Communications to the Editor

Design and Biosynthesis of Elastin-like Artificial Extracellular Matrix Proteins Containing Periodically Spaced Fibronectin CS5 Domains

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Synthetic polymers have attained a dominant position in materials science and technology largely on the basis of their excellent physical and mechanical properties. The more subtle chemical and biological properties of natural polymers, especially of the proteins and nucleic acids, have been difficult to capture in synthetic macromolecular materials, in part because these properties arise from microstructural features that cannot be controlled in statistical polymerization processes. We describe herein the use of artificial genes to direct the synthesis of polymers of precisely controlled architecture,[1] in which biological function—specifically, the capacity to support attachment of vascular endothelial cells—is the primary object of the design. A long-term objective of this work is the development of improved materials for the regeneration, replacement or repair of vascular tissue.

Surgical reconstruction of small- and medium-diameter blood vessels is exceedingly difficult. The material of choice for vascular reconstruction in the lower leg is autologous saphenous vein if it is available and healthy; unfortunately, the success rates for such reconstructive procedures are generally only about 70% after 5 years.[2] Poly(tetrafluoroethylene) and poly(ethylene terephthalate) have also been used for small- and medium-caliber grafts; however, patency rates for these materials are even lower than those for saphenous vein.[2b,3] Failure most often occurs through thrombosis and occlusion of the graft or through neointimal hyperplasia at the junction between the graft and the surrounding tissue. New materials are needed for the construction of improved vascular prosthetics.

In an attempt to address this need, we report herein the preparation of artificial extracellular matrix proteins[4] comprising two kinds of elements: (i) a repeating unit structure $(GVPGI)_x$[5] related to mammalian elastin and (ii) a cell-binding domain (designated CS5) derived from the natural extracellular matrix protein fibronectin.[6] Our choice of the elastin-like repeating unit was based on the extensive work of Urry and co-workers[7] on the family of polypentapeptides represented as $-(GVPGZ)_x-$, where Z can be any of a wide variety of amino acid residues; the specific choice of Z = I was dictated by the anticipated thermal transition behavior of the polymer (vide infra). Urry has suggested the use of elastinlike polypeptides in a vascular graft design in which the intimal layer bears peptide signals for endothelial cell attachment.[8] The results reported here relate directly to this proposal, in that the CS5 region of fibronectin contains the REDV sequence previously shown to support attachment and spreading of endothelial cells, but not smooth muscle cells or platelets, on artificial surfaces.[9] We describe here the microbial expression of artificial extracellular matrix proteins carrying CS5 domains, and we demonstrate that such proteins do in fact support attachment and spreading of vascular endothelial cells.

The target polymers can be represented by **1a** and **1b**, wherein *CS5* designates the eicosapeptide sequence **2**.

$MG[LDCS5(GVPGI)_x]_yLE$ **1a:** $x = 40$, $y = 3$

b: $x = 20$, $y = 5$

−GEEIQIGHIPREDVDYHLYP− **2**

Oligonucleotides encoding the CS5 and $(GVPGI)_5$ domains were synthesized via the phosphoramidite method,[10] purified, annealed, sequenced, and assembled into full-length sequences encoding **1a** and **1b**.[11] The coding sequences were ligated into the unique XhoI site of plasmid pET28ap.[12] The resulting vectors, designated pET28ap[CS5$(GVPGI)_{40}]_3$ and pET28ap[CS5$(GVPGI)_{20}]_5$ respectively, were used to transform the *Escherichia coli* expression host BL21(DE3)pLysS.[13] Protein expression, under control of a bacteriophage T7 promoter, was accomplished in 2xYT medium containing chloramphenicol (34 mg/mL) and kanamycin (34 mg/mL). The cultures were grown to $OD_{600} = 0.8$ at 37 °C and protein expression was induced by the addition of isopropyl *β*-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. Cells were harvested after 3 h by centrifugation (5000*g*, 15 min, 4 °C) and frozen prior to purification.

Protein purification was achieved through a simple procedure involving selective precipitation of contaminating proteins below the lower critical solution temperatures (or "inverse temperature transitions," T_t) of the target proteins.[14] Yields of 60 mg/L of **1a** (molecular weight 59 765) and 40 mg/L of **1b** (molecular weight 56 984) were obtained.

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Table 1. Analysis of Target Protein Purity As Determined by Amino Acid Analysis

	1a		**1b**	
amino acid	theor (mol %)	exptl (mol %)	theor (mol %)	exptl (mol %)
Asp/Asn	1.34	2.31	2.44	2.22
Glu/Gln	1.94	3.25	3.42	3.03
Ser		0.64		0.37
Gly	36.9	33.9	34.4	34.0
His	0.9	0.95	1.63	1.19
Arg	0.45	0.82	0.81	0.89
Thr		0.01		0.35
Ala		1.11		0.72
Pro	18.8	16.9	16.3	17.3
Tyr	0.9	1.09	1.63	1.34
Val	18.4	17.6	17.1	17.7
Met	0.15	0.21	0.16	0.16
Ile	19.2	17.8	18.7	18.1
Leu	1.04	1.79	1.79	1.92
Phe		0.30		0.21
Lys		0.62		0.44

^{1}H and ^{13}C NMR spectra (obtained in formic acid on a Bruker AMX-500 instrument) of **1a** and **1b** could be readily assigned on the basis of the predominant −(GVPGI)− repeating unit sequence. The observed spectra are in excellent agreement with those reported by McPherson et al. for recombinant $(VPGVG)_x$ with the exception of changes due to substitution of isoleucine for valine in the repeating pentapeptide.[15] Amino acid analysis results for **1a** and **1b** are consistent with the expected polymer compositions within the uncertainty of the measurement, and with the presence of the CS5 regions at the proper levels (Table 1).

Surface coatings of **1a** and **1b** were prepared on 12 mm glass coverslips for subsequent culture of human umbilical vein endothelial cells (HUVECs, Clonetics). Coverslips were coated with **1a** or **1b** from solutions in formamide or water by spreading 40 mL of a 1 mg/mL protein solution over the surface of the coverslip with the tip of a pipet. Fibronectin was coated similarly by using 20 mL of a 100 mg/mL solution in water. Coated coverslips were dried in vacuo at 55 °C, incubated for 2 h at 37 °C in sterile water (0.5 mL) to remove loosely bound protein, and dried again at 55 °C for 2 h. Scintillation counting of coverslips coated with radiolabeled protein (^{3}H-glycine) confirmed that ca. 2 mg of **1a** and 0.5 mg of **1b** remained on the surface after six 30-min washes with phosphate-buffered saline (PBS) solution. Uniformity of the protein coating was verified by scanning electron microscopy.

Cell culture was performed in serum-free M199 medium (Gibco). HUVECs in passages 2−10 were used for all cell counting experiments. HUVECs were harvested from tissue culture polystyrene using 0.02% EDTA in PBS and resuspended in serum-free M199 at a density of 10^4 cells/mL. Aliquots (1 mL) of the cell suspension were transferred to 24-well dishes containing protein-coated coverslips. Cells were maintained at 37 °C and 5% CO_2 for 4 h. Coverslips were removed from the wells, washed three times with PBS plus $CaC1_2$ (0.1 g/L) and $MgC1_2$ (0.1 g/L) and placed in new 24-well plates. For cell counting, endothelial cell medium (phenol red free, 0.4 mL, Clonetics) and 0.2 mL of XTT cell counting reagent (Boeringer Mannheim) were added to each well. Cells were returned to the CO_2 incubator for 20 h and the absorbance of the medium was read at 460 nm. Calibration curves were prepared for each cell culture experiment, with the number of cells per well varied from 250 to 10 000. Table 2 shows the results of cell counting for each protein surface. Substrates coated with the artificial protein **1b** or with the natural extracellular matrix protein fibronectin bound essentially all plated cells. Significantly fewer cells attached to **1a** or to the uncoated glass surface used as the negative control. Figure 1 compares the morphologies of HUVECs plated on glass and on **1b**, and confirms that attachment to the protein substrate is accompanied by significant cell spreading. It is interesting to note that the areal density of CS5 ligands in the **1b** film (assuming all sites are available for binding) is approximately 10^5 sites per square micron. This compares to the reported value of 5.8×10^6 sites for the corresponding receptor on the endothelial cell surface.[9] Preliminary measurements show that attachment of HUVECs to polymer **1b** is inhibited by the soluble CS5 analogue GREDVDY (Research Genetics), consistent with receptor-mediated cell adhesion to the artificial extracellular matrix protein. Control experiments with the soluble CS5 analogue revealed no inhibition of the attachment of HUVECs to fibronectin. The latter result is expected in view of the fact that fibronectin presents several cell-binding domains in addition to CS5.[8]

Table 2. Adhesion of HUVECs on Glass and Protein Surfaces[a]

protein/cast from	no. of cells on surface
glass	2880 ± 1670
1a/formamide	1150 ± 360
1a/water	2590 ± 570
1b/formamide	9170 ± 1800
1b/water	11700 ± 760
fibronectin/water	10140 ± 290

[a] 4 h in serum-free medium; 10 000 cells plated per well. Adherent cells given as mean ± standard deviation (n = 4).

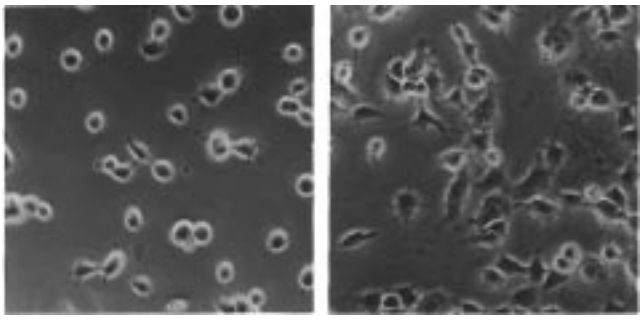

Figure 1. Phase contrast micrographs of HUVECs plated on glass (left panel) and on **1b** (right panel). Cells were plated in 24-well dishes at a density of 5×10^4 cells/well, and maintained at 37 °C and 5% CO_2 for 4 h before fixing in 3.7% formaldehyde. The width of the field in each panel is ca. 300 μm.

Although endothelial cell attachment to **1b** was anticipated on the basis of the known adhesive properties of REDV peptides,[9] the successful attachment reported here stands in apparent contradiction to an earlier observation made by Urry and co-workers.[16] In an exploration of the adhesive properties of polypeptides of repeating unit structure (GVPGV), Nicol et al. found that incorporation of short REDV peptides (at approximately the same frequency as in **1b**) failed to promote endothelial cell attachment above background.[16] Several potential explanations for these different results may be proposed: (i) the longer CS5 region may be a more effective adhesion ligand than the short REDV inserts examined by Nicol et al.,[16] owing to differences

in conformation or in interactions between the adhesion receptor and flanking residues, (ii) the $(GVPGI)_x$ background used here may facilitate ligand−receptor interactions that are weak or absent in the $(GVPGV)_x$ polymers, (iii) the radiative cross-linking step used by Nicol et al. may have damaged the adhesion ligand, or (iv) the use of trypsin by Nicol et al. in the harvest of their endothelial cells may have damaged the adhesion receptor.[17] We believe that this last explanation is most likely, since we found markedly reduced adhesion on **1b** when the test cells were harvested with trypsin rather than with EDTA.

Further studies of the biological and mechanical properties of artificial extracellular matrix proteins are underway.

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(5) The single-letter amino acid code is as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

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(12) pET28ap was constructed by insertion of an 18-base pair linker between the NcoI and XhoI sites of pET28a (Novagen). Insertion eliminated the XhoI site of pET28a, but created a new XhoI site with flanking translational start and stop signals. This construction encodes minimal N- and C-terminal fusion sequences in polymers **1a** and **1b**.

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(14) **1a** exhibits a T_t of 13 °C (40 mg/mL in H_2O); for **1b** T_t is 12 °C. These values are in excellent agreement with the T_t of 10 °C predicted by Urry for polymers of the $(GVPGI)_x$ repeating unit sequence (Urry, D. W. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 819). The choice of Z = I, with T_t below room temperature, allows handling of the polymer at room temperature and at 37 °C without complications associated with the dimensional changes that occur at T_t.

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