unstable tissue fragments. With thermoresponsive PIPAAm modified culture dishes, however, the cells are detached and can be recovered fairly easily in noninvasive fashion only by reducing the temperature from the culture temperature of 37°C to 20°C, the temperature at which the PIPAAm hydrates. Not only do the cells retain their intercellular connections, but the ECM proteins on the bottom of the cells is also released as a monolayer of contiguous tissue.[17), 18)] Furthermore, ECM proteins also act as an "adhesive" when transferring the cell sheets to a new culture dish or when forming multiple layers of tissue. For successful cell culture and recovery of the cell monolayers from the thermoresponsive surfaces, the previously mentioned PIPAAm graft amounts (or thicknesses) are extremely important and with a grafting density of 1.5~2.0μg/cm^2, at 37°C the cells adhere and can then be spontaneously recovered once the temperature drops below the transition temperature of PIPAAm molecules. At grafting densities of any less than this range, the cells will adhere and proliferate, but these cells will not be remained adhered even through the culture temperature was decreased below 32°C. Conversely, if the grafting density exceeds 2.0μg/cm^2 then the seeded cells will not adhere at all even at 37°C where the surface grafted PIPAAm molecules are in dehydrated and aggregated conformation. It is no exaggeration to say that this is the key to surface design that achieves cell adhesion and detachment control accompanying changes in temperature. With hepatic parenchymal cells and glia cells, irreversible cell damage occurs as a result of trypsin treatment, resulting in a major decrease in survival rate and cell function. On the other hand, it is of worth noticing that no such cell damage occurs when tissue is detached and recovered from the thermoresponsive culture dishes using low temperature processing. Furthermore, it has also been discovered that apoptosis does not occur during low temperature processing (from a few minutes through to approximately 2 hours at 20°C) with this method.[19)]

Considering "cell sheets" manufactured in this way as a single unit of tissue formation, using the strategy shown in **Fig. 3**, we are also investigating single layer tissue, multiply stratified tissue formed from the same cells and multiple layer tissue formed from more than 2 different kinds of cell sheets.

Single Layer Tissue: Epidermis cell sheets and corneal epithelial cell sheets are already being provided for transplantation immediately after recovery from culture dishes using low temperature processing in clinical applications, such as for treating burns and alkali burns. Usually, with corneal transplants only one cornea can be removed from each donated eyeball and stitches are necessary for transplantation of corneal tissues. By contrast, with transplants using corneal epithelium cell sheets, only a few square millimeters of limbal tissue are required to produce a single corneal epithelial cell sheet. Furthermore, as ECM proteins exist

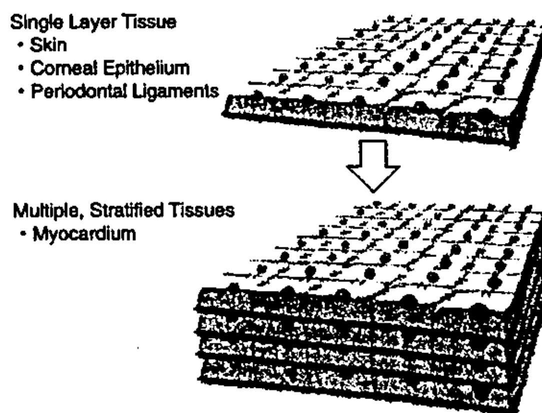


Fig. 3 Schematic drawings of the new concept of regenerative medicine to construct monolayer tissue and stratified tissues based on "cell sheet engineering" using thermoresponsive culture dishes

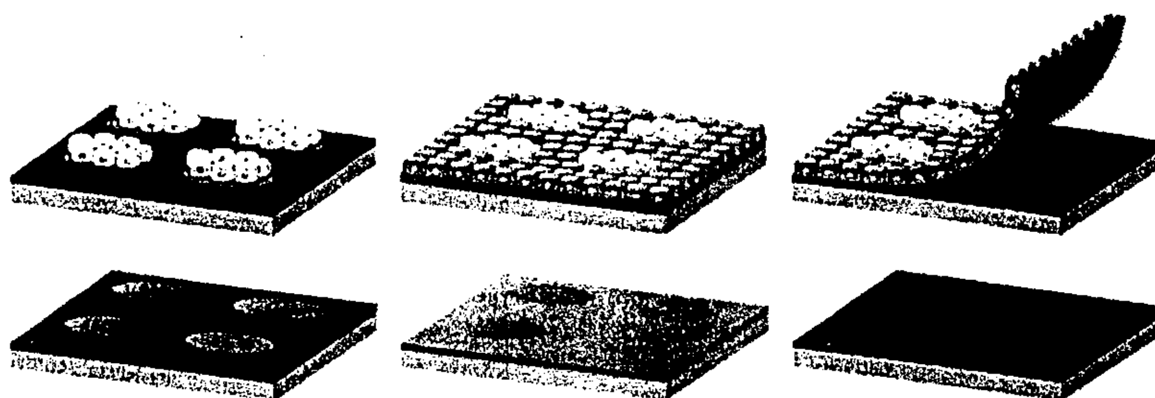


Fig. 4 Patterned dual thermoresponsive culture dishes for patterned co-culture of heterotypic cell types on one-dish surfaces

Two types of thermoresponsive polymers with different transition temperatures are fixed in an organized pattern. Cells are selectively attached to the hydrophobized domains at a temperature between the transition temperatures of the two polymers (left). Next, another cell type is seeded at a temperature higher than the transition temperature of both domains (center). Finally, a single co-cultured cell sheet is released and recovered at a lower temperature than either of the transition temperatures (right).

on the basal side of the cell sheets, it only takes about five minutes to complete the transplantation process without any stitches.[20] In addition, with cell sheet transplantation, as intercellular connection is maintained, this acts as a highly effective barrier immediately after transplantation.

Periodontal disease, such as alveolar pyorrhea, is a major problem in an aging society. At present, it is extremely difficult to regenerate gum tissue, meaning that the reliance is still on symptomatic treatment. We cultured gum tissue cells known as periodontal ligaments and made them into a single cell sheet. When this was transplanted to the gums, favorable regeneration was achieved with no periodontal pockets being formed.

Multiple Layer Tissue: We are considering the regeneration of the myocardiac tissue as an innovative treatment for heart attack victims. It is already possible to culture myocardial cells that have the ability to beat by themselves in synchronized fashion. If single cell sheets of these cells are formed into multiple layers, it is possible to regenerate heart tissue-like constructs to such a degree that one can see the cell sheets beating with the naked eye. Gap junctions are formed between multiple layers of myocardial cell sheets and we confirmed that the heart beat of multiple cell sheets was completely synchronized.[21),22]

When multilayered myocardiac cell sheets were transplanted onto the surfaces of the heart as a cardiac muscle patch, it started to beat in time with the heart of the host rat with myocardial infarction. Furhtermore, a remarkable improvement in heart function is achieved with tansplanted double-layered myocardial tissues.

This is sharp contrast with tissue transplantation techniques that have so far been investigated, such as injecting cardiac muscle cells in suspension, the proposed myocardial tissue transplantation may be useful as a new form of treatment with higher success rate and without cell rejection by the host.

Stomach and intestine tissue are currently used for the regeneration of the bladder in clinical situation, however, complications arise due to the secreting characteristics of epithelium of such tissue. However, when a bladder epithelium cell sheet was transplanted on to stomach tissue flaps from which the epithelium had been removed, none of the above-mentioned complications arose and the cells regenerated into tissue that is very similar to actual bladder tissue.[23]

Cell Patterning and Co-Cultured Cell Tissue: Electron beams used in the surface fixation of PIPAAm can easily be shut out using an extremely thin film of plastic or glass cover slips. Using such materials we fixed the PIPAAm molecules in a specific pattern and succeeded in culturing two different kinds of cells by controlling the culture temperature.[24),25] This kind of co-culture does not occur simply by seeding two different kinds of cells at the same time but by first seeding and culturing one kind of cell on a specific area and then seeding the second cell types. Applying such co-culture systems, we prepared patterned dual thermoresponsive surfaces with two kinds of thermoresponsive domains with different transition temperatures by copolymerization of a hydrophobic monomer to PIPAAm.[26] Co-cultured tissue was thus formed by culture each cell types in selective domains through temperature control and the kind of cells seeded (see **Fig. 4**, article accepted for publication). The co-cultured tissue was recovered into a single sheet at low temperature below the transition temperature of both domains.

It was noteworthy that the patterned co-cultured hepatic parenchymal cells showed significant functional increments in production of more albumin and ammonia metabolism to release urea as compared with those of monoculture hepatocytes.

This was made clear for the first time when we used patterned thermoresponsive culture dishes and we are currently analyzing this matter in more detail.

5 Conclusion

In this article we have provided an overview of part of our approach to tissue regeneration and the formulation of a separation system using a matrices modified with thermoresponsive polymers. Those thermoresponsive polymers greatly change their interaction with water in response to changes in temperature. The proposed new tissue regeneration technique, achieved by controlling both the graft densities and amounts of the thermoresponsive polymers, and the chromatography system that achieves high efficiency separation only in an aqueous environment, will become increasingly more important. Currently we are leading further researches aimed at achieving even further development. Although we have developed a new technique for activating cell function and carrying out tissue formation by utilizing polymer modification technology to achieve strict control on the nanometer level of polymer grafting and immobilization of bioactive sequences, we have decided to provide an explanation of such matters at a different time.

Researches we have introduced in this article is the results of the extensive research works of a large number of dedicated staff at this research institute, including Associate Prof. Masayuki YAMATO and Assistant Prof. Tatsuya SHIMIZU. The achievements mentioned in this article are the result of joint research with Prof. Motohiro NOZAKI and Research Assistant Prof. Kazutaka SOEJIMA of Plastic Surgery Department, Tokyo Women's Medical University regarding skin transplantation, Prof. Yasuo TANO and Assistant Prof. Koji NISHIDA of Osaka University regarding corneal grafting, Dr. Masaki HASEGAWA and Prof. Atsushi ISHIKAWA of Faculty of Dentistry, Tokyo Medical and Dental University for periodontal tissue regeneration, Dr. Shigeru MIYAGAWA, Associate Prof. Yoshiki SAWA and Prof. Hikaru MATSUDA of School of Medicine, Osaka University for cardiomyocyte sheet transplantation, and Dr. Yoshiyuki SHIRAYANAGI and Prof. Hiroshi TOMA of the Urology Department of Tokyo Women's Medical University for bladder regeneration, to all of whom we are exceptionally thankful.

[References]

1) Langer, R.; Vacanti, J. P. Science 1993, 260, 920-926.
2) Vacanti, J. P.; Vacanti, C. A. In Principles of Tissue Engineering; Lanza, R. P.; Langer, R.; Vacanti, J., Eds.; Academic Press: San Diego, 2000, p 3-7.
3) Okano, T.; Bae, Y. H.; Jacobs, H.; Kim, S. W. J. Control. Rel. 1990, 11, 255-265.
4) Okano, T.; Kikuchi, A.; Sakurai, Y.; Takei, Y.; Ogata, N. J. Control. Rel. 1994, 36, 125-133.
5) Matsukata, M.; Takei, Y.; Aoki, T.; Sanui, K.; Ogata, N.; Sakurai, Y.; Okano, T. The J. Biochem.: Biotechnol. 1994, 116, 682-686.
6) Matsukata, M.; Aoki, T.; Sanui, K.; Ogata, N.; Kikuchi, A.; Sakurai, Y.; Okano, T. Bioconjugate Chem. 1996, 7, 96-101.
7) Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Sakurai, Y.; Okano, T. Macromolecules 1994, 27, 6163-6166.
8) Yakushiji, T.; Sakai, K.; Kikuchi, A.; Aoyagi, T.; Sakurai, Y.; Okano, T. Langmuir 1998, 14, 4657-4662.
9) Yamada, N.; Okano, T.; Sakai, H.; Karikusa, F.; Sawasaki, Y.; Sakurai, Y. Makromol. Chem., Rapid Commun. 1990, 11, 571-576.
10) Kanazawa, H.; Yamamoto, K.; Matsushima, Y.; Takai, N.; Kikuchi, A.; Sakurai, Y.; Okano, T. Anal. Chem.1996, 68, 100-105.
11) Kanazawa, H.; Kashiwase, Y.; Yamamoto, K.; Matsushima, Y.; Kikuchi, A.; Sakurai, Y.; Okano, T. Anal. Chem. 1997, 69, 823-830.
12) Kanazawa, H.; Sunamoto, T.; Matsushima, Y.; Kikuchi, A.; Okano, T. Anal. Chem.2000, 72, 5961-5966.
13) Kobayashi, J.; Kikuchi, A.; Sakai, K.; Okano, T. J. Chromatogr. A 2002, 958, 109-119.
14) Kobayashi, J.; Kikuchi, A.; Sakai, K.; Okano, T. Anal. Chem. 2003, 73, 2027-2033.
15) Yoshizako, K.; Akiyama, Y.; Yamanaka, H.; Shinohara, Y.; Hasegawa, Y.; Carredano, E.; Kikuchi, A.; Okano, T. Anal.Chem. 2002, 74, 4160-4166.
16) Yamanaka, H.; Yoshizako, K.; Akiyama, Y.; Sota, H.; Hasegawa, Y.; Shinohara, Y.; Kikuchi, A.; Okano, T. Anal. Chem 2003, 75, 1658-1663.
17) Kikuchi, A.; Okuhara, M.; Karikusa, F.; Sakurai, Y.; Okano, T. J. Biomater. Sc., Polym. Ed. 1998, 9, 1331-1348.
18) Hirose, M.; Kwon, O. H.; Yamato, M.; Kikuchi, A.; Okano, T. Biomacromolecules 2000, 1, 377-381.
19) Kushida, A.; Yamato, M.; Konno, C.; Kikuchi, A.; Sakurai, Y.; Okano, T. J. Biomed. Mater. Res. 2000, 51, 216-223.
20) Nishida, K.; Yamato, M.; Hayashida, Y.; Watanabe, K.; Isoi, Y.; Maeda, N.; Watanabe, H.; Nagai, S.; Kikuchi, A.; Tano, Y.; Okano, T. Transplantation 2004, 77, 379-385.
21) Shimizu, T.; Yamato, M.; Kikuchi, A.; Okano, T. Tissue Eng. 2001, 7, 141-151.
22) Shimizu, T.; Yamato, M.; Isoi, Y.; Akutsu, T.; Setomaru, T.; Abe, K.; Kikuchi, A.; Umezu, M.; Okano, T. Circul. Res. 2002, 90, e40-e48.
23) Shiroyanagi, Y.; Yamato, M.; Yamazaki, Y.; Toma, H.; Okano, T. Tissue Eng. 2003, 9, 1005-1012.
24) Yamato, M.; Kwon, O. H.; Hirose, M.; Kikuchi, A.; Okano, T. J. Biomed. Mater. Res. 2001, 55, 137-140.
25) Yamato, M.; Konno, C.; Koike, S.; Isoi, Y.; Shimizu, T.; Kikuchi, A.; Makino, K.; Okano, T. J. Biomed. Mater. Res. 2003, 67A, 1065-1071.
26) Tsuda, Y.; Kikuchi, A.; Yamato, M.; Sakurai, Y.; Umezu, M.; Okano, T. J. Biomed. Mater. Res. 2004, 69A, 70-78.

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT•

Journal of Controlled Release 101 (2005) 69–84

journal of
controlled
release

www.elsevier.com/locate/jconrel

Nanostructured designs of biomedical materials: applications of cell sheet engineering to functional regenerative tissues and organs

Akihiko Kikuchi*, Teruo Okano

Institute of Advanced Biomedical Engineering and Science, Center of Excellence (COE) Program for the 21st Century, Tokyo Women's Medical University, 8-1 Kawadacho, Shinjuku, Tokyo 162-8666, Japan

Received 5 July 2004; accepted 30 August 2004

Abstract

Biomaterials surface design is critical for control of cell–materials interactions. Materials surface characteristics important to cell–materials interactions are the following: (a) nonfouling surfaces where cells cannot interact; (b) surfaces that interact with cells but do not alter cell morphology or metabolism (passive adhesion processes); and (c) surfaces that strongly interact with cells and cell–surface receptors to alter cell shape after metabolic interactions (active adhesion). In this paper, we briefly discuss the relationship between materials surface characteristics and cells for biomaterials designs in these categories. We have extensively investigated the thermoresponsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), as grafted surfaces allowing recovery of confluent cell monolayers as contiguous living cell sheets for tissue engineering applications. Cellular interactions with PIPAAm-grafted surfaces can be regulated vertically using the thickness of the PIPAAm-grafted layers in nanometer-scale levels, as well as laterally (spatially) using nano-patterned PIPAAm chemistry on various other surface chemistries. PIPAAm-grafted surfaces with 15–20-nm thick layers exhibit temperature-dependent cell adhesion/detachment control, while surfaces with PIPAAm layer thicknesses of more than 30 nm do not support cell adhesion. These changes in cell adhesion are explained by the limited mobility of the surface grafted polymer chains as a function of grafting, hydration, and temperature.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Cell-adhesive surfaces; Cell non-adhesive surfaces; Cell detachment; Hydrophilic surfaces; Nanophase separated surfaces; Thermoresponsive polymer; Cell sheet engineering

1. Introduction

Over several decades, an enormous array of materials has been produced for possible applications in biomedical research and clinical devices. Each material exhibits numerous, often uncontrolled interactions with proteins and cells both in vitro and in vivo, leading to various conclusions and determinations regarding

* Corresponding author. Tel.: +81 3 3353 8111x30232; fax: +81 3 3359 6046.

E-mail addresses: kikuchia@abmes.twmu.ac.jp (A. Kikuchi), tokano@abmes.twmu.ac.jp (T. Okano).

0168-3659/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2004.08.026

materials "biocompatibility". Protein adsorption and subsequent cell adhesion behavior are frequently disadvantageous for desired functions of these materials. In biosensor devices, protein adsorption significantly reduces sensing abilities, leading to fibrosis, inflammatory complications, decreasing sensing efficacies and duration, with users often requiring implantable sensor replacements within several day periods. Furthermore, blood-contacting materials like artificial hearts and artificial vessels currently used in clinical treatments are limited to temporary, replaceable applications and/or replacement only of arterial vessels with relatively large diameters. Thus, most blood vessels with diameters smaller than 3 mm are replaced only with the natural vessels taken from cadaver donors, or as autografts. In these limited examples, but also nearly every application of materials in medicine, interfacial performance between materials and biological components, particularly cells and proteins, remains an unsolved problem and limitation to improved clinical response.

Cell–materials interactions are schematically illustrated in Fig. 1. Three categories of cell–materials interactions are defined. The first regards nonfouling surfaces where cells fail to interact with the surface over desired periods of time determined by each application. Such surface design is witnessed repeatedly in multiple reports of highly hydrophilic surfaces modified with poly(ethylene glycol) (PEG) [1–3], and polyacrylamide (PAAm) [4,5] as typical examples. The second category of interactions is "passive adhesion", in which interfacial response is controlled by physicochemical interactions between the material surface, adsorbed proteins and adhering cells. Surfaces in this category inhibit cellular metabolic changes from passive to active, receptor-mediated adhesion, but adherent cells remain intact and are readily detached from these surfaces with minimal or negligible damage [6–9]. This cell–surface interaction occurs reversibly and without metabolic signaling within the cell. A final interaction is categorized as "active cell adhesion" followed by passive adhesion, where cells resident on surfaces spontaneously activate receptor-mediated processes, change their morphology to spread and commence signaling processes typically of attachment-dependent phenotypes. Distinct metabolic processes using ATP and initiating cell signaling pathways are characteristic of this response. Spread cells rarely detach spontaneously from these surfaces without reversing events associated with morphology changes from spread to round cell shapes and release of surface-engaged receptors. Thus, enzymatic digestion of extracellular matrix proteins (e.g., using trypsin) and chelation of divalent cations as Ca^{2+} ions to de-activate cell integrin receptors are typically used to detach actively adherent cells. Several physicochemical characteristics of materials affecting cell-materials interaction are summarized in Table 1.

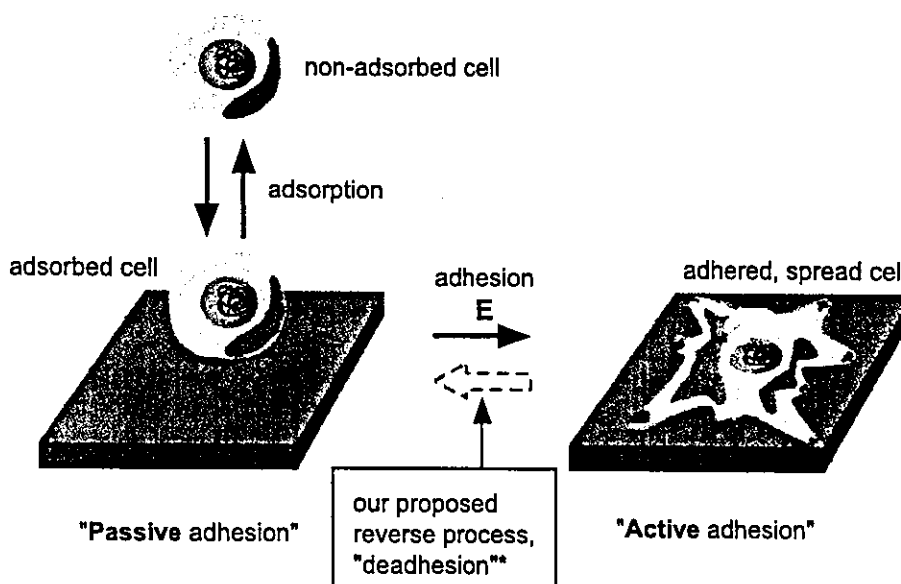


Fig. 1. Schematic diagrams for possible interactions of materials surfaces with cells.

Table 1
Physicochemical characteristics of materials affecting cell–materials interactions

• Surface roughness	
• Hydrophilic	PEG, PAAm, PVA
• Hydrophobic	PSt, PTFE
• Charges: positive	Poly(ethylene imine)
Negative	PAAc, Polystyrene sulfonate
• Nano-, micro-phase separation:	
Hydrophilic/hydrophobic	PHEMA–PSt–PHEMA [6,64,65], Pluronics [66]
Charge/non-charges	PHEMA-g-polyamine [67–69]
Crystalline/amorphous	Polyether-segmented polyamide [70–72]
Soft/hard	Polyether-segmented polyurethaneurea [73–77]
• Combination of above characteristics	[1,18,78]

In 1990, we found that many attachment-dependent cell types adherent on culture surfaces modified with nanometer-thick poly(*N*-isopropylacrylamide) (PIPAAm)-grafted chains at 37 °C can be spontaneously detached from these surfaces simply by reducing culture temperature below 32 °C for 30 min [10]. Such cell adhesion–detachment modulation on the surfaces grafted with thermoresponsive PIPAAm chains is a novel concept—no enzymes or chelators are required. Passive–active surface adhesion processes in cells can be experimentally activated using mild applications of cycling culture temperature. Over the past decade, we have extensively investigated the mechanisms of cell detachment from these surfaces [11,12], and reported possible applications in tissue engineering using the "cell sheet engineering" concept for several tissue-like cultures [13–15].

In the present paper, we briefly review the above-mentioned three categories of cell–materials interactions. Then, temperature-controlled cell adhesion/detachment behavior on the PIPAAm-grafted surfaces is reviewed in this cell–surface context, and finally opportunities to exploit this technology in tissue engineering applications will be summarized.

2. Cell non-adhesive surfaces

Cell non-adhesive surfaces, or nonfouling surfaces, have long been sought for clinical applications, especially for blood-contacting materials including artificial blood vessels, valves, and artificial heart devices, blood preservation bags, and catheters, but also in contact and intraocular lenses, shunts, implantable sensors, and many other device categories. These biomaterials seek largely to provide a clinical function while remaining largely 'inert' to adverse biological reactions. Attempts to attain these specific favorable characteristics on materials surfaces, highly hydrated, nonionic polymers are frequently immobilized, forming hydrophilic, diffuse layers on materials interfaces. Hydrophilic nonionic polymers used for this purpose include poly(ethylene glycol) (PEG) [1–3,16], poly(acrylamide) (PAAm), poly(*N*,*N*-dimethylacrylamide) (PDMAAm) [4,5,17], and poly(vinyl alcohol) (PVA) [4,18]. Nonfouling characteristics are often demonstrated in both in vitro

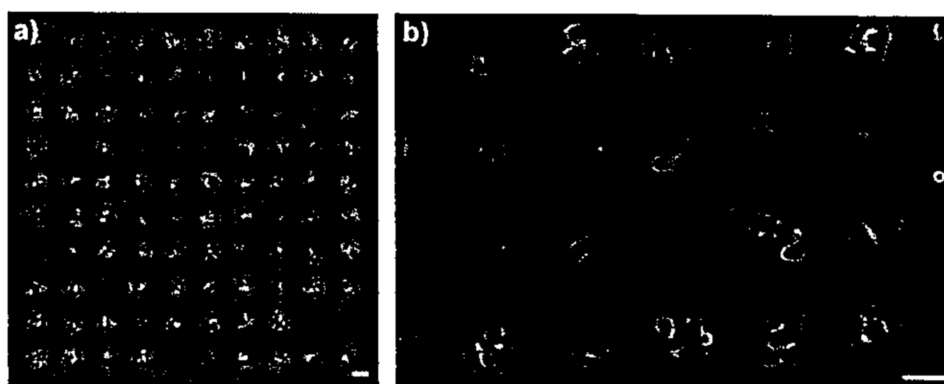


Fig. 2. Microscopic observations of selective cell adhesion on hydrophobic domains created on PAAm-grafted surfaces: (a) low magnification; (b) high magnification. Scale bar: 50 μm.

and in vivo experiments. However, it is difficult, if not impossible, to maintain long-term cell-resistant properties, especially when these surfaces are placed in static compartments (e.g., subdermal), or small veins where blood flow is very slow. Performance problems may derive from surface imperfections, insufficient density of grafted hydrophilic polymer chains, and gradual accumulation of small adsorbent molecules on the base surface. Thus, alternative methods for reliable, high density polymer grafting should be developed.

Utilizing cell nonfouling characteristics with grafted hydrophilic polymers, we recently developed new patterned cell culture dish surfaces [19]. Thermoresponsive PIPAAm-grafted surface domains grafted into desired shapes were fabricated using AAm monomer solution spread onto PIPAAm-grafted surfaces and followed by electron beam irradiation through a mask. Such modification allows us to obtain patterned surfaces for seeding cultured cells and subsequently generate cell sheets with appropriate gross morphology. Furthermore, we also modified solid surfaces with grafted PAAm followed by limited laser ablation to obtain surface patterns for creating cell arrays [20]. Fig. 2 shows a typical example of cell arrays made on PAAm grafted surfaces using limited excimer laser ablation. As seen in this figure, cells seeded on patterned surfaces adhered only within the ablated surface domains with no cells resident on non-ablated, hydrophilic PAAm surfaces. Such surfaces may be exploited to investigate basic biology of cell attachment, and drug screening in drug development and discovery.

3. Exploiting surface nano-topologies to prevent cell transitions from passive to active adhesion

We have extensively investigated the effects of surface microphase separation (cf. actually nanophase separation) of block copolymer surface coatings on their interactions with cells and biomolecules [6–9,21,22]. Triblock ABA types of the block copolymer, poly(2-hydroxyethyl methacrylate) (PHEMA)-b-polystyrene (PSt)-b-PHEMA was synthesized either by coupling bifunctional PSt and monofunctional PHEMA, or by living anionic polymerization of hydroxyl-protected HEMA and styrene (Fig. 3a). Block copolymer-coated surfaces display lamellae-type phase-separated structures with nanometer-scale domain widths (Fig. 3b). Such nanodomain sizes and morphologies can be regulated by the composition of two polymer units and their molecular weights. On these PHEMA–PSt–PHEMA surfaces, platelets adhere from platelet-rich plasma; however, adherent platelets do not change their morphologies or

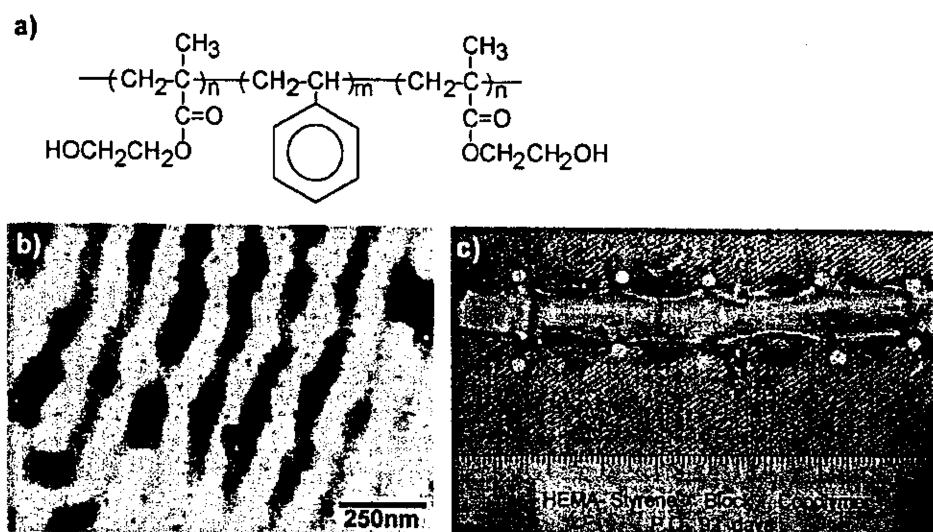


Fig. 3. Nanophase separated triblock copolymer surfaces with antithrombogenic characteristics. (a) Structural formula of the triblock copolymer, PHEMA-b-PSt-b-PHEMA. (b) Nanophase separation of the cast polymer observed by transmission electron microscopy. (c) Appearance of artificial vessels on the triblock copolymer-coated Dacron® graft 3 months after implantation into canine carotid artery.

degranulate to release reaction products, serotonin and 5-HT. Microscopic observations of these platelets on the PHEMA–PSt–PHEMA triblock copolymer surfaces showed that adherent platelets retain active motion-rolling, detachment, oscillatory vibrations, and directional changes—on these surfaces over controls [9]. These cell movements continued for more than 20 min and were distinct from those for inanimate PSt latex model particles. Treatments of platelets with an ATP synthesis inhibitor, sodium azide, or a membrane skeleton disrupting agent, dibucaine, significantly reduce platelet movement, indicating that platelets on PHEMA–PSt–PHEMA surfaces require metabolic process consuming ATP, and actively recruit the structural dynamics of their membrane cytoskeleton. Such energy-consuming movements may explain the observed low platelet activation on the triblock copolymer surfaces.

A triblock copolymer-coated 3-mm-diameter Dacron® graft was then evaluated in ex vivo experiments in canine carotid arteries [23]. On control PEG-grafted Biomer® surfaces, thrombus formation was apparent after 1-month implantation, while PHEMA–PSt–PHEMA surfaces were clear with no thrombus even after 3-month duration (Fig. 3c). These small diameter triblock copolymer-coated vessels were patent for more than a year. By transmission electron microscopic observations, multi-layer adsorption and conformational disruption of plasma proteins were apparent on PEG-grafted Biomer® surfaces. In contrast, only a monolayer of adsorbed albumin was present on triblock copolymer-coated surfaces, maintaining a native-like protein conformation [7,8]. Furthermore, this monolayer of adsorbed serum protein was maintained for extended periods.

We maintain that the unique surface nanostructure of PHEMA–PSt–PHEMA triblock copolymer surfaces dynamically modulates both serum protein composition and adsorption as well as adherent cells to produce nonthrombogenic characteristics in vivo.

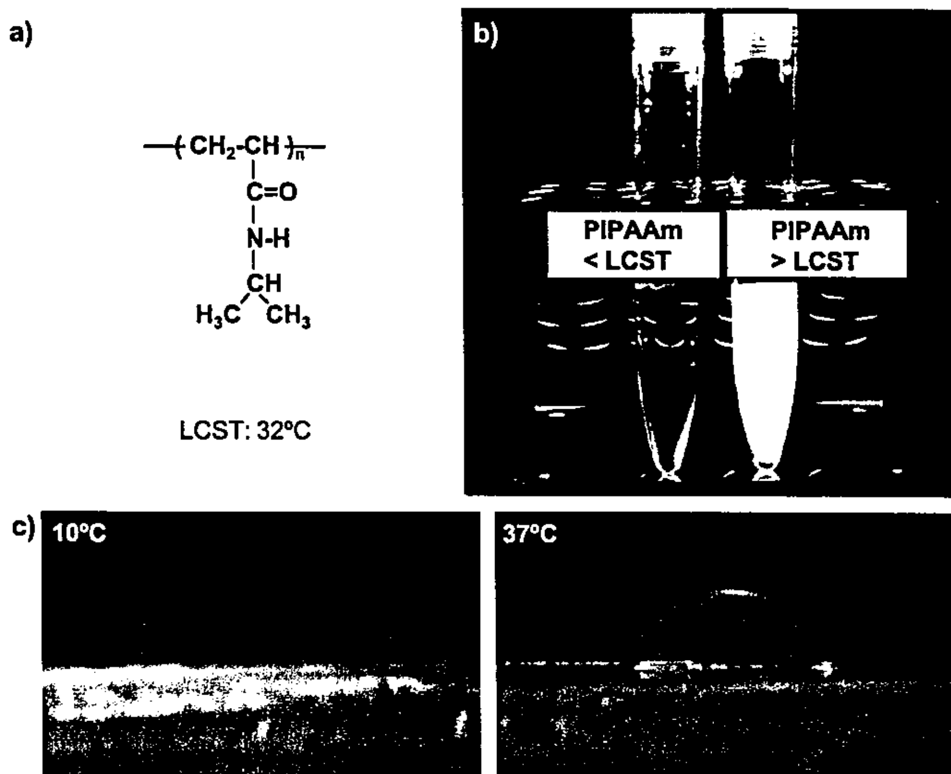


Fig. 4. Structure and properties of thermoresponsive PIPAAm in solution and on surfaces. (a) Structural formula for PIPAAm. (b) Thermoresponsive soluble/insoluble changes for PIPAAm in aqueous solution. (c) Temperature-dependent wettability changes for PIPAAm-grafted surfaces at 10 and 37 °C.

4. Using intelligent surfaces for reversible cellular adhesion and detachment

PIPAAm is now well-known to exhibit temperature-responsive soluble/insoluble changes in aqueous solution [24,25] (Fig. 4). This property can also be useful for PIPAAm-grafted surfaces. We have focused on temperature-responsive surfaces that demonstrate controlled hydrophilic/hydrophobic alterations, namely PIPAAm-grafted surfaces, as 'intelligent surfaces' controlled by external modulation of temperature [26,27]. Typical water contact angles on such surfaces change reversibly with temperature as shown in Fig. 4c. At temperatures below 32 °C, PIPAAm molecules are highly hydrated, and thus PIPAAm-grafted surfaces are hydrophilic. Above 32 °C, extensive PIPAAm dehydration occurs, resulting in an abrupt transition to hydrophobic surfaces. This change is completely reversible with temperature. Such surface characteristic changes have been exploited for new aqueous separations of bioactive compounds, including steroid hormones [28,29], polypeptides and proteins [30–33], and nucleic acids [34]. In addition to such regulation of molecular interaction with thermoresponsive polymer-modified surfaces, we have also achieved temperature-modulated cell adhesion detachment control [10]. In this system, commercially available tissue culture grade polystyrene (TCPS) dish surfaces are graft-modified with PIPAAm. Briefly, appropriate amounts of 2-propanol solutions of IPAAm monomer are spread uniformly over TCPS dish surfaces, followed by electron beam irradiation with a dose of 0.3 MGy at 150 kV. During this procedure, IPAAm monomer is both polymerized and simultaneously grafted covalently onto the TCPS surfaces [35]. These PIPAAm-modified surfaces demonstrate temperature-dependent wettability changes, being hydrophilic at low temperatures below 32 °C, and became hydrophobic above this temperature. Cells in culture respond to these surface changes.

Various types of cultured cells adhere and proliferate on PIPAAm-grafted culture dish surfaces under normal culture conditions at 37 °C with 5% CO_2 [10–12,36–41]. These adhesion–proliferation processes are comparable to that on conventional TCPS dishes. Such processes correspond to metabolically "active adhesion" detailed in Fig. 1, where cells actively consume ATP, move receptors on their membrane surfaces, and trigger cell signaling to change their morphologies from rounded to flattened, both for survival and to express phenotypic metabolic functions (gene and protein expression). Active cell adhesion and proliferation on the PIPAAm-grafted surfaces are observed on surfaces modified with PIPAAm amounts ranging from 1.5 to 2.0 $\mu g/cm^2$ [35,42]. On PIPAAm-grafted surfaces with PIPAAm lower than 1.5 $\mu g/cm^2$, cells adhere and proliferate appropriately, but these cells never detach from the surfaces by lowering the temperature below 32 °C unless a digestive enzyme (trypsin) is used. Likewise, cells do not adhere to surfaces with more than 2.0 $\mu g/cm^2$ PIPAAm or with a graft thickness of more than 30 nm [42]. This cell behavior can be correlated with similar surface presence of the cell-adhesive protein, fibronectin (FN) on the PIPAAm-grafted surfaces with different PIPAAm graft amounts. Because these modified surfaces are completely covered with grafted PIPAAm molecules as determined by angular-dependent X-ray photoelectron spectroscopic (XPS) analyses [43], retention of cell adhesion on PIPAAm-modified surfaces with lower amounts of grafted PIPAAm below 32 °C is not due to exposure of cell-adhesive TCPS PSt substrates, but to intrinsic PIPAAm interaction with adherent cell-adhesive components. Additionally, the observed failure of cultured cells to adhere to surfaces grafted with PIPAAm at high density at 37 °C cannot be attributed to a different graft chemistry. PIPAAm simply cast onto these TCPS surfaces often leaves several mg/cm^2 of PIPAAm molecules on these surfaces. However, such surfaces do not support cellular adhesion even though the seeded cells are highly adherent like fibroblasts (unpublished results). Thus, the grafting density of these chains is critical to performance in this context; the mechanistic ability of PIPAAm-grafted density to influence cell adhesion/detachment changes in culture is better considered in terms of its reversible alteration of surface wettabilities and grafted PIPAAm chains molecular mobility.

Surface wettabilities have been determined by captive bubble methods in thermostated Milli-Q water, and these data are summarized in Table 2 [42]. As seen in the table, all PIPAAm-modified surfaces show hydrophilic/hydrophobic property alterations with temperature changes between 37 and 20 °C. However,