Studies towards Photochemical nanopatterning using a Caged Peptide

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Abstract: Patterning of cell adhesion molecules onto surfaces has become an important technique in the construction of cellular assemblies for biosensors, tissue engineering applications, and studies for cell-cell and cell-substrate interaction. The ability to study cell behavior at a biomaterial surface requires control of material surface chemistry. In this project the GRGDS (Gly-Arg-Gly-Asp-Ser) and GRGES (Gly-Arg-Gly-Glu-Ser) were immobilized by conjugation to surface bound azides to afford cell-receptive modified surfaces. The peptides were synthesized using Fmoc-solid phase peptide synthesis (SPPS) and purified and characterized by high performance liquid chromatography (HPLC). The modified surfaces were characterized using X-ray photoelectron spectroscopy (XPS) and the effect of these surfaces on fibroblast adhesion and spreading were examined at several time points. Our results demonstrate a higher degree of cell attachment and spreading on RGD modified surfaces compared to unmodified and control surfaces. Photoprotection of the carboxyl side-chain of Asp using an ortho-nitrobenzyl protecting group, this will then be extended to form the desired RGD sequence using SPPS, before being attached to the surface via a linker to develop a photo-addressable surface that will facilitate surface patterning.

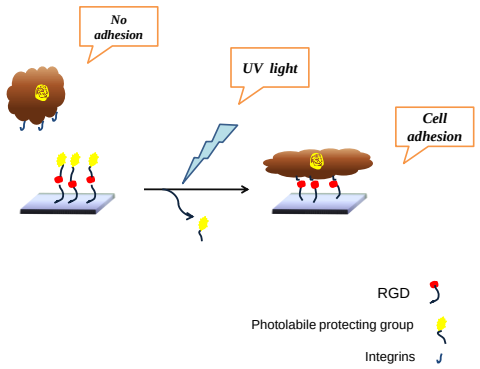
Keywords: RGD peptides, cell adhesion, photoprotecting groups, tissue engineering

Introduction

Cell adhesion is of fundamental importance to a cell; it provides anchorage, cues for migration, and signals for growth and differentiation, mediated by Integrins and extra-cellular matrix proteins (1). Cellular binding sites, like RGD (Arg-Gly-Asp) peptides, have been reported to play a major role in mediating cell adhesion through integrins, which transduce information to the nucleus through cytoplasmic pathways (3, 4). If the surface for cellular decoration does not contain reactive functional groups, then it is necessary to modify it in order to allow covalent immobilization of the peptide to the surface (1). The favorable response of cells to a material's surface is a governing factor in designing functional biomaterials (1, 2). Therefore, it is important to develop a technique to decorate biomaterial surfaces with the peptide in a way that retains the peptide's conformation and activity (2). Surface patterning is modifying surface properties so that one region of the substrate promotes cell attachment and the other region

prevents adhesion (5). Light-controlled cellular attachment requires the development of photo-sensitive molecules able to mediate cellular adhesion and whose activity changes upon irradiation (5, 6). For this Figure 1, shows the presence of the cage on the RGD prevents the cell adhesion which then upon iridation releases the cage and the cells adhere.

Study: we selected the RGD cell-adhesive peptide, and modified it by introducing a photolabile caging group on the carboxylic acid side chain of the apartic acid residue (7, 8). The presence of the caging group prevents recognition of the peptide by the



integrin receptors (10 - 13). The goal of this project was to synthesis the peptide with an azide terminal and imobilize it to premodified surface with an alkyne terminal *via* click chemistry. Then surface patterning using GRGDS peptide modified with a photolabile caging group which upon irradiation with *uv light* by using photolithographic techniques will expose the cell adhesive tripeptide, RGD, in a predetermined pattern.

Materials and Methods

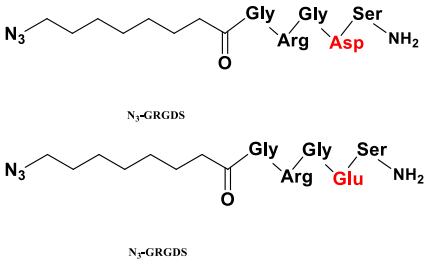
Chemicals and solvents were purchased from standard suppliers and used without further purification. Commercially available amino acids, coupling reagents and resins were purchased from Novabiochem. Round cover slips 12 mm. no. 1 in thickness were used for all surface chemistry manipulations. Mouse fibroblasts (Swiss 3T3 cells) were a gift from the tissue engineering group, fibronectin from bovine plasma was purchased from Sigma Aldrich used in cell culture.

The azide was synthesized using CEM Discover labmate microwave synthesis system and both the azide and alkyne synthesis was monitored by thin layer chromatography on commercially available precoated aluminium backed plates (Merck Kieselgel 60 F254). Visualization was by examination under UV light (254 and 366 nm).
Mass spectra (TOF ES +/-) were recorded on a waters 2795 separation module/micromass LCT platform.
1H-NMR spectra were recorded on a Bruker-AV at 400.13 MHz. Waters HPLC – 2525lc system equipped with a dual wavelength absorbance detector 2487, an auto-sampler injector and mass-lynx v 4.1 was used for HPLC analysis and purification procedures.

Surface analysis was performed using the Kratos AXIS ULTRA XPS and data analysis was carried out using the CASAXPS software with kratos sensitivity factors to determine atomic %values from peak areas.

Results and discusion

Peptide synthesis. The GRGDS and GRGES peptides were synthesized by Fmoc solid peptide synthesis (SPPS) and further derivatized with a pre-synthesised azide linker. The azide-derivatized peptides were therefore chemicaly armed for immobilization to the partner surface–immobilised substrates *via* Cu(I) catalysed chemistry.

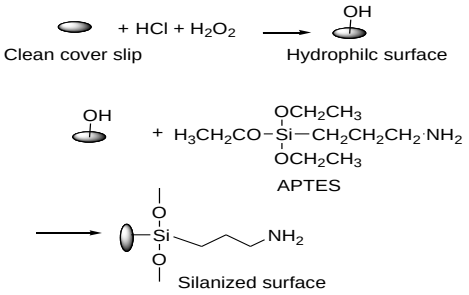


Scheme 1: The modified GRGDS peptide alongside the negative control GRGES.

Surface modification

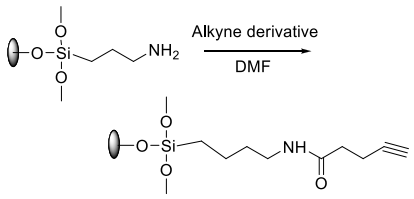
Preparation of substrates. The modification began with the removal of all contaminants; glass cover slips were cleaned then treated with freshly prepared Piranha solution, this way the cover slips were enriched with surface hydroxyl groups and were therefore suitable for functional silanization.

Silanization of substrate. This was achieved by using 3-aminopropyl-triethoxysilane (APTES with an $–NH_2$ functional group).



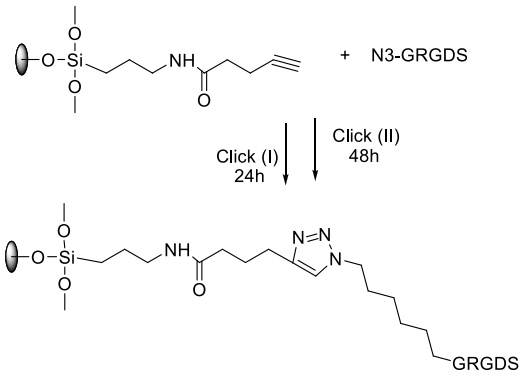
Scheme 2: The piranha treated and silanized cover slips

Alkyne addition to the substrate. The alkyne derivatized linker was then attached to the surface amine to yield a surface possessing an increasing amount of alkyne groups.



Scheme 3: Alkyne addition to the surface

Attachment of the N_3-GRGDS and N_3-GRGES. The reaction can be performed by copper (I)-mediated azide-alkyne cycloaddition



Scheme 4: Peptide immobilization using click chemistry, click (I) using Cu (I) directly and click (II) using Cu (II) sulphate

Table1: Average XPS atomic composition in percentagesandelemental ratios:

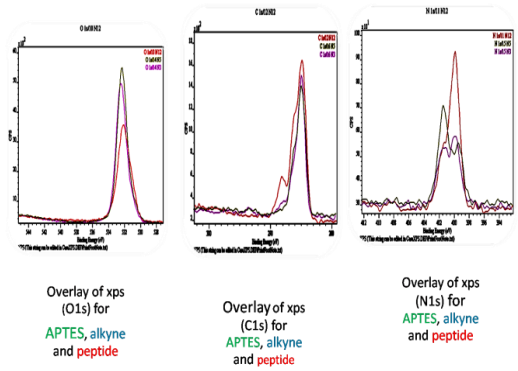
Sample	Concentration/ %									
	O	C	N	Si	F	S	Cl	N/ C	O/ C	N/ Si
Piranha	56.40	16.90		21.55	0.20				3.32	
APTES	44.00	27.70	5.90	19.60	1.10	0.70	1.10	0.21	1.58	0.30
Alkyne	40.00	33.30	5.70	18.10	1.00	1.10	0.50	0.17	1.20	0.30

Sample	Concentration/ %									
	O	C	N	Si	F	S	Cl	N/ C	O/ C	N/ Si
Peptide	27.80	52.90	9.50	7.90	0.30	1.00		0.18	2.92	1.20

XPS Results

XPS, X-ray photoelectron spectro-scopy, analysis was performed on the modified surfaces to ensure the presence of the silane, alkyne derivative and the peptides.

Figure 2: High resolution scans for modified surfaces Piranha treated glass

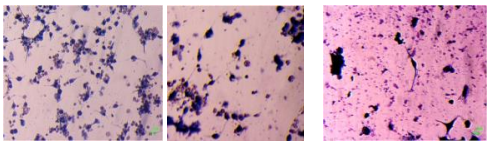


cover slips show no nitrogen detected on the surface, and show largest percentage of oxygen, silicon and other glass components, the carbon presence is due to impurities from processing. Analysis of the APTES coupled surface reveals 5.9% of surface nitrogen, which suggests significant incorporation of the desired silane, the amount of carbon increased signafically by 3.32% to 1.58% decreasing the oxygen to carbon ratio (see Table 1), this is due to the presence of the long hydrocarbon chain in APTES. Addition of the alkyne resulted in an increase in the % of carbon atoms (Table 1). Addition of the peptide using Click I and Click II reactions resulted in an increase in surface nitrogen as can be witnessed from N:Si ratio in Table 1 and observed from tha high resolution scans. The relative amount of nitrogen to silicon increases from 0.21% afteraddition of the silane to 1.20% using Click II, where the peptides are attached to the surface.Once the peptides had been successfully immobilized onto the glass substrate surface they were ready for trials of cell adhesion.

Cell adhesion

The cells were incubated on each surface and viewed at several time points (0.5, 2, 3, 6, 24, 48 hour) under x100 and x400 magnification as shown in the images below. The microscope pictures reveal the response of cells on each surface, and they clearly demonstrate that the coated surfaces with fibronectin and the GRGDS peptide adhered fibroblasts more than the GRGES peptide which showed almost no cell adhesion and also the previous unmodified and modified surfaces which showed no cell adhesion.

Pirahna, APTES Alkyne



Fibronectin GRGDS GRGES

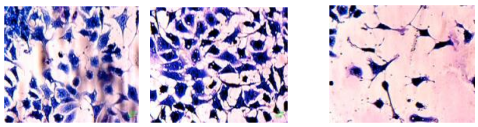


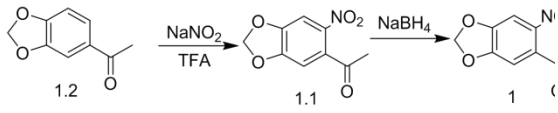
Figure 3: 3T3 mouse fibroblasts before addition of peptides and after adding the peptides compared to fibronectin (positive control) and GRGES (negative control).

Synthesis of photolabile protecting groups

The most common PLPG's are the ortho-nitrobenzyls, the first of which, 6-nitroveratroyloxycarbonyl group (NVOC), was introduced by Patchornik, Amit and Woodward in 1970 (14 - 16). Photo-isomerisation through a radical reaction forms the ortho-nitrobenzaldehyde, esters are therefore converted into a carboxylic acid and a ketone or aldehyde. A major advantage of this group is that the conditions needed for cleavage are exceptionally mild and even tryptophan, the most sensitive amino acid is unaffected (17 - 19).

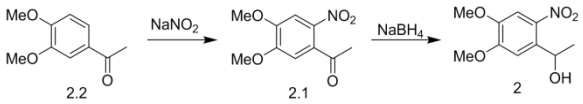
The synthesis of the photolabile protecting groups was carried out via a two-step pathway; nitration followed by reduction.

Methylnitropiperonyl alcohol (9)



(1)

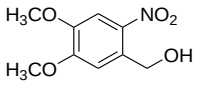
1-(4,5-Dimethoxy-2-nitrophenyl) ethanol



(2)

Sheme 5: step (1) nitration using sodium nitrite ($NaNO_2$). Step (2) reduction from the ketone to the alcohol using sodium borohydride (NaBH4).

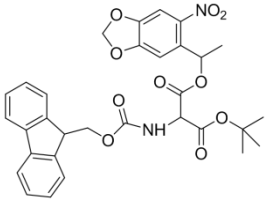
In addition to the synthesised protecting groups, we also employed a comercialy available protecting group (4,5-dimethoxy-2-nitrobenzyl alcohol).



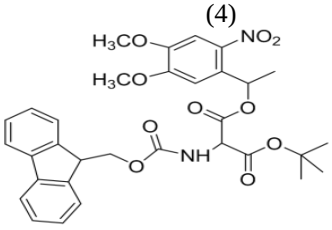
(3)

Linking the PLPGs to Fmoc-Asp(OtBu)-OH

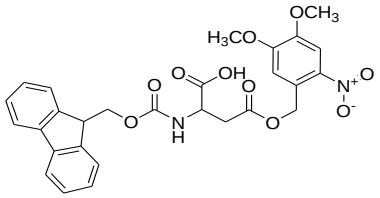
Linking the PLPG's to the terminal carboxylic acid of the amino acids was carried out by esterification using DCC and DMAP and further purification by appropriate flash column chromat-ography affords the pure compound in good yields.



(4)



(5)



(6)

Scheme 6: The modified acid will then be extended to form the desired GRGDS sequence using SPPS, with the combination of the above techniques to develop a photo-addressable surface that will facilitate surface patterning using UV light.

In conclusion: XPS data verified the presence of the chemical elements obtained during the stages of glass-surface chemical modification, including the final step of peptide attachment. 3T3 mouse fibroblasts were clearly sensitive to the differences in surface chemistry and functional groups of the substrate and the cells on each surface generated different morphological, cytoskeleton and adhesion signals.

The work outlined in this report has shown that there is a simple and effective method of synthesizing two different PLPG's; Methylnitro-piperonyl alcohol (1) and 1-(4,5-Dimethoxy-2-nitrophenyl) ethanol (2) through a two step synthesis from commercially available starting materials. The photolabeling protecting groups were linked to the aspartic acid *via* esterification, it was noticed that by using the comercialy available PLPG (3) which is less stericly hinderd produced in high yield than the synthasized groups (1, 2).

References

1. Lee KY and Mooney DJ (2001) Hydrogels for tissue engineering. Chem Rev. 101(7): 1869-1880.
2. Shin H, Jo S and Mikos AG (2003) Biomimetic materials for tissue engineering. Biomaterials. 24(24): 4353-4364.
3. Comoglio PM, Boccaccio C and Trusolino L (2003) Interactions between growth factor receptors and adhesion molecules: breaking the rules. Curr Opinion Cell Biol. 15(5): 565-571.
4. Humphries MJ (1990) The Molecular-Basis and Specificity of IntegrinLigand Interactions. J Cell Sci. 97: 585-592.
5. Humphries JD, Byron A and Humphries MJ (2006) J Cell Sci. 119: 3901.
6. Hersel U, Dahmen C and Kessler H (2003) Biomaterials. 24: 4385.
7. Tatsu Y, Nishigaki T, Darszon A and Yumoto N (2002) FEBS Lett. 525: 20-24 and Wood CD, Nishigaki T, Tatsu Y, Yumoto N (2007) Dev Biol. 306: 525-537.
8. Dechantsreiter M, Planker E, Matha B and Kessler H (1999) J Medicinal Chem. 42: 3033-3040
9. Michael C.Pirrung., (1996) J Bioconjugate Chem. 7: 317-321
10. Bochet CG (2002) Photolabile protecting groups and linkers. J Chem Soc Perkin Transactions. 1(2): 125-142.
11. Wang L, Corrie JT and Wootton JF (2002) Photolabile precursors of cyclic nucleotides with high aqueous solubility and stability. J Org Chem. 67: 3474-3478.
12. Ohmuro-Matsuyama,Y and Tatsu Y (2008) Photocontrolled Cell Adhesion on a Surface Functionalized with a Caged Arginine-Glycine-Aspartate Peptide. Angewandte Chemie International Edition. 47(39): 7527-7529.
13. Govindaraju T, Jonkheijm P, Gogolin L, Schroeder H, Becker CF, Niemeyer CM and Waldmann H (2008) Surface immobilization of biomolecules by click sulfonamide reaction. Chem Communic. (32): 3723-3725.
14. Patchornik A, Amit B and Woodward RB (1970) Photosensitive protecting groups. J Am Chem Soc. 92(21): 6333-6335.
15. Dillmore WS, Yousaf MN and Mrksich M A (2004) Photochemical Method for Patterning the Immobilization of Ligands and Cells to Self-Assembled Monolayers. Langmuir. 20(17): 7223-7231.
16. Matsuda T and Sugawara T (1995) Development of surface photochemical modification method for micropatterning of cultured cells. J Biomed Material Res. 29(6): 749-756.
17. Cheng N and Cao X (2010) Photoactive SAM surface for control of cell attachment. J. Colloid Interface Sci. 348(1): 71-79.
18. Pelliccioli AP and Wirz J (2002) Photoremovable protecting groups: reaction mechanisms and applications. Photochem Photobiol Sci. 1: 441-458.
19. Walker JW, McCray JA and Hess GP (1986) Photolabile protecting groups for an acetylcholine receptor ligand. Synthesis and photochemistry of a new class of o-nitrobenzyl derivatives and their effects on receptor function. Biochem. 25: 1799-1805.