

4.2 細胞シートの回収・積層化

2次元的に結合した細胞シートを回収するために、多孔質メンブレン上で細胞シートを構築した後に、単細胞の場合と同じように電位印加により剥離させた。さらに、剥離した細胞シートを別の基板上で形成したシートに重ね、2層を結合させた後、基板に電位を印加することで2層の積層シートが構築可能となる。この操作を繰り返すことで、3次元的な厚みを持った多層化細胞シートを形成させた (Fig. 8)。

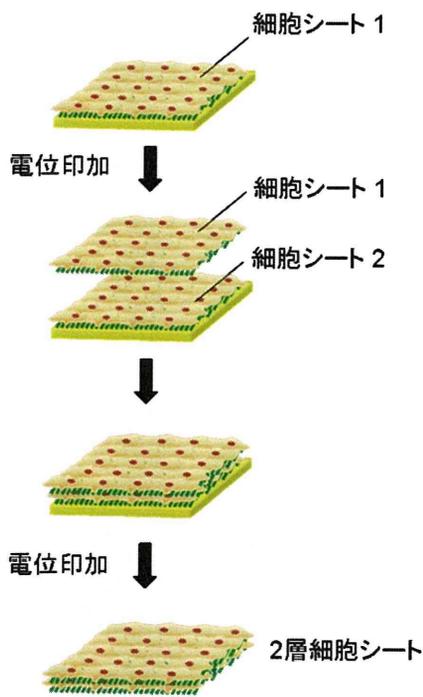


Fig. 8 細胞シートの積層化

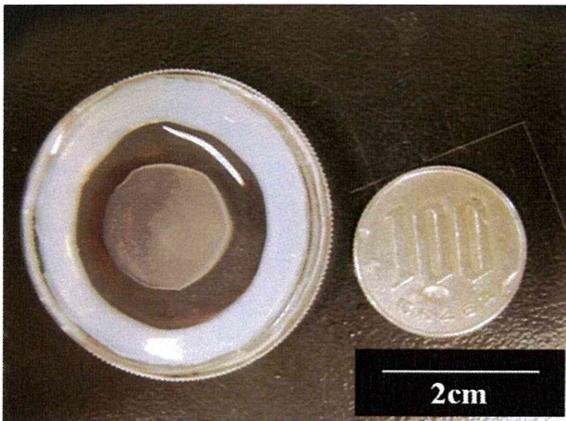


Fig. 9 回収した細胞シートの全体写真

多孔質メンブレン上でマウス線維芽細胞を2週間培養し、電位を5分間印加して回収した細胞シートの写真をFig. 9に示す。回収前の細胞シートのサイズは、メンブレンと同様に直径25 mmであるが、回収後細胞シートは自発的に収縮した。

また、細胞の生存状態を確認するために、回収した細胞シートを生細胞/死細胞染色をおこない、蛍光顕微鏡で観察した。FDA/EB染色では、生細胞はFDAによって緑に、死細胞はEBによって赤に染まる。Fig. 10の上が位相差顕微鏡、下が蛍光顕微鏡で観察した細胞シートであるが、ほとんどの細胞が緑に染まっている、すなわち生存状態であることが確認された。

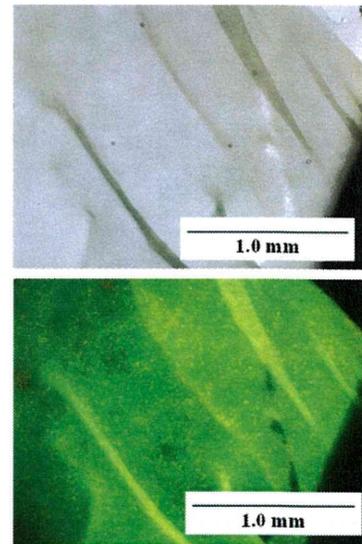


Fig. 10 回収した細胞シートの生死染色 (上)位相差顕微鏡写真、(下)蛍光顕微鏡写真

さらに、回収した細胞シートをディッシュに入れ、その上から新たな細胞シートを重ね、37°C、5% CO₂-Airの雰囲気下で30分間インキュベートした。その後、さらに新たな細胞シートをその上から重ね、同様にインキュベートさせることで、積層化された細胞シートを作製した。回収した細胞シートの下面には、脱離したオリゴペプチドの

他に、細胞自身が生産した細胞外マトリックスが存在する。よって、細胞シート同士は単純に重ねることによって、30分ほどで完全に接着した。回収した細胞シートを次々に重ねていくことで、多層化細胞シートを作製した。1~3層を積層化した際の細胞シート断面を、ヘマトキシリン-エオジン(H.E.)染色したものをFig. 11に示す。H.E.染色では生細胞しか染色されないが、Fig. 11(A-C)のようにシート中央部もきちんと染まっている、すなわち生存状態であることが分かった。Fig. 11(D)は、実際にシートを重ねている様子である。細胞シートの端の部分が自発的に収縮している様子が確認できる。従来の手法によって3枚積層化した細胞シートと比較すると、本手法では約7倍の厚みである140 μm の細胞組織を得ることができた (Fig. 11(C))。

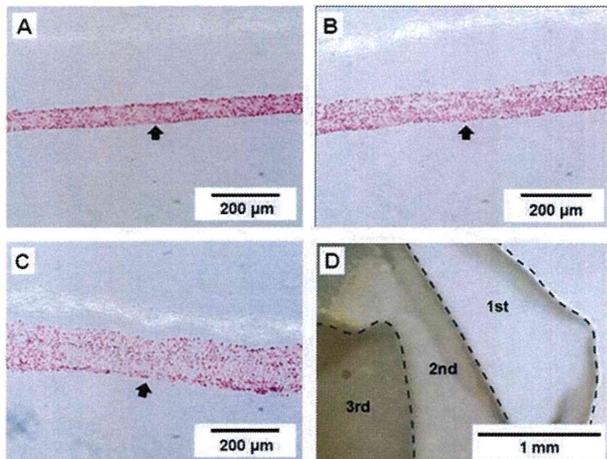


Fig. 11 積層化した細胞シートの断面 (H.E.染色, (A)1層、(B)2層、(C)3層。(D)3枚重ねた際の位相差顕微鏡写真

さらに同様の操作を繰り返してシートを重ねることによって、5枚の多層化シートを構築した際の断面をFig. 12に示す。H.E.染色の様子からも分かる通り、細胞シート間の境目が曖昧になる程にそれぞれの細胞シートが強固に結合し、細胞が蜜になっている

様子が確認できた。このことから細胞の形態や細胞間の結合が損なわれることなく、組織が構築することができたと考えられる。また、主要な細胞外マトリックスであるフィブロネクチンの免疫染色を行うことで、細胞シート内のフィブロネクチンの発現を観察した (Fig. 13)。この結果は、細胞シートの積層化プロセスが短い理由を説明するのみならず、移植時にも生体への高い生着性が期待できる。

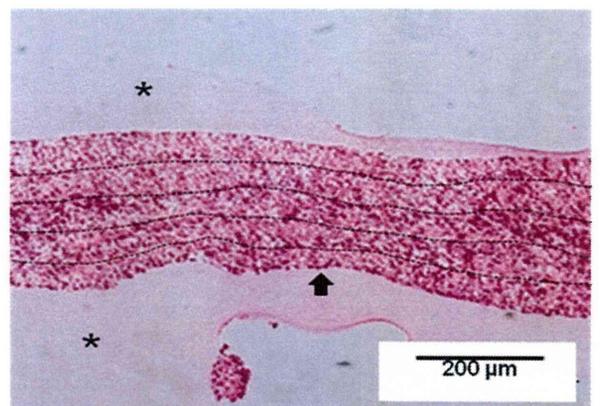


Fig. 12 積層化した細胞シートの断面 (H.E.染色, 5層)

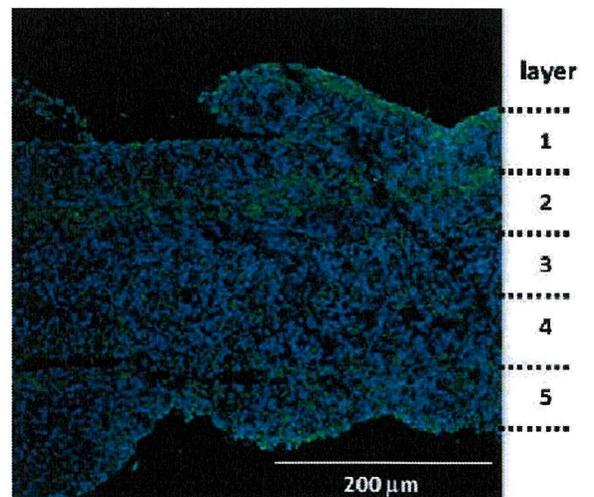


Fig. 13 積層化した細胞シートの断面 (フィブロネクチン蛍光染色/DAPI, 5層)

D. 結論

メンブレン上で電気化学的原理に基づいて、単細胞の脱離を評価した結果、-1.0 V(vs Ag/AgCl)の負電位を5分間印加することでほとんどの細胞が脱離可能であり、脱離後も良好な生存状態および増殖能を維持していることを確認した。次に、メンブレン上で細胞シートを培養することによって、通常の培養基板に比べて厚みのある細胞シートを構築することができた。また、オリゴペプチドを用いた電気化学的原理に基づいて、多孔質メンブレン上で線維芽細胞を2週間程度培養することで構築した細胞シートを回収する技術を確立した。また、それらを重ね合わせることによって均質な多層化細胞シートを構築することが可能であることを示した。

E. 健康危険情報

該当なし

F. 研究発表

1. 論文発表

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2. 学会発表

国内学会

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G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（再生医療実用化研究事業）
分担研究報告書

ヒアルロン酸ゲルの作製
研究分担者 伊藤 大知 東京大医学部

研究要旨

細胞外マトリックスの構成成分を原料とし、これらの二液を混合するのみにて10秒程度でゲル化するスマートバイオマテリアルを開発した。さらに、これらスマートバイオマテリアルと電気化学的な細胞脱離技術を組み合わせることで、接着細胞を基板からゲル側へ素早く転写可能であることを示した。

A. 研究目的

細胞シートの機械的強度は小さいため、細胞シートを積層化したり移植する際には一般に操作が難しくなる。そこで、生体材料を用いたハイドロゲルを利用し、機械的な強度を補助するとともに、移植部位に確実に固定する工夫を行った。

生体の細胞外マトリックスは主にコラーゲン、グリコサミノグリカン、糖たんぱく質からなる。本研究では、この中からグリコサミノグリカンの一種であり、保水力を持つヒアルロン酸 (HA)と、細胞外マトリックスの骨格を形成し細胞の接着因子を持つコラーゲンの部分変性体であるゼラチン (Gelatin) の2つをベースとし、ハイドロゲルを作製した。具体的には、ヒアルロン酸に過ヨウ素酸ナトリウムを用いた開環反応によりアルデヒド基 (CHO)を導入し、ゼラチンにはEDC (1-エチル-3-(3-ジメチルアミノプロピル)カルボジイミド塩酸塩) 試薬を用いることによって、脱水縮合反応でヒドラジド基 (ADH)を修飾した。これにより、HA-CHO、Gelatin-ADHの二つのハイドロゲル原料を作製し、二つのゲル原料の配合を変更することによって、強

度などの性質を容易に変更できる化学修飾ハイドロゲルを開発した。このゲルを用いることで、細胞シートを容易に取り扱えるようにすることが本研究の目的である。

B. 研究方法

1. ハイドロゲルの合成と評価

アルデヒドとヒドラジドを用いたハイドロゲルの原料となるHA-CHOの合成を行った。ヒアルロン酸を原料に用いて、過ヨウ素酸ナトリウムを用いた開環反応によって、HA-CHOを作製した。評価として、¹HNMRで解析を行った。

1.1 実験装置と試薬・材料

[試薬]

- Hyaluronic acid (FCH-200) : キッコーマンバイオケミファ
- 過ヨウ素酸ナトリウム Sodium periodate (M=213.89) : 和光純薬
- エチレングリコール Ethylene glycol (M=62.07) : 和光純薬
- Gelatin from porcine skin typeA : シグマアルドリッチ
- 1-エチル-3-カルボジイミド

1-ethyl-3-carbodiimido (EDC) (M=191.7) :
和光純薬

• 1-hydroxybenzotriazole (HOBT) (M=135.12) :
和光純薬

• アジポヒドラジド Adipohydrazide (ADH)
(M=174.20) : 和光純薬

• ジメチルスルホキシド Dimethyl sulfoxide
(DMSO) (M=78.14) : 和光純薬

• 1M HCl : 和光純薬

• 1M NaOH : 和光純薬

• 塩化ナトリウム sodium chloride (NaCl)
(M=58.44) : 和光純薬

• エタノール ethanol (M=46.07) : 和光純薬

• トリニトロベンゼンスルホン酸 (TNBS) :
和光純薬

• t-BC : 和光純薬

[装置]

• 透析膜 MWCO 6-8000 : ThermoFisher

• 凍結乾燥機 : EYELA , FDU-1200

• 紫外可視光吸光度計 : SHIMAZU ,
UVmini1240

• ダブルシリンジ : アステラス製薬

1.2 実験手順

1.2.1 HA-CHOの合成 (Fig. 1)

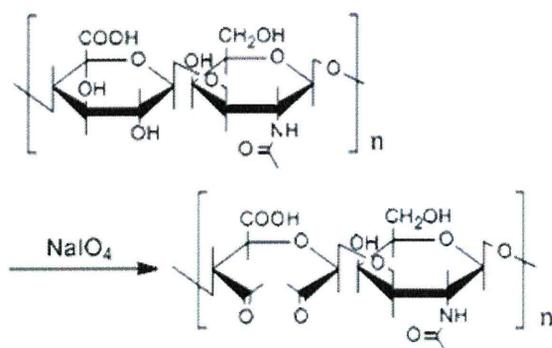


Fig. 1 HA-CHOの合成スキーム

1) ヒアルロン酸 (HA)の溶解

300 mlナスフラスコに1.5 gのFCH-200を加

え、150 mlのmilliQ水で1晩攪拌させる。常温
で反応させた。

2) 過ヨウ素酸ナトリウム溶液の滴下による 開環反応

過ヨウ素酸ナトリウムを0.8038 g (1等量)
を10 mlのmilliQ水に溶解させ、これをフラス
コに滴下し、加えた。遮光のため、アルミホ
イルでフラスコを包み、2時間攪拌を行った。
2時間後、未反応物除去のためにエチレング
リコール 0.2 mlを加え、15分攪拌した。

3) 透析

MWCO=6-8000の透析膜で7日間透析を
行った。(1日2回透析水を交換した)

4) 凍結乾燥

溶液を分注したのち凍結乾燥機を用いて、
7日間凍結乾燥を行った。

5) NMRでの測定

核磁気共鳴装置を用いて¹H NMRスペク
トルを測った。

6) HA-CHOの保存

4 °Cのフリーザーで保存した。

1.2.2 Gelatin-ADHの合成 (Fig. 2)

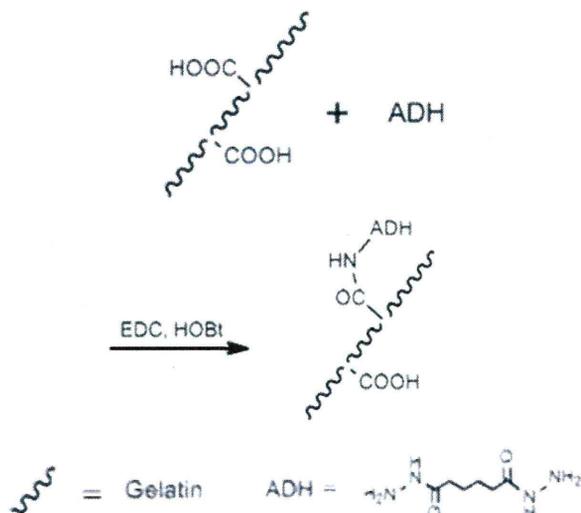


Fig. 2 Gelatin-ADHの合成スキーム

1) Gelatinの溶解

300 mlナスフラスコに1.0034 gのGelatin type-Aを加え、100 mlのmilliQ水で1晩攪拌して溶解させた。

2) ADHとSchiff base reaction

Gelatin溶液にアジポアルデヒド1.41718 gを入れ、透明になるまで1時間攪拌した。

3) EDC試薬による反応

1時間後、NaOH, HClを用いて、pHを7程度に調整し、HOBt溶液 (HOBt 0.15 gにDMSO 1.4 ml, milliQ 1.0 mlを混合)をパスツールピペットで全量滴下した。その後、同様にEDC溶液 (EDC 0.15 gにDMSO 0.6 ml, H₂O 0.6 mlを混合)をパスツールピペットで全量滴下し、pHを5~6の間に調整した。(以後、4時間、30分おきにpH調整を行った)さらに、1晩攪拌を行った。

4) 透析

翌日から、透析膜 (MWCO=6-8000)で7日間透析を行った。

5) 凍結乾燥

溶液を分注したのち凍結乾燥機を用いて、7日間凍結乾燥を行った。

6) Gelatin-ADHの溶解

凍結乾燥をしたものを5 wt%のNaCl溶液に溶かした。(Gelatin-ADH濃度5-10 mg/ml)

7) エタノール再沈殿

Ethanol 1 L中にNaClに溶解したGelatin-ADH溶液を攪拌しながら滴下し、Gelatin-ADHを再沈殿させた。30分攪拌させたのち、これを遠心分離機で2500回転、4分間遠心分離することでGelatin-ADH再沈殿を回収した。

8) 透析

回収したGelatin-ADHを100 mlのmilliQ水に加え2時間攪拌及び溶解したのち、再度、7日間透析を行った。

9) 濾過

透析後 (透析開始7日目)、ろ紙による沈殿

の濾過を行った。

10) 凍結乾燥

50 ml遠心管に移し、7日間凍結乾燥を行った。

11) NMRでの測定

核磁気共鳴装置を用いて¹HNMRスペクトルを測った。

12) HA-ADHの保存

4°Cのフリーザーで保存した。

13) TNBS溶液の調整

トリニトロベンゼンスルホン酸 (TNBS) 63.06 mgを1 mlのmilliQ水に溶解し5 wt%のTNBS溶液 (179.57 mM)を作製した。この5 wt%のTNBS溶液200 mlを800 mlのmilliQ水に溶解し、1 wt%のTNBS溶液を作製し、さらに、この1 wt%のTNBS溶液200 mlを9.8 mlのmilliQ水に加え、0.02 wt%のTNBS溶液 (0.72 mM)を作製した。

14) t-BC溶液の調整

t-BC 132.16 mgを10 mlのmilliQに加える (100 mM)。この100 mMの t-BC 溶液100□□lを9.9mlのmilliQ水に溶解し、1 mMのt-BC 溶液を作製した。

15) Gelatin溶液の調整

Gelatin 40 mgを2 mlのmilliQ水に溶解し、2 wt%のGelatin溶液を作製した。

16) Gelatin-ADH溶液の調整

Gelatin-ADH 40mgを2 mlのmilliQ水に溶解し、2 wt%のGelatin-ADH溶液を作製した。

17) サンプルの調整

24wellプレートにt-BC溶液100 wt%、50 wt%、25 wt%、12.5 wt%、6.3 wt%、3.2 wt%、1.0 wt%、0.5 wt%、0.25 wt%、0.125 wt%、0.063 wt%、0.032 wt%をそれぞれのwellに1 mlずつ加えた。同様に、Gealtin 溶液およびGelatin-ADH溶液においても、0.6 wt%、0.5 wt%、0.4 wt%、0.25 wt%、0.2 wt%、0.125 wt%、0.1 wt%、0.062 wt%、0.05 wt%をそれぞれの

wellに1 mlずつ加えた。

18) TNBS試薬との反応

それぞれのwellにTNBS溶液を1 mlずつ加え、37°Cのインキュベータで半日間、反応させた。

19) 吸光度測定

334 nmの吸光波長で吸光度測定を行った。この時、Gelatin-ADH溶液は同じ濃度のGelatin溶液を0点補正に用いた。

1.2.3 Gelatin-ADHとHA-CHOのゲル化 (Fig. 3)

1) Gelatin-ADH, HA-CHOの溶解

Gelatin-ADHを5 wt%でPBSに溶かす。同様にHA-CHOを1 wt%、2 wt%、3 wt%、4 wt%でPBSに溶かした。

2) Gelatin-ADHとHA-CHOのゲル化

この2つの原料をダブルシリンジを使ってゲル化させた。

3) Gelatin-HAハイドロゲルの凍結乾燥

作製したハイドロゲルを-80 °Cのフリーザーで凍結させ、凍結乾燥を行った。

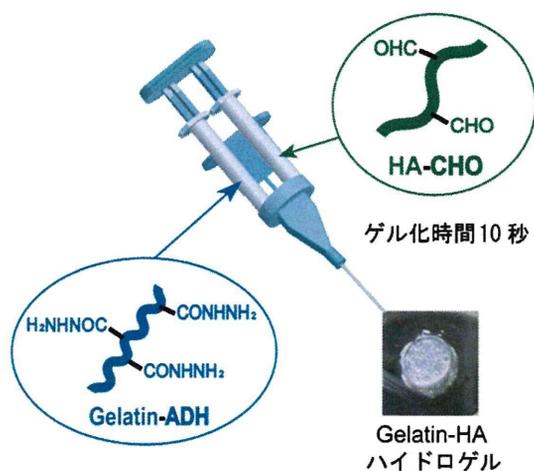


Fig. 3 ダブルシリンジを用いたゲル化

2. ハイドロゲルへの細胞転写

合成したHA-Gelatinハイドロゲルが足場材

料として有用であるか評価を行うために、Gelatin-ADHとHA-CHOの細胞毒性評価を行い、さらに、2液の混合比を変化させ、細胞の接着率を評価した。そして、電気化学的な細胞脱離と組み合わせ、細胞をゲル側に転写可能かどうか評価した。

2.1 実験装置と試薬・材料

[試薬]

- ・ヒト臍帯静脈血管内皮細胞 HUVEC : 理研Cell bank
- ・血管内皮細胞用増殖培地 EBM-2・添加因子セット : 三光純薬
- ・リン酸緩衝生理食塩水 Phosphate Buffered Saline (PBS) : GIBCO.
- ・フルオロセインジアセテート (FDA)
- ・臭化エチジウム (EB)
- ・白金板 : ニラコ
- ・PDMS
- ・PDMS硬化剤

[装置]

- ・位相差蛍光顕微鏡 : IX-71, Olympus.
- ・クリーンベンチ : 三洋電機
- ・遠心分離機 : 三洋電機製
- ・インキュベータ : 三洋電機製
- ・超純水製造装置 (Milli-Q Advantage A10) : MILLIPORE
- ・Double Barrel Syringe
- ・48wellプレート
- ・スパッタデポジション装置 CFS-4ES-231 : Shibaura Electec.
- ・ポテンシヨスタット/ガルバナスタット HA-151 : Hokuto denko.
- ・Ag/AgCl参照電極#2080A : HORIBA.

2.2 実験手順

2.2.1 ハイドロゲル原料の細胞毒性評価

1) GFP-HUVEC播種

GFP-HUVECを48wellプレートに 1×10^4 cells/dishで播種し、1日間培養した。

2) HA-CHO, Gelatin-ADH溶液の調整

凍結乾燥したHA-CHO、Gelatin-ADHをPBSで5 wt%、4 wt%、3 wt%、2 wt%、1 wt%、0.5 wt%、0.25 wt%、0 wt%で希釈した。

3) HA-CHO, Gelatin-ADHの混濁

溶解した2液をそれぞれwell内に5 wt%、4 wt%、3 wt%、2 wt%、1 wt%、0.5 wt%、0.25 wt%、0 wt%で0.5 ml加え、CO₂ インキュベータ内で培養を行う。

4) EBによる死細胞染色

臭化エチジウム (EB)によって染色を行う。

5) 細胞接着性の観察

3h後の初期接着を観察したのち、1日おきに観察を行った。

2.2.2 HA-CHO, Gelatin-ADHゲルの細胞接着評価

1) HA-CHO, Gelatin-ADH溶液の調整

凍結乾燥したHA-CHOをPBSで1 wt%、2 wt%、3 wt%、4 wt%に希釈し、Gelatin-ADHをPBSで2.5 wt%、5 wt%、7.5 wt%、10 wt%で希釈した。

2) HA-CHO, Gelatin-ADHゲルの作製

溶解した2液をダブルシリンジを用いて混合することでゲル化させ、φ35 mm dishをコーティングした。

3) GFP-HUVEC播種

GFP-HUVECを 1×10^5 cells/dishで播種した。

4) 細胞接着性の観察

3 h後の初期接着を観察したのち、1日おきに観察を行った。(培地交換は24 hごとに行い、PBSで1回washしたのち、EBM-1を2 mlずつ加えた)

2.2.3 電気化学を用いた細胞転写 (Fig. 4)

1) 基板洗浄

ガラス基板を25 %アンモニア水 : 30%過酸化水素水 : 純水 = 1 : 1 : 4の沸騰水溶液に5分間浸漬させ、沸騰した純水にてすすぎを2回それぞれ5分間行い、自然乾燥させた。

2) Au/Cr層のスパッタリング

Au/Cr層のスパッタリングを行った。(出力100 W、アルゴン雰囲気0.3 PaにてCr : 1分間、Au : 2分間)

3) 金表面へのRGDペプチドの修飾

RGDペプチドを表面に修飾するために、純水を用いて1.0 mMに希釈したRGDペプチド溶液に、予めアセトンで洗浄した金ガラス基板をそれぞれ一晚浸漬させた後、純水で洗浄した。更にクリーンベンチ内で70%エタノール内に5分間浸漬を二回行い、滅菌した純水でよくすすいだ。

4) 細胞播種

5×10^5 cells/mlの細胞懸濁液1 mlを金ガラス基板上に垂らし、インキュベータ内で1日間培養を行った。

5) ハイドロゲルの作製

培養後、基板上の細胞混濁液を吸い取り、すぐにPDMSで作った囲いをガラス基板にセットし、ダブルシリンジを用いて、HA-CHO 2 wt% , Gelatin-ADH 5 wt%の化学架橋ハイドロゲルを作製した。

6) 負電位印加

対極に白金、参照極に銀-塩化銀電極を接続し、この金ガラス基板にポテンシオスタットを用いて-1.0 Vの負電位を5分間印加後、ゲルをゆっくり金ガラス基板からはがし、φ35 mm dishに培養液を加え培養した。

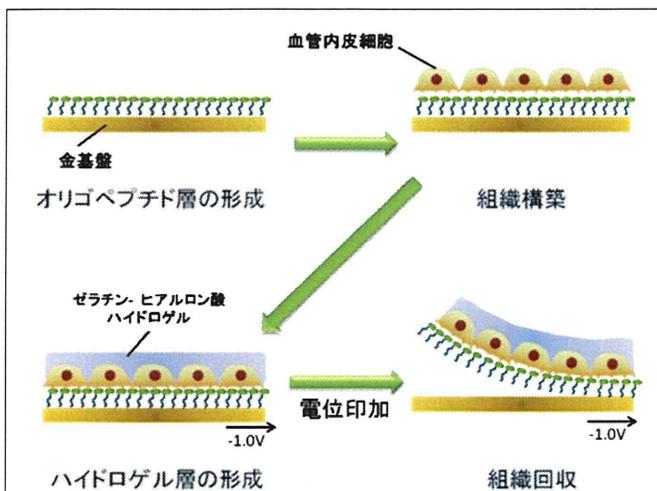


Fig. 4 ハイドロゲルへの細胞転写

C. 研究結果と考察

3. ハイドロゲルの合成と評価

3.1 HA-CHOの合成

ヒアルロン酸 (HA) と合成した HA-CHO の ^1H NMR スペクトルの結果を比較すると、 2.00 ppm に新たなピークが出現した。このピークはアルデヒドの水素原子によるものであり、アルデヒド基の修飾が確認された。また、この積分値から修飾率は75%であると見積もった。

3.2 Gelatin-ADHの合成

HA-CHO の場合と異なり、 ^1H NMR スペクトルの解析では判断が困難であったため、TNBSアッセイを用いた。このTNBSアッセイにより、ゼラチンのカルボキシル基へのアジポヒドラジドの修飾率は27%と見積もられた。この値は一般に高くないが、ゼラチンの細胞接着配列である、R (Arg)-G (Glu)-D (Asp) のD (Asp) はカルボキシル基を側鎖に持っているため、すべて反応してしまうとRGD配列が少なくなり、細胞接着率の低下につながりかねない。そのため、この程度の修飾率が適当であると判断した。

3.3 Gelatin-ADHとHA-CHOのゲル化

ダブルシリンジを用いて、Gelatin-ADHとHA-CHOを混合させることによって、常温下において10秒程度でゲル化する様子が確認された。さらに、Gelatin-ADHとHA-CHOの濃度を変化させることで容易にゲル強度の変更が可能であることを示した。



Fig. 5 Gelatin-ADHとHA-CHOの混合ゲル

4. ハイドロゲルへの細胞転写

4.1 ハイドロゲル原料の細胞毒性評価

ゲル原料の細胞毒性を調べたところ、死細胞の割合がGelatin-ADHでは、5wt%未満で10%程度、HA-CHOでは、1wt%未満で10%程度と示された (Fig. 6、Fig. 7)。また、Live-Dead assay によって HA-CHO と Gelatin-ADH を混合したゲルの細胞毒性は非常に少なく (Fig. 8)、細胞の足場材料としての利用に適していることが示された。

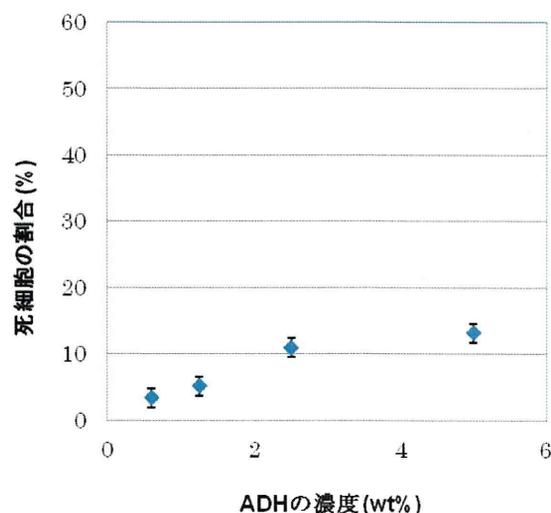


Fig. 6 Gelatin-ADHの細胞毒性

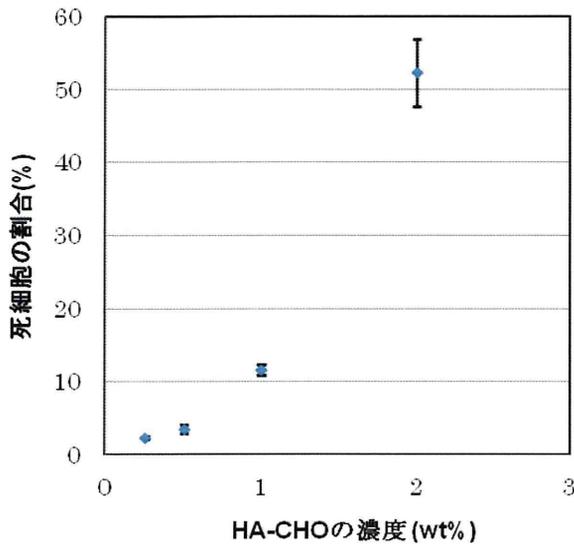


Fig. 7 HA-CHOの細胞毒性

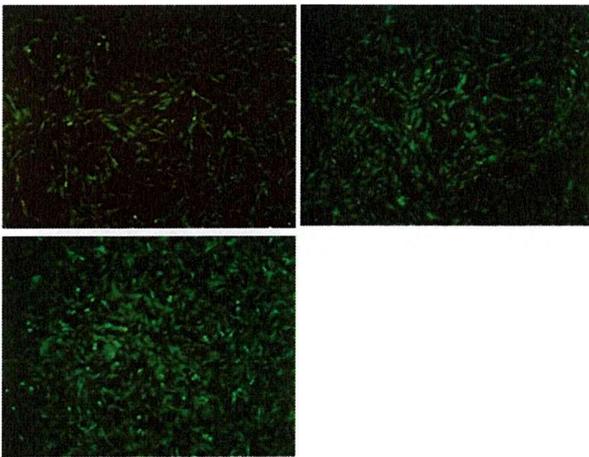


Fig. 8 HA-Gelatinハイドロゲルの細胞毒性 (Gelatin-ADH 5wt% HA-CHO 2wt%)。左上：培養1日目、右上：3日目、左下：5日目。緑色：生細胞、赤：死細胞

4.2 HA-CHO、Gelatin-ADHゲルの細胞接着評価

細胞接着評価を行ったところ、混合比は変化させずに濃度を上げていくと、細胞接着率は増加した(Fig. 9)。これは、ゼラチンやヒアルロン酸に含まれる細胞接着因子の量が増加し、さらに、濃度上昇により、ゲル強度が、

上昇したためと考えられる。また、濃度を上げていくと、ゲル化時間も早くなり、強度も強くなるが、ハイドロゲルの膨潤が大きくなり、より早く分解した。このことから、ゲルの濃度を変化させると初期の細胞接着率は上昇するが、長期的な培養では、他の要素も踏まえて最適なゲル濃度を決定する必要があることが分かった。

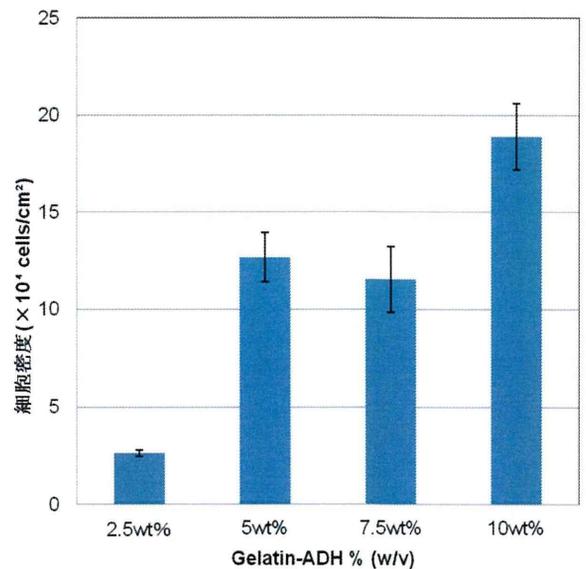


Fig. 9 ゲル濃度と初期細胞接着数の関係

4.3 電気化学を用いた細胞転写

電位印加によって細胞はHA-CHO、Gelatin-ADHゲルに転写され、蛍光写真での生存も確認された(Fig. 10)。また、電位印加後の生存状態は良好で転写した細胞は増殖し、ゲル上を覆っていった。さらに、細胞の脱離の変化を電位印加の時間やペプチドの有無によって評価したところ、5分間の電位印加によってほとんどの細胞が回収できることが示された。

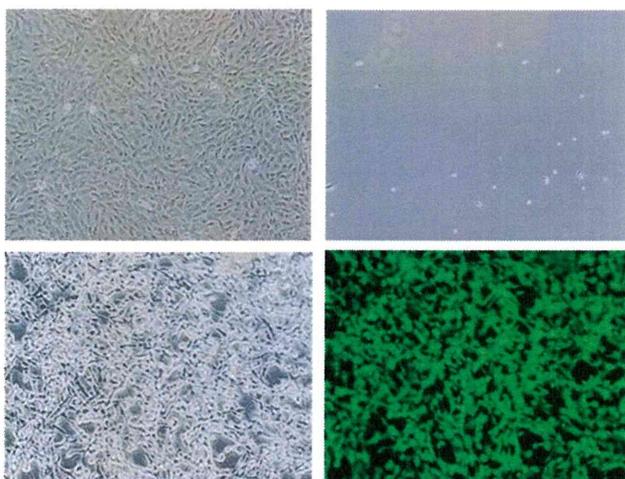


Fig. 10 HA-CHO, Gelatin-ADHハイドロゲルへの電気化学細胞脱離を用いた細胞転写。左上：電位印加前の金基板、右上：電位印加後の金基板、左下：電位印加後のゲル、右下：電位印加後の蛍光写真

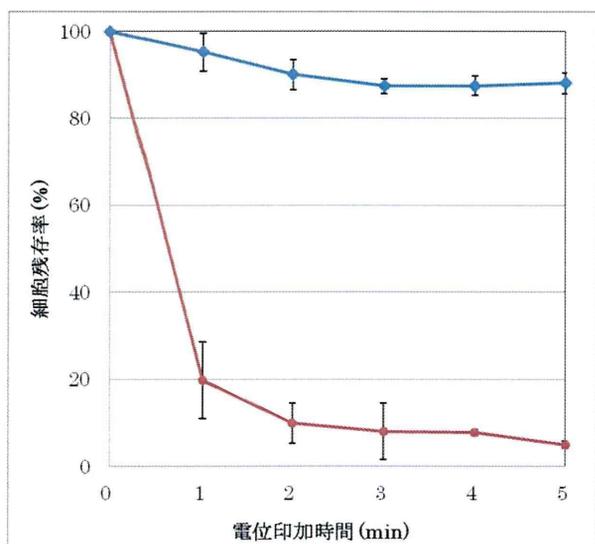


Fig. 11 電位印加時間と脱離率。赤：ペプチド修飾金基盤ゲルコーティング、青：ペプチドなし金基盤ゲルなし

D. 結論

ヒアルロン酸とゼラチンを用いて、化学修飾ハイドロゲルを作製した。細胞接着評価により、2液の混合濃度を変更することによって、容易に異なる性質に変更できることを示

した。さらに、ゲル化時間が10秒以内ということも示された。細胞毒性評価及び、細胞接着性評価を行い、HA-Gelatinハイドロゲルが高い生体適合性を持つことを示した。さらに、オリゴペプチドを修飾した金基板上に接着した細胞を電気化学的に脱離させハイドロゲルへ転写可能であった。

E. 健康危険情報

該当なし

F. 研究発表

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2. 学会発表

国際学会

なし

国内学会

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- 2) in situ架橋ゲルからのシスプラチン徐放挙動の解明と腹膜播種への応用 須原宜史・鈴木幸光・亀井隆雄・山口博紀・石神浩徳・北山丈二・伊藤大知 第8回医工連携研究会 東京大学医学部教育研究棟13階 東京

2011年12月17日

3) ラット肝切除術後癒着モデルを用いた癒着防止材料の有効性の比較研究 大道清彦・清水篤志・須原宜史・伊藤大知・長谷川潔・國土 典宏 第8回医工連携研究会 東京大学医学部教育研究棟13階 東京 2011年12月17日

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5) 膜乳化法を用いた人工赤血球の開発 佐藤真優・鈴木幸光・赤松憲樹・中尾真一・酒井康行・伊藤大知 膜シンポジウム2011 沖縄 2011年11月18-19日 208

6) 医用in situ架橋ゲルの開発－新規バイオマテリアル創製から腹膜癒着・腹膜播種治療まで－ 伊藤大知 生物工学会セルプロセッシング評価研究部会 2011年9月 (招待講演)

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8) 癒着防止開発事例～癒着防止ゲル～ 伊藤大知 情報技術協会 2011年5月 (招待講演)

G. 知的財産権の出願・登録状況

1. 特許取得

特願2011-122183

伊藤大知、鈴木幸光、高橋彬、清水篤志
東京大学「ハイドロゲル及びその製造方法」

2. 実用新案登録

なし

3.その他

なし

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
該当なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
N. Mochizuki, T. Kakegawa, T. Osaki, N. Sadr, NN. Kachouie, H. Suzuki, J. Fukuda	Tissue Engineering Based on Electrochemical Desorption of an RGD-Containing Oligopeptide	Journal of Tissue Engineering and Regenerative Medicine	in press	Doi: 10.1002/te.rm.519	2012
N. Sadr, M. Zhu, T. Osaki, T. Kakegawa, Y. Yang, M. Moretti, J. Fukuda, A. Khademhosseini	SAM-based cell transfer to photopatterned hydrogels for microengineering vascular-like structures	Biomaterials	32(30)	7479-90	2011
J. Fukuda, Y. Kameoka, H. Suzuki	Spatio-temporal detachment of single cells using microarrayed transparent electrodes	Biomaterials	32(28)	6663-9	2011



Tissue engineering based on electrochemical desorption of an RGD-containing oligopeptide

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Abstract

This paper describes a non-invasive approach for efficient detachment of cells adhered to a gold substrate via a specific oligopeptide. Detachment is effected by an electrical stimulus. The oligopeptide contains cysteine, which spontaneously forms a gold–thiolate bond on a gold surface. This chemical adsorption reaches 95% equilibrium within 10 min after immersion of a gold-coated substrate in a solution containing the peptide. The peptide is reversibly desorbed from the surface within 5 min of application of a negative electrical potential. By taking advantage of this simple adsorption and desorption mechanism, cells can be grown on an oligopeptide-functionalized gold surface and can be efficiently detached as single cells or cell sheets by application of a negative electrical potential. This approach was also applied to the surface of gold-coated microrods. Capillary-like microchannels were formed in collagen gel by transferring endothelial cells to the internal surfaces of the microchannels. During subsequent perfusion culture, the enveloped endothelial cells migrated into the collagen gel and formed luminal structures, which sprouted from the microchannels. This technique has the potential to provide a fundamental tool for the engineering of thick cell sheets as well as vascularized tissues and organs. Copyright © 2011 John Wiley & Sons, Ltd.

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Keywords cell sheet; blood vessel; oligopeptide; electrochemistry; gold–thiolate bond; fibroblast; human umbilical vein endothelial cells; collagen

1. Introduction

A major approach in tissue engineering is the use of scaffolds composed of biodegradable synthetic polymers or extracellular matrix (Langer and Vacanti, 1993). This approach has proved to be beneficial for the reconstruction of several types of tissues and has been increasingly translated to successful clinical applications (Atala, 2009). However, some issues remain to be addressed, including mismatches between scaffolds and native matrices, spatial and temporal differences between cell growth and scaffold degradation, a lack of proper vasculature and the tissue-like organization of different cell types (Kohane and Langer, 2008; Sung *et al.*, 2004; Khademhosseini *et al.*, 2006). Another promising approach

is the use of cell-dense aggregates, such as cell sheets and spheroids (Ohashi *et al.*, 2007; Mironov *et al.*, 2009; Nichol and Khademhosseini, 2009; Fukuda *et al.*, 2006a, 2006b). The manipulation and transplantation of such cell aggregates rely on innovative strategies for non-invasive cleavage of cell-to-culture substrate connections while preserving cell-to-cell connections (Inaba *et al.*, 2009). For example, cell sheets adhered to a thermo-responsive polymer can be detached by controlling the hydrophilicity of the surface with temperature modulation (Ohashi *et al.*, 2007; Okano, 2008). This technique was used to fabricate oral mucosal epithelial cell sheets for reconstruction of corneal tissue in a clinical trial (Nishida *et al.*, 2004). Similarly, microwells consisting of a thermoresponsive hydrogel have been used to generate spheroids that were subsequently released by altering the temperature (Tekin *et al.*, 2010). However, a drawback of these approaches is that relatively long time periods are required to detach cell sheets or spheroids from the substrates (Kwon *et al.*, 2003). Thus, the development of biocompatible and easily applicable approaches for rapid collection of

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cell-dense constructs is desired to assist the process of building *in vivo*-like structures for tissue-engineering applications.

In this paper, we present an electrochemical method for non-invasive detachment of cells from a surface (Figure 1). Cells were cultured on a layer of synthetic oligopeptide adsorbed onto a gold surface. The peptide contained RGD (arginine–glycine–aspartate) in the centre and cysteine residues at both ends. The peptide adsorbed onto the gold surface via a gold–thiolate bond, which mediates cell adhesion to the substrate. The peptide was then reductively desorbed from the gold substrate by the application of a negative electrical potential. This caused the cells to detach from the gold surface in a rapid and reliable manner while maintaining cell–cell connections (Seto *et al.*, 2010; Sadr *et al.*, 2011). In this study, we showed that as a first possible application, this technology can be used to fabricate cell sheets and microcapillary-like structures.

2. Materials and methods

2.1. Materials and reagents

Swiss 3 T3 murine fibroblasts (RCB1642) were purchased from Riken Cell Bank, Japan. Human umbilical vein endothelial cells (HUVECCC-2517A), endothelial basal medium-2 (EBM-2, CC-3156) and SingleQuots growth supplement (CC-3162) were purchased from Cambrex Bio Science, USA. The other reagents used for cell culture and tests included Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS; Invitrogen, USA); phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, Japan); collagen type I (Cellmatrix Type I-A; Nitta Gelatin, Japan); fluorescent diacetate (FDA) and ethidium bromide

(EB; Wako Pure Chemical Industries, Japan); DAPI (Sigma-Aldrich); and rhodamine–phalloidin (Cytoskeleton, USA).

The materials used in the fabrication of culture substrates were as follows: glass wafers (No. 7740; diameter 3 inches; thickness 500 μm ; Corning, USA); glass rods (diameter 600 μm ; length 3.2 cm; Hirschmann Laborgeräte, Germany); and synthetic oligopeptide (CCRRGDWLC; Sigma-Aldrich). All other chemicals were purchased from Wako Pure Chemical Industries, unless otherwise indicated.

2.2. Modification of gold substrates with the oligopeptide

The oligopeptide CCRRGDWLC contains an RGD domain in the centre and cysteine residues at both ends (Figure 1A). The oligopeptide is designed such that cells adhere onto a gold surface via the oligopeptide and are detached by an electrical stimulus. A gold surface was prepared by sputter-coating a layer of Cr a few nanometers thick and a 40 nm layer of Au on either a glass wafer or a glass rod (diameter 600 μm). The wafer was then cut into 15 \times 10 mm pieces. These gold surfaces were modified by immersing them into 0.5 μM aqueous solutions of the oligopeptide overnight at 4 $^{\circ}\text{C}$. After washes with double-distilled water, the substrates were sterilized with 70% ethanol before cell culture.

2.3. Monitoring of adsorption and electrochemical desorption of the oligopeptide

The adsorption of the peptide onto the gold surface was monitored using a quartz crystal microbalance (QCM, AFFINIX-QN; Initium, Tokyo, Japan). Gold electrodes on

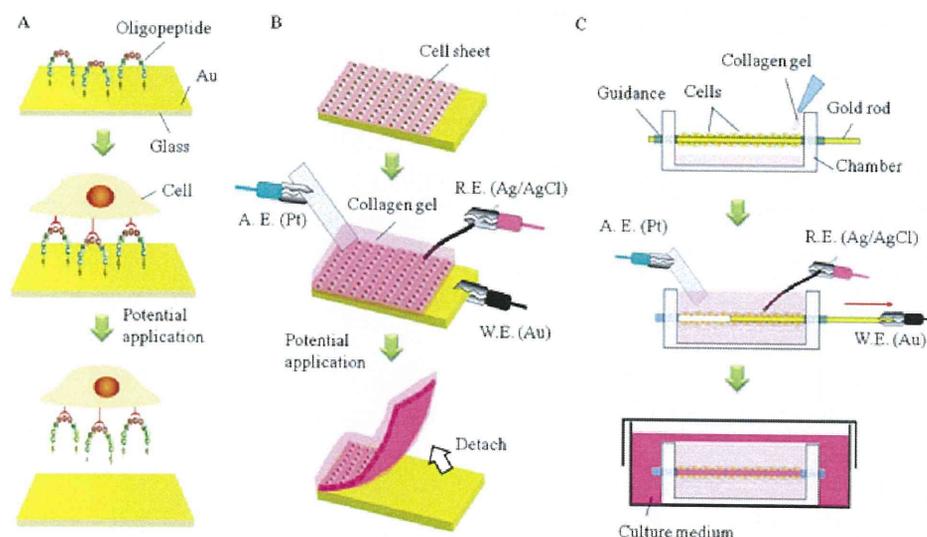


Figure 1. Detachment of cells by application of an electrical potential. (A) Principle of cell detachment: cells adhered to the gold surface via the adsorbed oligopeptide, CCRRGDWLC, were detached during the reductive desorption of the oligopeptide. (B) Cell sheet: cells were grown to form a cell sheet on the gold surface. After addition of a collagen gel to facilitate handling, the cell sheets were detached from the surface by the application of an electrical potential and collected as single entire cell sheets. (C) Capillaries: thin gold rods covered with HUVECs were aligned in a chamber. Upon application of a potential and extraction of the rods from the chamber, the cells were transferred onto the surface of capillaries in collagen gel. Subsequent culture induced migration and sprouting in the gel

Electrical cell detachment

the QCM (250 ± 50 nm layer of gold) were cleaned with piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$, 3:1) and 1% sodium dodecyl sulphate. The QCM was then set in 8 ml pure water, and 4 μL of a 1 mM aqueous solution of the oligopeptide (final concentration: 0.5 μM) was added with stirring. The amount of adsorbed oligopeptide was determined by the change in resonance frequency according to Sauerbrey's equation.

Cyclic voltammetry was employed to determine the reductive potential for desorption of the oligopeptide adsorbed on the gold surface. Immediately before cyclic voltammetry was performed, an electrolyte solution containing 0.5 M KOH was deoxygenated by bubbling nitrogen gas for 20 min. The oligopeptide-modified gold substrate, a Ag/AgCl reference electrode (No. 2080 A; Horiba, Tokyo, Japan), and a platinum auxiliary electrode were set in the electrolyte solution and connected to an electrochemical measurement system (Autolab; Metrohm Autolab, The Netherlands). A cyclic voltammogram was recorded at the scanning rate of 20 mV/s in the range 0–1.0 V three times. All potential values refer to those measured with respect to a Ag/AgCl electrode.

Changes in surface topography after adsorption and desorption of the oligopeptide were determined by atomic force microscopy (AFM; 5500 AFM, Agilent Technologies, CA, USA) using the acoustic AC mode with a single crystal Si tip with a resonant frequency of 290.3 kHz and a scan speed of 1 $\mu\text{m}/\text{s}$. All images were obtained under atmospheric pressure at room temperature. Images were analysed using commercial DI software; PicoImageBasic (Agilent).

2.4. Preparation of cells

Swiss 3T3 murine fibroblasts were maintained in DMEM supplemented with 10% FBS. HUVECs were maintained in EBM-2 supplemented with SingleQuots growth supplement. HUVECs from passages 3–8 were used for experiments. The media were changed every other day. Cell passage was conducted with a solution of 0.25% trypsin and 0.02% ethylenediamine tetra-acetic acid (EDTA) after 3–4 days.

2.5. Detachment of single cells

Fibroblasts (0.5×10^5 cells/ml) in 2 ml culture medium were seeded on the flat substrate modified with oligopeptide and were cultured for 2 days at 37 °C in 5% CO_2 in a humidified incubator. The substrates were then washed with PBS three times and connected to a potentiostat (HA-151; Hokuto-Denko, Tokyo, Japan). A Ag/AgCl reference electrode and a platinum auxiliary electrode were also set in the PBS solution and connected to the potentiostat. After application of a potential of –1.0 V for 5 min, the substrates were gently washed with PBS and the attached cells were counted under a microscope. The solution containing the detached cells was transferred into a conventional culture dish to investigate whether

the reseeded cells maintained their ability to grow after electrochemical detachment.

Phase-contrast images of the cells on the gold coated surface were obtained using an Olympus inverted microscope (IX-71, Olympus Co., Japan). Shape features (projected area, perimeter) of individual cells were extracted from the phase-contrast images using image analysis software (Photoshop, Adobe Systems, CA, USA). Cell circularity was calculated using the following formula:

$$\text{circularity} = 4\pi(\text{area})/\text{perimeter}^2$$

as previously described (Kazmers *et al.*, 2009). A circularity of 1.0 indicates perfect circular morphology and 0.0 indicates a line.

2.6. Scanning electron microscopy

To observe cells on the glass substrate under a scanning electron microscope (SEM), we washed the culture three times with PBS and fixed with a mixed solution of 2.5% glutaraldehyde and 2% formaldehyde in PBS for 1 h at room temperature. Thereafter, the culture was washed with PBS and fixed with 1% osmium tetroxide in PBS for 1 h at 4 °C. The culture was washed with purified water and dehydrated with a graded ethanol series in the range 30–90% on ice and absolute ethanol substitution three times at room temperature. The solution was further substituted with 100% *t*-butanol, which was frozen at 4 °C and dried by vacuum freeze drying. The cells were observed under an SEM (TM-5000; Hitachi, Japan) operated at 15 kV.

2.7. Detachment of cell sheets

Fibroblasts (1.25×10^5 cells/ml) in 2 ml culture medium were seeded onto the flat substrates. The cells were cultured for 7 days until the cells covered the entire surface of the substrate. The cell sheets were then detached by application of a voltage of –1.0 V for 10 min. Cell viability after the detachment was evaluated using a live/dead fluorometric assay with FDA and EB.

To stain a cross-section of cell sheets with haematoxylin and eosin (H&E), a few drops of type I collagen solution (0.24% w/v) were poured onto the sheet and gelled in a humidified incubator for 30 min at 37 °C to facilitate the subsequent manipulations. Subsequently, by application of a potential of –1.0 V for 10 min and peeling off the gel layer, the cell sheet covered with collagen gel was collected from the culture surface (Figure 1B). The sheet was fixed with 3.7% formaldehyde in PBS, embedded in paraffin and sectioned. H&E staining was performed after the general procedure.

2.8. Fabrication of capillary-like structures

The chamber used for the formation of capillary-like structure (Figure 1C) was fabricated with a poly(methyl

methacrylate) plate by using computer-aided laser machining (Laser PRO C180; GCC, Taiwan). The volume of the chamber was 0.5 ml. The chamber had three pairs of holes of 800 μm diameter at intervals of 500 μm for the guidance of the gold rods.

The gold rods (600 μm in diameter) modified with the oligopeptide were placed in a non-adherent 35 mm dish (Techno Plastic Products, Switzerland) and seeded with HUVECs (3.0×10^5 cells) in 2 ml culture medium. The cells, which adhered to the gold rods, were cultured and grown to reach confluence for 3–4 days.

The gold rods with cells were transferred to the chamber and 0.5 ml of the collagen solution was poured into the chamber and gelled in an incubator at 37 $^{\circ}\text{C}$. Subsequently, a potential of -1.0V was applied for 5 min and the rods were carefully extracted. Once the construct was generated, the chamber was either immersed in the culture medium for stationary culture or connected to a syringe pump for perfusion culture. After 3 days of culture, the cells were stained with DAPI and rhodamine-phalloidin to visualize the coverage of the internal surface of the channel. After 3 days of culture, PMA was added to the culture medium at a final concentration of 20 ng/ml to

accelerate spontaneous vascularization (Gamble *et al.*, 1993; Montesano and Orci, 1985). The culture was continued for an additional 14 days and luminal structures were observed using a phase-contrast fluorescent microscope (IX-71; Olympus, Japan).

3. Results and discussion

3.1. Dynamics of adsorption and desorption of the oligopeptide

A QCM was used to monitor the adsorption of the oligopeptide on a gold surface over time. The adsorption reached 95% of the final equilibrium value within 10 min (Figure 2A). This adsorption time is consistent with the results of previous studies on adsorption of alkanethiol molecules (Uosaki *et al.*, 1991; Yamada and Uosaki, 1997). The amount of adsorbed oligopeptide at 30 min was estimated to be 0.18 nmol/ cm^2 . This amount is sufficiently high for the adhesion of cells because typical cases require a maximum of ~ 20 pmol/ cm^2 (Houseman

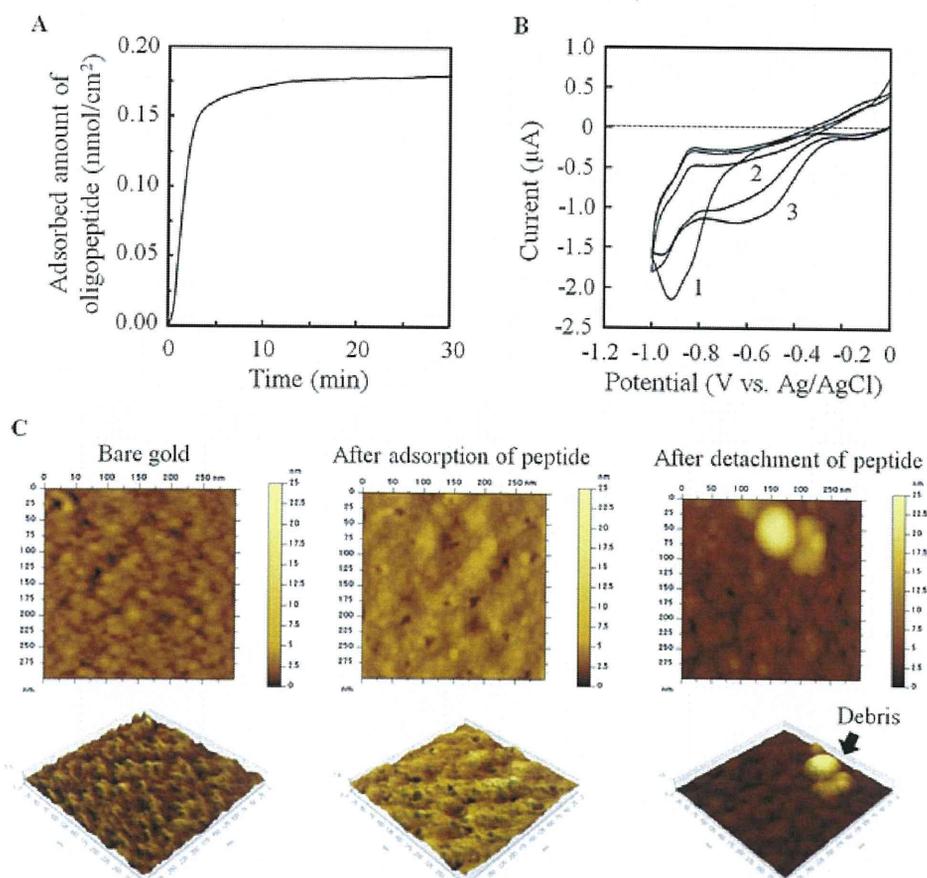


Figure 2. Dynamics of adsorption and desorption of the oligopeptide. (A) Adsorption of the oligopeptide monitored using a QCM: the adsorption reached 95% of the final equilibrium value within 10 min. (B) Cyclic voltammogram obtained during the reductive desorption of the oligopeptide. The labelled digits indicate the scan number. Cyclic voltammograms were recorded at a scanning rate of 20 mV/s with respect to a Ag/AgCl reference electrode. The working electrode area was 4.9 mm². (C) AFM images of the bare gold surface and the gold surface after adsorption and desorption of the oligopeptide. The bottom row shows 3D representations of each image. The scanning areas are 300 × 300 nm

Electrical cell detachment

and Mrksich, 2001). Meanwhile, cyclic voltammetry was used to evaluate the desorption of the oligopeptide from the gold surface. The peak potential for reductive desorption of the oligopeptide was -0.88V in the first scan (Figure 2B). In the second and third scans, the apparent peak at -0.88V disappeared, thus indicating that the oligopeptide was readily desorbed by the first potential scan. Broad peaks emerged in the vicinity of -0.6V in the second and third scans. This is likely attributable to penetration of exogenous oxygen during the measurements. On the basis of the results, a potential of -1.0V was selected for subsequent cell detachment experiments.

AFM analysis was carried out to investigate changes in surface topography after the adsorption and desorption of the oligopeptide (Figure 2C). The analysis showed that the bare gold surface was rough and irregular, with a maximal height difference of $\sim 25\text{nm}$. This topography is probably attributable to the selection of a very thin gold layer (40 nm height) to allow cell observation by phase-contrast microscopy. A longer sputtering period and annealing processes may be required to obtain a smoother surface at the nanometer level. It is important to note that the processes of adsorption and desorption of the peptide are affected by gold substrate topography, as previously reported for thiolate coatings (Cortes *et al.*, 2009). It should also be noted that the gold layer on the QCM is

thicker (250 nm height) but not treated by thermal annealing. The topography of the gold surface and its relationship to cell detachment will be our focus of attention in future investigations. After immersion in the peptide solution, the oligopeptide became spontaneously adsorbed across the entire surface with some small defects, which were visible as black portions (Figure 2C). After application of the electrical potential, the peptide was desorbed from the surface. Some debris was identified in aggregates (arrow) on the surface (Figure 2C). This appears to be a part of adsorbed oligopeptide. Further examinations will be required to clarify the details of this observation.

3.2. Detachment of single cells by electrochemistry

Fibroblasts became readily attached and were dispersed on the surface (Figure 3A). After 2 days of culture, the cells were detached on application of the potential for 5 min. The cells were gradually detached and appeared bright and round (Figure 3B). In this process, the projection area of the cells decreased to 48% and circularity increased from 0.39 to 0.67 (Figure 4A). After 5 min of application of the potential, most of the cells ($82 \pm 7.4\%$)

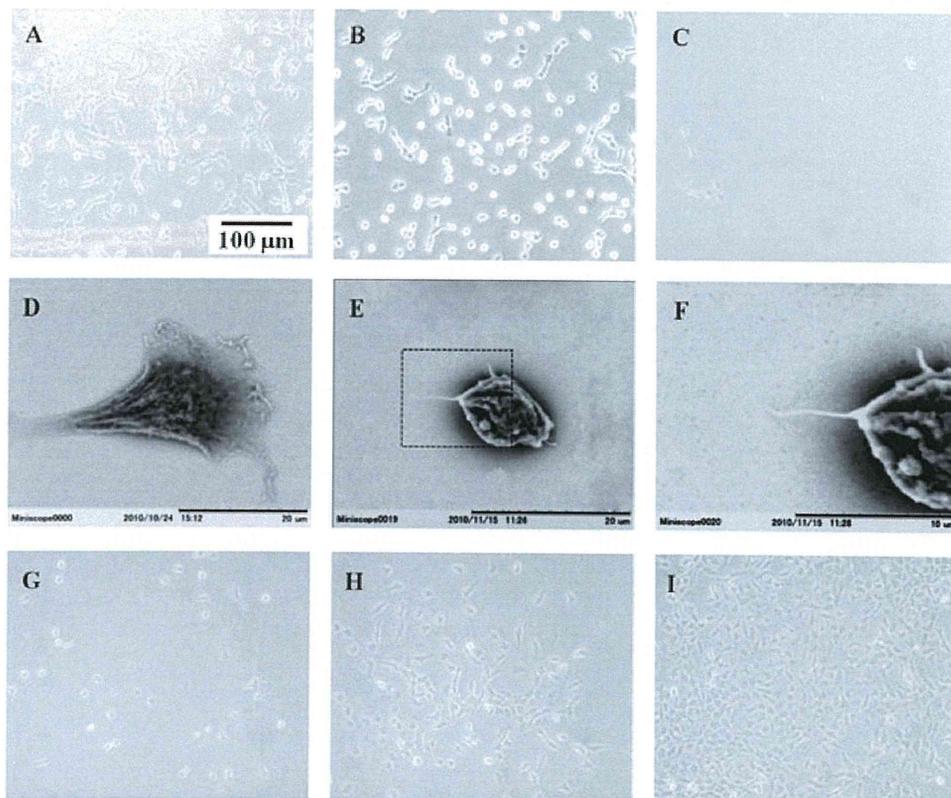


Figure 3. Fibroblasts on the gold surface modified with the oligopeptide. (A) Cells cultured for 2 days. (B) Cells after application of -1.0V for 5 min; the cells appeared bright and round. (C) The gold surface after gentle rinsing. (D – F) SEM images of fibroblasts before (D) and after application of the potential (E). (F) A magnified view of the square in (E). (G – I) Proliferation of cells that were reseeded in a culture dish 1 (G), 2 (H) and 3 days (I) after application of the potential

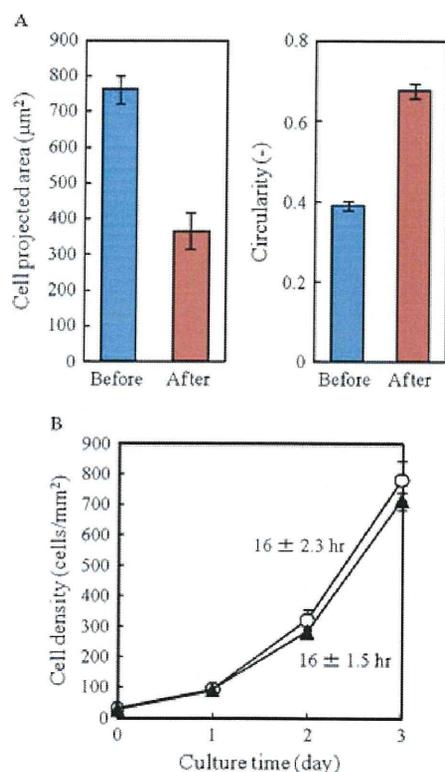


Figure 4. Quantitative characterization of cells detached by application of the potential. (A) Changes in the area and circularity of cells before and after potential application. (B) Proliferation of cells detached and reseeded on a conventional dish. White circles, electrically detached cells; black triangles, trypsinized cells; error bars, standard deviations (SDs) calculated from three independent experiments for each plot. Digits in graph indicate average doubling times after 3 days of culture

were withdrawn into a micropipette and found to be detached (Figure 3C). SEM images also indicated that fibroblasts were dispersed on the surface and appeared round after the potential was applied (Figure 3D, E). In addition, this analysis showed that the rounded cells were not completely detached and remained loosely anchored on the surface with filopodium-like structures. However, these connections were readily disrupted by gentle pipetting.

Approximately 18% of the remaining cells did not significantly detach, even when the time of application of the potential was extended to 7 min. This suggests that these cells were non-specifically adhered to the surface via adsorbed proteins or to the defective portions of the peptide coating identified in the AFM analysis. We have previously shown that fibroblasts seeded onto a gold surface modified with an alkanethiol self-assembled monolayer could be detached by the application of potential (Inaba *et al.*, 2009). In this previous study, $99 \pm 0.3\%$ of the cells were detached. Thus, the dense alkanethiol monolayer has superior characteristics in terms of cell retrieval. Recently we further demonstrated that the alkanethiol monolayer on micropatterned electrodes can be used to detach cells in succession in a spatially controlled manner with single-cell resolution (Fukuda *et al.*, 2011). However, it is important to consider that alkanethiol and

similar organic chemicals may remain in the detached tissues and potentially cause inflammatory responses or other adverse reactions after transplantation of the engineered tissue. The use of oligopeptides is expected to be a promising alternative to avoid this biocompatibility issue. Indeed, this class of molecule is naturally present in the human body and can thus be metabolized to amino acids, either by means of enzymatic processes or by simple hydrolysis. To optimize the process of cell retrieval, new oligopeptide designs are now under investigation with the objective of designing dense self-assembled monolayers on a surface that would avoid unspecific protein adsorption.

To evaluate whether cell viability is affected by the electrochemical desorption process, cells were withdrawn using a pipette and transferred to a conventional culture dish, where they were subsequently cultured. These cells dispersed on the surface and proliferated (Figure 3G–I). The proliferation rate of the detached cells (doubling time, 16 ± 2.3 h) was comparable to the doubling time of a typical subculture (16 ± 1.5 h) (Figure 4B) and the doubling time reported in the literature (16.6 h) (Rossow *et al.*, 1979). These results indicate that the proposed oligopeptide and electrical detachment did not exert a significant adverse effect on the cells.

3.3. Detachment of cell sheets

Fibroblast sheets were also detached from the surface by the application of a negative potential (Figure 1B). Fibroblasts were cultured and grown to confluence for 7 days on the gold substrate with the oligopeptide. Application of -1.0 V to the surface caused detachment of a single-layered cell sheet (25×25 mm) within 10 min (Figure 5A). Live/dead fluorescent staining showed that all of the cells in the harvested cell sheet were viable, with negligible amounts of dead cells (Figure 5B). The cross-section of the cell sheet was composed of a few layered cells with close cell–cell connections (Figure 5C). As we have previously reported with alkanethiols (Inaba *et al.*, 2009), the cell sheets were easily stacked with other cell sheets in succession to form a multilayered cell sheet (data not shown). We could find only one study concerning the use of electrochemical approaches for the detachment of cell sheets. In this reported study, a polyelectrolyte layer electrostatically adsorbed on an electrode was desorbed by application of a positive potential (Guillaume-Gentil *et al.*, 2008). The desorption mechanism was considered to rely on a local pH change for subsequent dissolution of the polyelectrolyte layer. With this technique, fibroblast sheets were detached by application of $+1.8$ V for 30 min. However, this approach may have a drawback. The pH change produced by application of a potential beyond the electrical potential window of the electrode for a long period time may exert harmful effects on the cells. More recently, the same group reported a faster and less invasive approach using mesenchymal stem cells, based on the same principle (Guillaume-Gentil *et al.*, 2011). We believe that the emerging electrical cell sheet

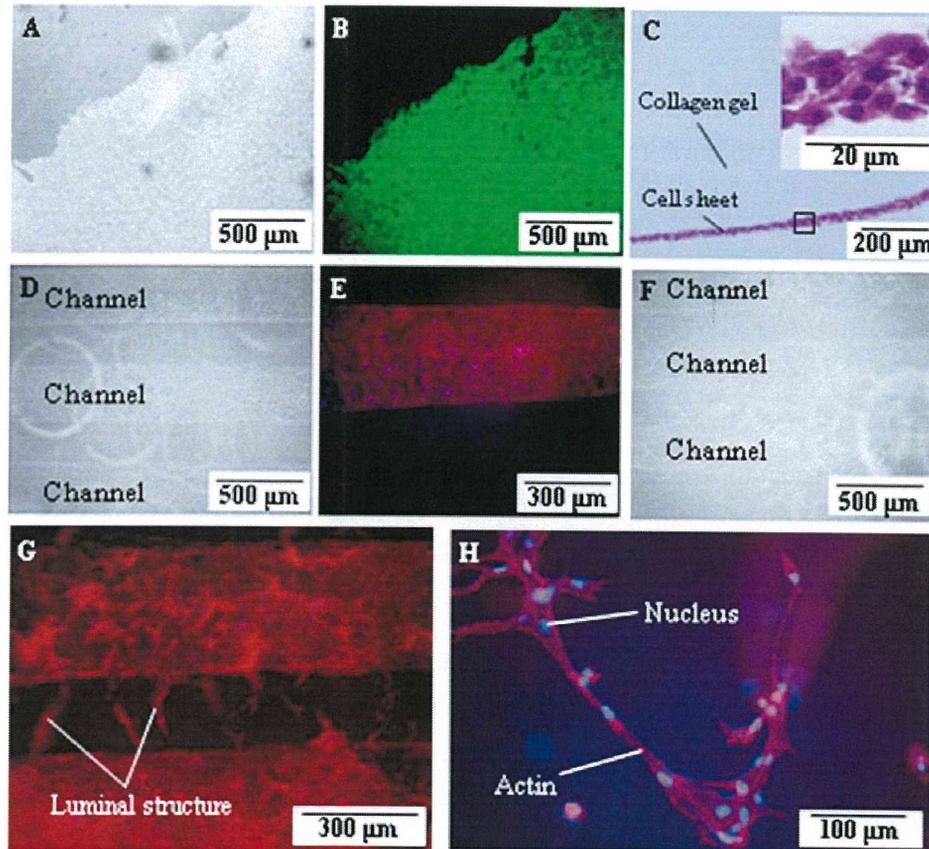


Figure 5. Detachment of cell sheets and formation of capillary-like structures. (A) Fibroblasts were grown to form a cell sheet for 7 days and were then detached by the application of electrical potential (-1.0 V for 10 min). (B) The fluorescent staining of live and dead cells shows that almost all of the fibroblasts in the sheet were viable (green) with a negligible number of dead cells (red). (C) Haematoxylin and eosin staining of the detached fibroblast sheet. Inset image shows the magnified view of the region enclosed by the square. (D) HUVECs were transferred from gold rods to the internal surface of capillary structures in a collagen gel. (E) Nucleus and F-actin staining of the HUVECs adhering to the internal surface. (F) HUVECs migrated and sprouted, and eventually formed luminal structures in the collagen gel after 17 days of culture; arrows indicate channels. (G) HUVECs stained with rhodamine-phalloidin after 7 days of perfusion culture. (H) Magnified view of luminal structures; double staining with rhodamine-phalloidin and DAPI

engineering approaches, including our proposed technology, hold great promise for future application in regenerative medicine.

3.4. Fabrication of capillary-like structures in collagen gel

One of the major obstacles in the engineering of thick tissue constructs is the need to fabricate vascular networks throughout tissues (Laschke *et al.*, 2006; Fukuda *et al.*, 2004, 2005). To address this issue, we modified our approach to fabricate capillary-like structures (Figure 1C). Rods covered with HUVECs were aligned in the chamber and a collagen solution was poured and allowed to form a gel. After application of a potential of -1.0 V for 5 min, the rods were carefully extracted from the chamber through guide holes. The cells were transferred from the gold rods to collagen, thereby creating capillary-like structures (Figure 5D). The distance between the capillaries

was $\sim 500\text{ }\mu\text{m}$ and the longitudinal length was 16 mm. The nuclei and F-actin of the cells were fluorescently stained, and the results showed that the internal surface of the capillary was densely covered with cells after 3 days of culture (Figure 5E). A culture medium was then perfused through the capillaries at a flow rate of $10\text{ }\mu\text{l}/\text{min}$. Although the structure was initially rough and primitive, angiogenesis occurred throughout the endothelial cells on the surface. This eventually induced connections between the adjacent capillary structures in perfusion culture (Figure 5F–H). This phenomenon is an example of the well-known angiogenic process, which occurs in collagen gel. The perfusion of culture medium was not necessarily required for oxygen supply to HUVECs in this experiment. However, the introduction of a second cell population (e.g. metabolically active hepatocytes) into the collagen would require a prompt initiation of culture medium flow to satisfy oxygen and nutrient demands. This approach can potentially be used for engineering of vascularized tissue structures.