Cell adhesive hydrogels synthesised by copolymerisation of arg-protected Gly-Arg-Gly-Asp-Ser methacrylate monomers and enzymatic deprotection†

Lynne Perlin,[ab] Sheila MacNeil*[b] and Stephen Rimmer*[a]

Received (in Cambridge, UK) 1st August 2008, Accepted 8th September 2008
First published as an Advance Article on the web 9th October 2008
DOI: 10.1039/b813392a

This work reports the synthesis of protected Gly-Arg-Gly-Asp-Ser functionalised hydrogels, which are deprotected (and activated for cell adhesion) by reaction with glutathione-S-transferase.

Functionalisation with peptides based on the arginine-glycine-aspartic acid (Arg-Gly-Asp) sequence is one of a range of key technologies for improving the cell adhesive properties of substrates for use as scaffolds in tissue engineering.[1] Two strategies are of general use for covalent attachment: copolymerisation with a suitable peptide monomer or direct reaction with the peptide onto activated surfaces. However, both of these strategies generally use the unprotected peptide so that it is essential to carefully consider the correct choice of reaction media including pH. Also, the Arg residue can bind metals, which can give rise to complications in controlled radical polymerisations. Arg-Gly-Asp containing methacrylate monomers have been previously prepared and polymerized[2–5] but these strategies always require the use of aqueous or highly polar reaction conditions. Side reactions involving Arg or other nucleophilic/basic peptides during polymerisation are possible due to the use of the non-protected amino acid. For example, Hern and Hubbell noted the occurrence of side reactions during acrylation of Arg-containing peptides.[4] Arg also acts as a basic catalyst and requires protection during solid-phase peptide synthesis. This latter aspect led us to investigate the possibility of polymerising Arg protected peptides with methacrylate end groups and then deprotecting the peptide on the potential cell-adhesive polymer. Deprotection of the peptide on the hydrogel has the added advantage of a simpler peptide purification procedure. Unfortunately, deprotection of the Arg protected with *tert*-butoxy oxycarbonyl (*t*BOC), the group usually used to protect Arg, requires reaction with high concentrations of trifluoroacetic acid (TFA). This procedure leads to hydrolysis of methacrylate esters on the polymers and in the case of polymer hydrogels cross-linked with ethandiol dimethacrylate led to undesirable cleavage of cross-links and dissolution. Thus, our search for an alternative protection–deprotection strategy in which the deprotection could be delayed until after the polymer had been synthesised led us to a previous report of using glutathione-S-transferase (GST) to liberate amines from aryl sulfonamides in the presence of the cosubstrate; reduced glutathione (GSH).[6,7] In a previous report Todd *et al.* used proteolytic enzymes to cleave a protected Phe from an Arg-Gly-Asp sequence tethered to a hydrogel and showed enhanced adhesion of osteoblasts after cleavage.[8] Also, previous enzymatic deprotections involving enzymes other than GST are covered in ref. 9. Here we report the use of a methacrylated GRGDS peptide with the Arg residue protected with an aryl sulfonamide, which can be removed post-polymerisation with GST, and a similar polymerisable peptide containing a spacer arm between the methacrylate and peptide sections.

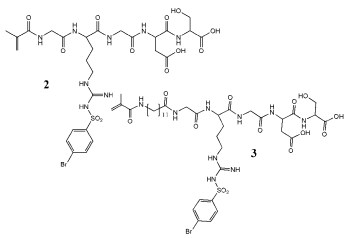
In order to examine the feasibility, of deprotecting aryl sulfonamides of Arg by application of GST we prepared Arg derivatives with the guanidine function protected with the 4-bromobenzene sulfonamide (4-Bbs). *N*-carbobenzoxy-L-arginine (*N*′-bromobenzene sulfonamide) (**1**) was synthesized using the reaction of *N*-carbobenzoxy-L-arginine with 4-bromobenzylsulfonyl chloride in acetone and 3 M sodium hydroxide solution. The syntheses of **1** were achieved with yields of ~50 and 89% purity as assessed by HPLC. The product was used to assess the removal of the 4-Bbs group by the GST. A range of concentrations (50, 100 and 200 μmol cm^{-3}) of **1** was incubated with two different concentrations of GST (0.05 and 0.5 mg cm^{-3}) at room temperature for 7 days. However, even after this extended reaction time the highest yield of the deprotected amino acid was only 40% ([**1**] = 200 μmol dm^{-3}, [GST] = 0.5 mg cm^3). Increasing the reaction temperature to 37 °C produced a large improvement in the yield of deprotected amino acid so that at this temperature conversions of 85% were achieved in 24 hours. ([**1**] = 50–200 μmol dm^{-3}, [GST] = 0.05–0.5 mg cm^3).

In general cells do not adhere to high water content hydrogels such as those based on poly(1,2-propandiol methacrylate) (PGMA) or poly(*N*-vinyl pyrolidinone) unless they are modified with cell-adhesive peptides, alkyl amines or hydrophobic sequences.[1–5,10–12] Thus, hydrogels based on these monomer units are a good choice for derivatisation with RGD as the effects of the peptide should not be complicated by the effects of non-specific protein (and cellular) adsorption. As far as we are aware, PGMA hydrogels functionalised with cell adhesive peptides have not been previously reported.

Two protected polymerisable peptides were prepared as shown in Scheme 1, by solid-phase peptide synthesis. **2** was used to directly attach the peptide to the polymer chain, and **3** provides for an alkyl spacer between the polymer chain and the peptide sequence. Synthesis of **2** and **3** was achieved by

[a] *Dept. of Chemistry, Univ. of Sheffield, Sheffield, South Yorkshire, UK S3 7HF. E-mail: s.rimmer@sheffield.ac.uk*
[b] *Dept. of Engineering Materials, The Kroto Research Institute, Univ. of Sheffield, Sheffield, South Yorkshire, UK. E-mail: s.macneil@sheffield.ac.uk*

† Electronic supplementary information (ESI) available: Further experimental details. See DOI: 10.1039/b813392a



Scheme 1 The structure of the two peptide GRGDS monomers.

using the fluorenylmethoxy carbonyl (FMOC) derivative of the Bbs-protected Arg, **4**. **4** was synthesised in 86% yield and 99% purity (HPLC). The FMOC group is not stable to the conditions required to add the 4-Bbs group. Therefore, the synthesis of **4** required a three-step process using an alternative strategy for protection of α-amine group, as shown in Scheme S1, ESI†. In the first attempt of this approach the 4-Bbs derivative of Arg with the α-amine protected with the carbobenzoxy group was used. However, removal of the carbobenzoxy group with the usual H_2/Pd procedure also removed the bromine from the 4-Bbs group.

Peptide monomers **2** and **3** contained identical peptide sequences (GRGDS) but **3** contained an alkyl spacer, which was designed to add some space between the integrin binding sequence and the polymer chain, whereas **2** does not. The alkyl chain was added to the peptide at the end of solid-phase synthesis by adding 12-(fluorenylmethyl carbamate)dodecanoic acid to the last amino acid. The final peptide monomers were prepared by removal of the FMOC and reaction of the free amine terminus with methacrylic acid.

Both peptide monomers were produced in 96% (HPLC) purity after preparative HPLC. Copolymers (P(GMA-*co*-EDMA) containing GMA, ethylene glycol dimethacrylate (EDMA) (2.5 wt% of the GMA) and various concentrations of each peptide were synthesised as films (60 μm) polymerised onto a PET backing sheet by UV-initiated radical polymerisation. The films were washed with ethanol and sterile phosphate-buffered saline, incubated with GST for 24 h at 37 °C and then primary human dermal fibroblasts were cultured on the surface for 24 h. Cell viability (and indirectly cell number) was assessed by the MTT assay and their morphology was examined by optical microscopy (Fig. 2). Fig. 1 shows the cell viability data from this set of experiments for both peptide monomers. Two observations can be made from these data. There is a substantial and significant increase in the MTT values at the highest nominal peptide concentrations, lower concentrations did not produce any improvement. Secondly there was no significant difference in the performance of the cells on the two peptides.‡ Cells on these materials also performed better, in these serum-free cultures, than on the tissue culture plastic (TCP) control wells.

The similarity in the performance of the polymers functionalised with **2** or **3** was unexpected as in general spacer arms, usually poly(ethylene glycol), are regarded as useful molecular features in a cell adhesion promoting monomer. However, the spacer used in this work was a hydrophobic moiety, which was attached to a highly swollen system. It appears that in this situation the use of a spacer does not provide any performance benefits.

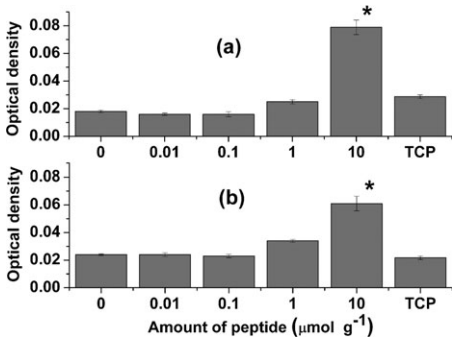


Fig. 1 Cell viability data (MTT assay) of fibroblasts cultured in serum-free media for 24 h on substrates containing various nominal amounts of GRGDS: materials prepared by copolymerisation with (a) **2** and (b) **3**. ANOVA showed a significant difference in both data sets ($p < 0.001$), * denotes results significantly different from the others (*post hoc* analysis using the Tukey procedure). TCP indicates cells cultured on tissue culture plastic as an internal positive control.

Phase-contrast images of the cells on PGMA functionalised with **2** are shown in Fig. 2. PGMA with the lowest nominal concentration of peptide was a poor substrate for cell culture, as shown in Fig. 2(a). Cells on non-functionalised PGMA do not adhere to any useful extent as we have previously reported in ref. 10 and 12. Here cells were clearly rounded and appeared to be poorly adhered to the substrate. In contrast, Fig. 2(b), (c) and (d) confirmed the viability data shown in Fig. 1 showing that the material produced with a nominal peptide concentration of 10 μmol g^{-1} provided an excellent substrate and the cells were observed to be well spread and were beginning to form cell–cell contacts. However, the micrographs also showed a morphological difference between the response of cells to material produced with a nominal peptide concentration of 0.01 μmol g^{-1} and the materials with peptide concentrations of 0.1 and 1 μmol g^{-1}. The viability data (Fig. 1) provide only a small indication that there is a difference but it is clear from the micrographs that increasing the amount of peptide from 0.01 to 0.1 μmol g^{-1} had a substantial effect on cell morphology.

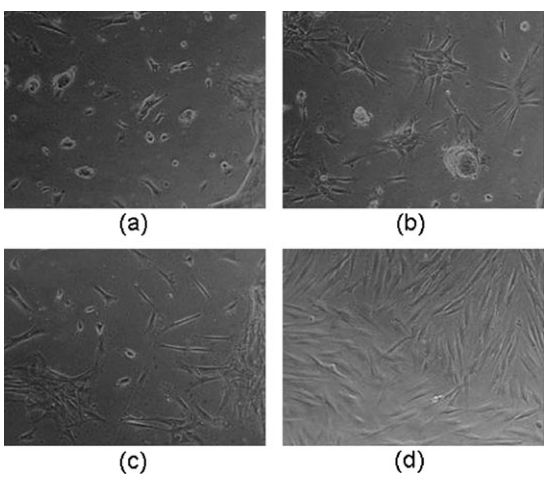


Fig. 2 Phase-contrast optical micrographs of fibroblasts cultured in serum-free media for 24 h on PGMA hydrogels containing: (a) 0; 0.01; (b) 0.1; (c) 1 and (d) 10 μmol g^{-1} (amount of peptide per mass of monomer feed).

This journal is © The Royal Society of Chemistry 2008

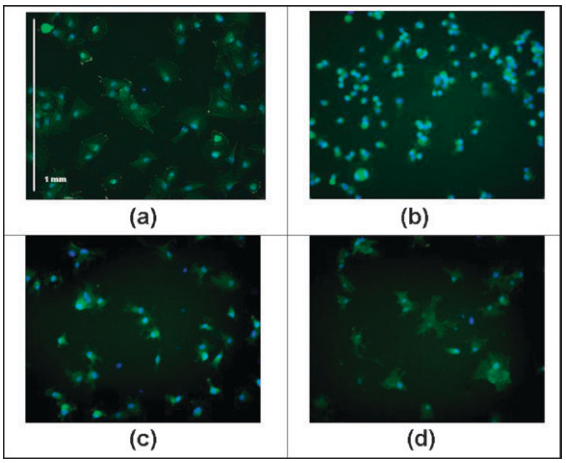


Fig. 3 Micrographs of fibroblasts cultured for 24 h on: (a) TCP; (b), (c) and (d) PGMA functionalised with **2** (10 μmol g^{-1}) and then used without deprotection with GST (b) or following 1 day (c) or 3 days (d) deprotection. Cell nuclei stained with DAPI (blue) and F-actin stained with FITC-phallodin (green).

Fig. 3 shows micrographs of cells cultured on a polymer functionalised with **2** (10 μmol g^{-1}). The nuclei are stained with DAPI and the cytoskeleton can be seen *via* staining of F-actin with FITC-phallodin. The control cells grown on TCP (a) displayed a clear and extensive network of actin at 24 h. In contrast, cells on (b)—no deprotection—were still quite rounded with no real cytoskeleton organisation. Cells on (c)—1 day deprotection—were more organised than cells on (b) and cells on (d) (material functionalised with **2** that had been reacted with GST for 3 days) were almost as organised as the cells cultured on TCP. While these results are qualitative an observer blind to the cell substrates would have had no difficulty distinguishing micrographs (b) and (c) from (a) but would not have reliably discriminated between (d) and (a). Thus cells grown on materials that had undergone only 1 day deprotection showed some F-actin formation but this was substantially less than that achieved by cells on materials deprotected for 3 days. The data provide good evidence for the release of the GRGDS functionality following deprotection of the guanidine moiety on the Arg unit.

Whilst the foregoing clearly show that these materials can provide better substrates (in terms of cell viability) for serum-free culture than TCP, the data do not provide direct evidence that the effects are due to GRGDS binding to cell surface integrins. In order to test this hypothesis GRGDS was added to compete with the polymer-bound peptide.[13] Fig. 4 shows phase-contrast micrographs of HDFs cultured on P(GMA-*co*-EDMA) functionalised with deprotected **2**, before and after addition of soluble GRGDS. The results, show the detachment of cells strongly suggesting that cells adhere to these materials by binding the polymer-bound peptide to cell surface integrins. In control experiments the cells cultured on tissue

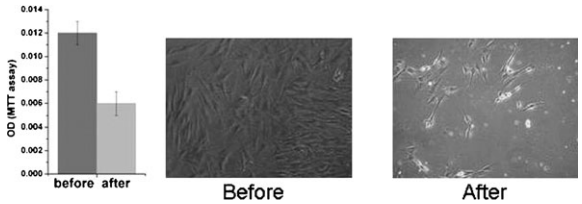


Fig. 4 MTT cell viability data and phase-contrast micrographs of fibroblasts cultured on PGMA functionalised with **2** (10 μmol g^{-1}): before application of soluble GRGDS and 60 min after the addition.

culture plastic (not shown) remained attached to the substrate when this soluble peptide was added.

In conclusion we have shown that P(GMA-*co*-EDMA) hydrogels, which are generally non-fouling and non-cell adhesive, can be modified by copolymerisation of GMA, EDMA and peptide containing methacrylate monomers. The Arg amino acid residue of these peptide monomers can be protected with 4-Bbs, which can be removed by reaction with the transferase, GST. Once deprotected these materials provide excellent substrates for cell culture, and HDFs appear to proliferate faster on the optimised materials than on TCP. In this work GST was added as a separate step and is clearly an effective methodology for deprotecting peptides without any adverse effects on the subsequent interaction of cells with these peptide-functionalised hydrogels.

Notes and references

‡ Using Student's-T test, there was no significant difference between the two TCP controls ($p = 0.12$) and no significant difference between the materials prepared with **2** or **3** at 10 μmol g^{-1} ($p = 0.26$).

1 L. Perlin, S. MacNeil and S. Rimmer, *Soft Matter*, 2008, DOI: 10.1039/b801646a.
2 L. M. Weber, K. N. Hayda, K. Haskinsb and K. S. Anseth, *Biomaterials*, 2007, **28**, 3004.
3 C. R. Nuttelman, M. C. Tripod and K. S. Anseth, *Matrix Biol.*, 2005, **24**, 208.
4 D. L. Hern and J. A. Hubbell, *J. Biomed. Mater. Res.*, 1998, **39**, 266.
5 C. E. Kang, E. J. Gemeinhart and R. A. Gemeinhart, *J. Biomed. Mater. Res.*, 2004, **71A**, 403.
6 Z. Zhao, K. A. Koeplinger, T. Paterson, R. A. Conradi, P. S. Burton, A. Suarato, R. L. Heinrikson and A. G. Tomasselli, *Drug Metab. Dispos.*, 1999, **27**, 992.
7 K. A. Koeplinger, Z. Zhao, T. Peterson, J. W. Leone, F. S. Schwende, R. L. Heinrikson and A. G. Tomasselli, *Drug Metab. Dispos.*, 1999, **27**, 986.
8 S. J. Todd, D. Farrar, J. E. Gough and R. V. Ulijn, *Soft Matter*, 2007, **3**, 547.
9 D. Kadereit and H. Waldmann, *Chem. Rev.*, 2001, **101**, 3367.
10 Y. Sun, J. Collett, N. J. Fullwood, S. Mac Neil and S. Rimmer, *Biomaterials*, 2007, **28**, 661.
11 L. Smith, S. Rimmer and S. MacNeil, *Biomaterials*, 2006, **27**, 2806.
12 R. Haigh, N. Fullwood and S. Rimmer, *Biomaterials*, 2002, **23**, 3509.
13 A similar procedure is described in: M. Ebara, M. Yamato, T. Aoyagi, A. Kikuchi, Ki. Sakai and T. Okano, *Biomacromolecules*, 2004, **5**, 505.