



Cite this: *Org. Biomol. Chem.*, 2016, **14**, 3120

## Enzymatic transhalogenation of dendritic RGD peptide constructs with the fluorinase†

Stephen Thompson,<sup>a</sup> Ian N. Fleming<sup>b</sup> and David O'Hagan<sup>\*a</sup>

The substrate scope of fluorinase enzyme mediated transhalogenation reactions is extended. Substrate tolerance allows a peptide cargo to be tethered to a 5'-chloro-5'-deoxynucleoside substrate for transhalogenation by the enzyme to a 5'-fluoro-5'-deoxynucleoside. The reaction is successfully extended from that previously reported for a monomeric cyclic peptide (cRGD) to cargoes of dendritic scaffolds carrying two and four cyclic peptide motifs. The RGD peptide sequence is known to bind upregulated  $\alpha_v\beta_3$  integrin motifs on the surface of cancer cells and it is demonstrated that the fluorinated products have a higher affinity to  $\alpha_v\beta_3$  integrin than their monomeric counterparts. Extending the strategy to radiolabelling of the peptide cargoes by tagging the peptides with [<sup>18</sup>F]fluoride was only moderately successful due to the poor water solubility of these higher order peptide scaffolds although the strategy holds promise for peptide constructs with improved solubility.

Received 28th January 2016,  
Accepted 15th February 2016

DOI: 10.1039/c6ob00239k

www.rsc.org/obc

### 1. Introduction

The fluorinase enzyme (E.C. 2.5.1.63) was originally isolated<sup>1</sup> from the soil bacterium *Streptomyces cattleya* and has recently been identified in a number of other bacterial species.<sup>2</sup> The enzyme shows promise as a biocatalyst for the incorporation of fluorine-18 into [<sup>18</sup>F]-labelled PET radiotracers, and has the singular attribute that the C–F bond forming reaction is carried out under neutral aqueous conditions.<sup>3–6</sup> The fluorinase catalyses the combination of fluoride ion and *S*-adenosyl-L-methionine **1** (SAM) to generate 5'-fluoro-5'-deoxyadenosine **2** (FDA) and *L*-(*S*)-methionine **3** (*L*-Met) (Scheme 1A).<sup>1,7</sup> We have recently demonstrated that the enzyme has a specificity tolerance at the 2-position of the adenine base.<sup>5</sup> Replacement of hydrogen with an acetylene group at this position, as shown in Scheme 1B, gave the modified substrate ClDEA **4** which was an efficient substrate for transhalogenation in the presence of *Se*-adenosyl-L-selenomethionine (SeSAM).<sup>7</sup> This transformation was utilised to prepare [<sup>18</sup>F]FDEA [<sup>18</sup>F]-**6** under ambient aqueous conditions. The pendant acetylene moiety was then efficiently reacted with an azido-cRGD (cyclic arginyl-glycyl-aspartyl) peptide using the CuAAC (Cu-catalysed alkyne–azide cycloaddition) click reaction, to generate a [<sup>18</sup>F]-radiolabelled cRGD construct.<sup>6</sup>

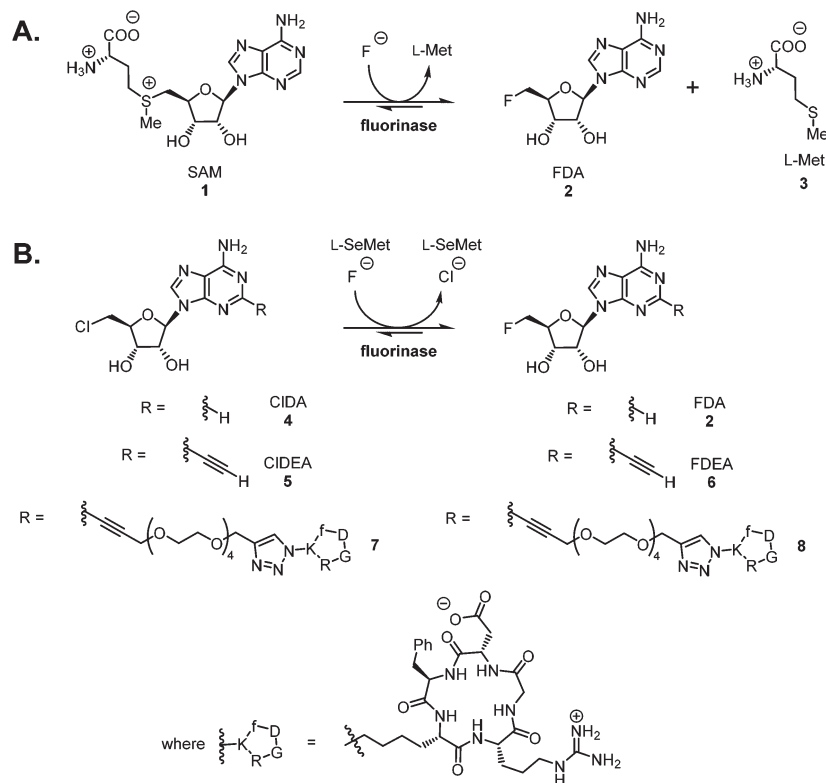
While two step strategies involving prosthetic groups remain widely used for radiolabelling of peptides and proteins with fluorine-18,<sup>8</sup> it is attractive to consider the development of straightforward, “last step” radiolabelling protocols. Such protocols need to be relatively rapid due to the short half-life of fluorine-18 ( $t_{1/2} = 109.7$  min), and be conducted under conditions that preserve the structural and functional integrity of the biomolecule.<sup>9</sup> The poor nucleophilicity of aqueous [<sup>18</sup>F]fluoride ion, the form most readily prepared on a cyclotron with an [<sup>18</sup>O]H<sub>2</sub>O target,<sup>10</sup> often precludes direct labelling of biomolecules using a “last step” approach. To circumvent the limited reactivity of aqueous [<sup>18</sup>F]fluoride, fluoride sequestering strategies have been explored. For example boron,<sup>11</sup> aluminium<sup>12</sup> and silicon<sup>13</sup> based methods have been developed in an effort to achieve “last step” radiolabelling of peptides by bioconjugation approaches which secure these elements to the peptide in advance. The strategies have proven successful and these technologies are being further developed,<sup>14,15</sup> but there are challenges as the conditions required to achieve fluoride ion sequestration require various combinations of low pH, organic solvents and heating. The fluorinase has the capacity to catalyse C–F bond formation at ambient temperature and near neutral pH. Within this context, we have previously demonstrated<sup>5</sup> a fluorinase enzyme-based system for “last step” radiolabelling of a small cRGD peptide **7**. The peptide was tethered to a 5'-chloro-5'-deoxynucleoside substrate through a PEG linker prior to enzymatic radiolabelling with [<sup>18</sup>F]fluoride, as shown in Scheme 1B. This proved successful and the radiolabelled construct [<sup>18</sup>F]-**8** was shown to be stable to defluorination *in vivo* (in a rat), and the cRGD moiety retained high affinity for  $\alpha_v\beta_3$  integrins. In order to explore

<sup>a</sup>School of Chemistry and Biomedical Sciences Research Centre, University of St Andrews, North Haugh, St Andrews KY16 9ST, UK. E-mail: do1@st-andrews.ac.uk

<sup>b</sup>Aberdeen Biomedical Imaging Centre, School of Medicine and Dentistry, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6ob00239k



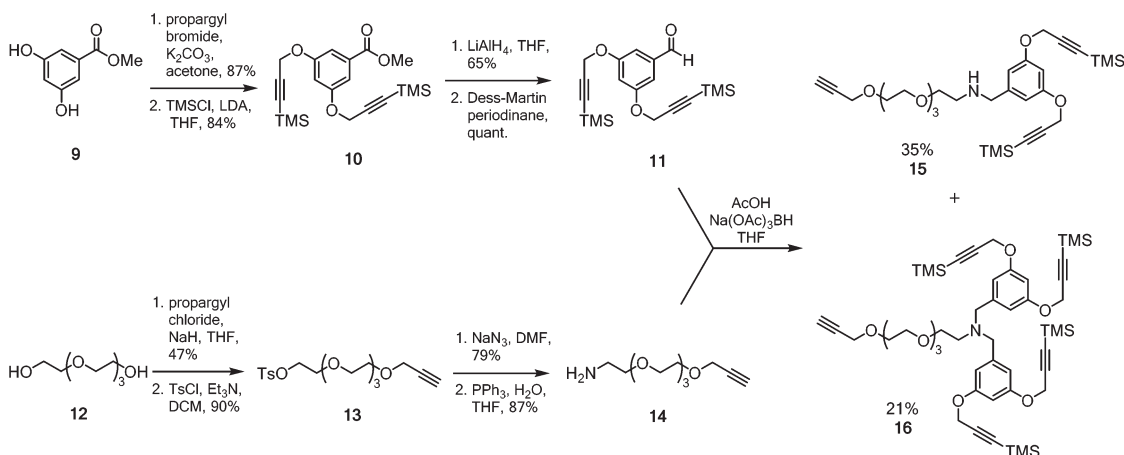


**Scheme 1** A. The native reaction catalysed by the fluorinase enzyme. B. Fluorinase catalysed transhalogenation reactions with C-2 modified substrates.

this approach further, we wished to examine whether the substrate scope at the C-2 position would extend to larger, more complex peptides.

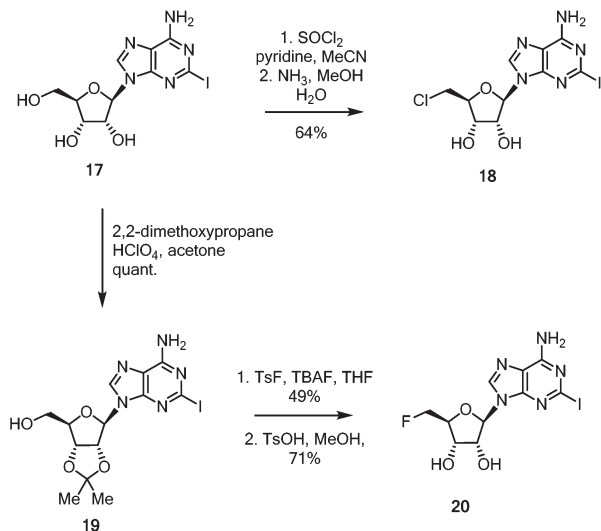
The search for high affinity ligands for biological receptors has led to the development of imaging agents based on multivalent antibody fragments<sup>16</sup> or multivalent small peptides.<sup>17</sup> Multimeric interactions play a key role in recognition events between biological ligands and receptors.<sup>18</sup> Weak non-covalent interactions between monovalent ligands and individual recep-

tors can be enhanced by attaching multiple ligands to a single scaffold.<sup>19,20</sup> Incorporating the RGD (arginyl-glycyl-aspartyl) tripeptide motif into cyclic pentapeptides gives cRGDs, a class of high affinity ligands for  $\alpha_v\beta_3$  integrin, a cell surface protein identified as a biomarker of angiogenesis and strongly associated with malignant tumours.<sup>21,22</sup> Multimeric cRGD peptides are validated constructs employed for PET imaging using either fluorine-18 or heavy metal PET isotopes.<sup>19,23,24</sup> Usually the cRGD peptide is conjugated to a metal chelating group for



**Scheme 2** Synthesis of protected aldehyde **11** and amine **14** for reductive amination to generate the desired alkynyl frameworks **15** and **16**.





**Scheme 3** Synthesis of halogenated 2-iodo coupling partners **18** and **20** for Sonogashira coupling.

co-ordination to radiometals, often at the final step in the synthesis of such a tracer. Fewer fluorine-18-based multivalent radiotracers have been developed, as incorporation of fluorine-18 into such scaffolds often requires a multi-step, prosthetic group-based strategy.<sup>25–28</sup> However, two multimeric cRGD-based radiotracers have been reported recently, synthesised

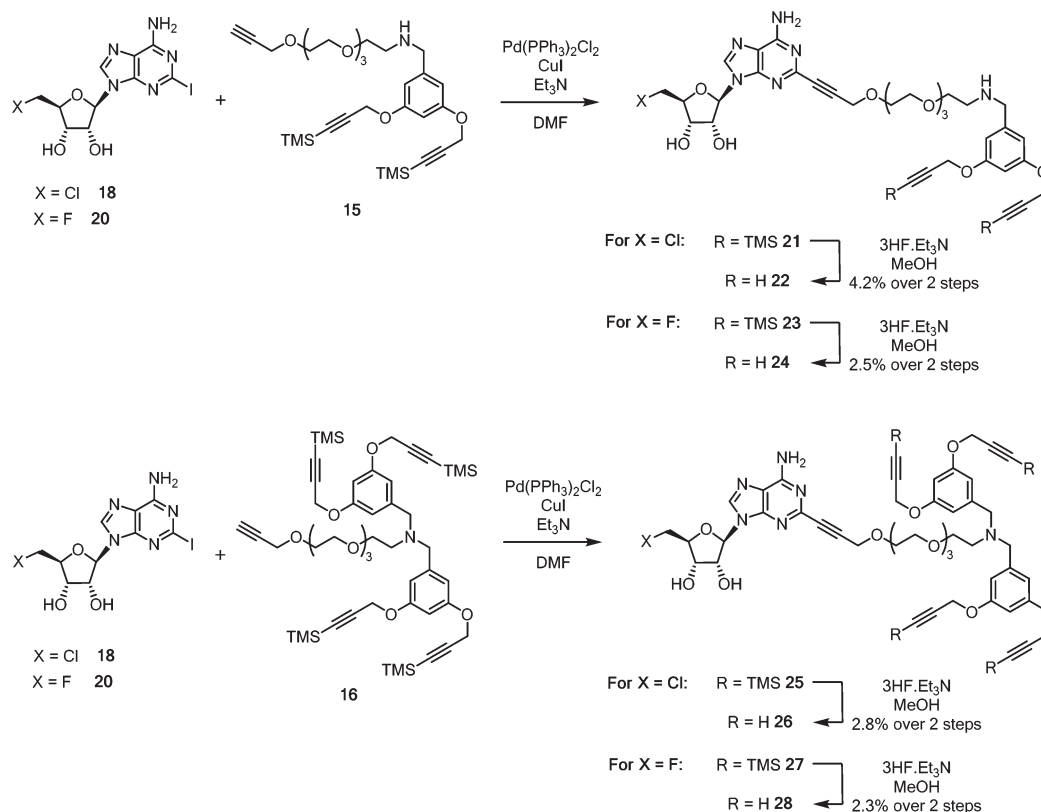
using a “last step” radiofluorination protocol. These are the aluminium chelates, [<sup>18</sup>F]alfatide I<sup>14,29</sup> and [<sup>18</sup>F]alfatide II<sup>15,30</sup> which were prepared using a kit-like<sup>31</sup> methodology and they have been evaluated in humans.<sup>14,15</sup> In addition, the CuAAC reaction between an alkyne and an azide-modified cRGD peptide has also proved useful for the assembly of multimeric cRGD peptides, which were subsequently labelled with radiometals.<sup>32–35</sup>

Herein, we report our initial results on “last step” fluorination of structurally complex multimeric cRGD peptides with the fluorinase enzyme. A primary objective at this stage was to establish if the fluorinase could tolerate higher molecular weight cargoes attached to the C-2 position of the adenine base. Although successful biotransformations could be achieved with fluoride, it became apparent that reduced solubility of higher molecular weight substrates proved challenging for efficient radiolabelled experiments, where the fluoride-18 concentrations are necessarily low.

## 2. Results and discussion

### 2.1. Synthesis of di- and tetra-alkynes for a CuAAC reaction

A multimeric peptide substrate for the fluorinase requires an enzyme recognition/reaction site attached to a linker to extend the peptide cargo away from the surface of the enzyme. In order to assemble such a substrate, we envisaged a strategy



**Scheme 4** Synthesis of pendant di- and tetra-acetylene cores attached to 5'-halo-5'-deoxyadenosine motifs.



similar to that used for the synthesis of **5** and **7** (Scheme 1B).<sup>5</sup> A Sonogashira coupling was identified for coupling a 2-iodo-nucleoside to an alkynyl-PEG linker, which had been pre-functionalised with either a protected dialkyne or a protected tetraalkyne. Following deprotection to the free terminal alkynes, a multi-CuAAC reaction with an azide bearing cRGD peptide, similar to that reported by Liskamp *et al.*,<sup>34</sup> should furnish multimeric cRGD substrates. The synthesis of the protected alkynes and halogenated 2-iodo-nucleosides is summarised in Schemes 2 and 3 respectively.

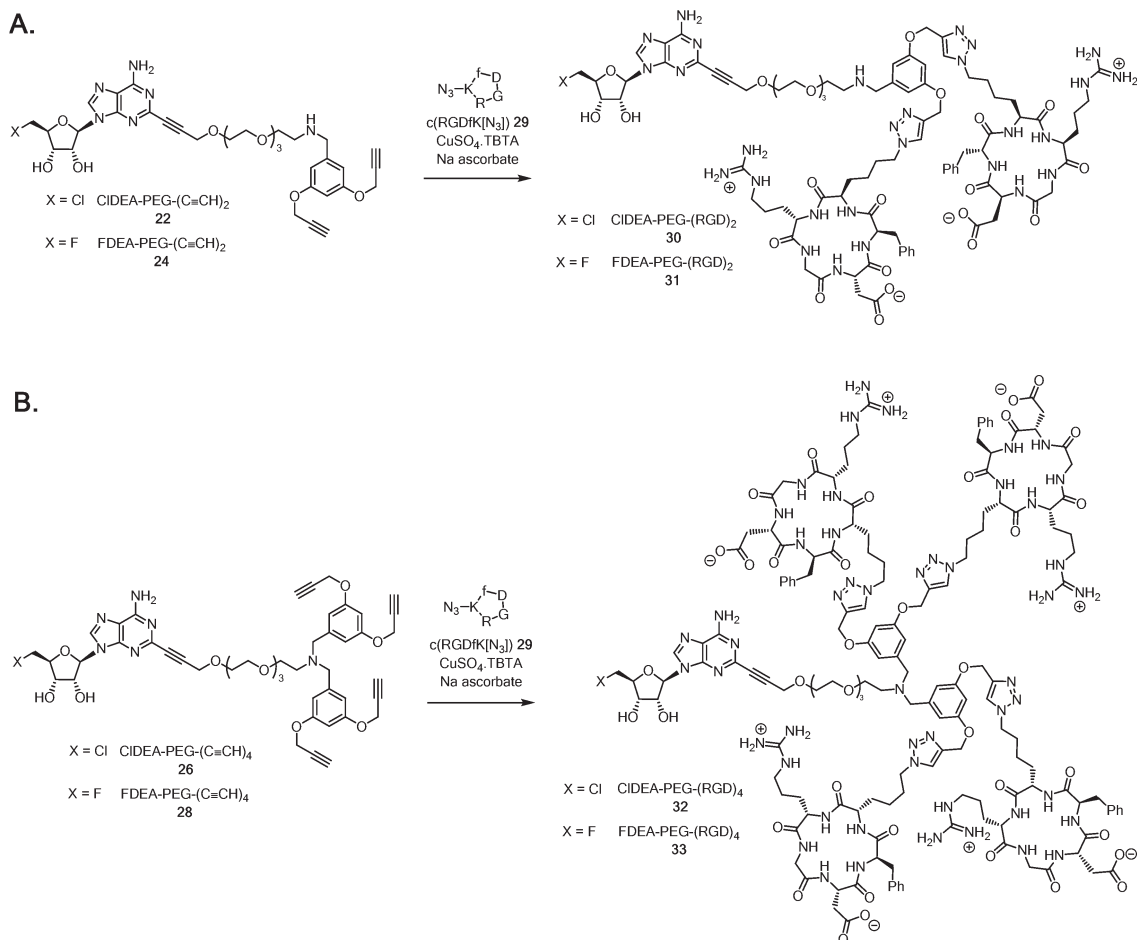
The dendrimeric core was prepared by dialkylation of methyl 3,5-dihydroxybenzoate **9** with propargyl bromide (Scheme 2).<sup>34</sup> The acetylenes of the newly installed propargylic ethers were then silylated to furnish ester **10**. Ester **10** was then reduced to the corresponding benzylic alcohol and periodinane oxidation<sup>36</sup> generated aldehyde **11** which was used directly in future reductive amination reactions without further purification. The linker was prepared by monoalkylation of tetraethylene glycol **12** with propargyl chloride. The free hydroxyl was converted to the corresponding tosyl ester and then nucleophilic displacement by azide gave the corresponding azido-PEG. Reduction under Staudinger conditions<sup>37</sup>

gave amine **14**. With both aldehyde **11** and amine **14** in hand, attention turned to coupling the linker to the multi-meric core.

The reductive amination after condensation of amine **14** with aldehyde **11**<sup>38</sup> generated both the mono- and di-alkylated products **15** and **16**. Separation of the two products by column chromatography gave the di-alkyne linker-core assembly **15** in 35% yield (based on aldehyde **11**), while the terta-alkyne linker-core assembly **16** was formed in 21% yield (based on aldehyde **11**).

With these linker-core assemblies in hand, attention turned to the synthesis of the iodinated coupling partners. 2-Iodo-adenosine **17**,<sup>5</sup> was treated with thionyl chloride in pyridine, followed by ammonia in aqueous methanol to furnish the 5'-chloro-5'-deoxy adenosine **18**, as shown in Scheme 3.<sup>39</sup> For the synthesis of the fluorinated nucleoside, 2-iodoadenosine **17** was protected as its 2',3'-acetonide **19** before being fluorinated by the action of TsF and TBAF in refluxing THF. Deprotection to generate **20** was achieved in good yield with catalytic anhydrous TsOH in refluxing MeOH.<sup>40</sup>

Sonogashira couplings<sup>41–43</sup> of the halogenated 2-iodo-nucleosides **18** and **20** with acetylenes **15** and **16** were now



**Scheme 5** A. Synthesis of CIDEA-PEG-(RGD)<sub>2</sub> **30** and FDEA-PEG-(RGD)<sub>2</sub> **31**. B. Synthesis of CIDEA-PEG-(RGD)<sub>4</sub> **32** and FDEA-PEG-(RGD)<sub>4</sub> **33**.

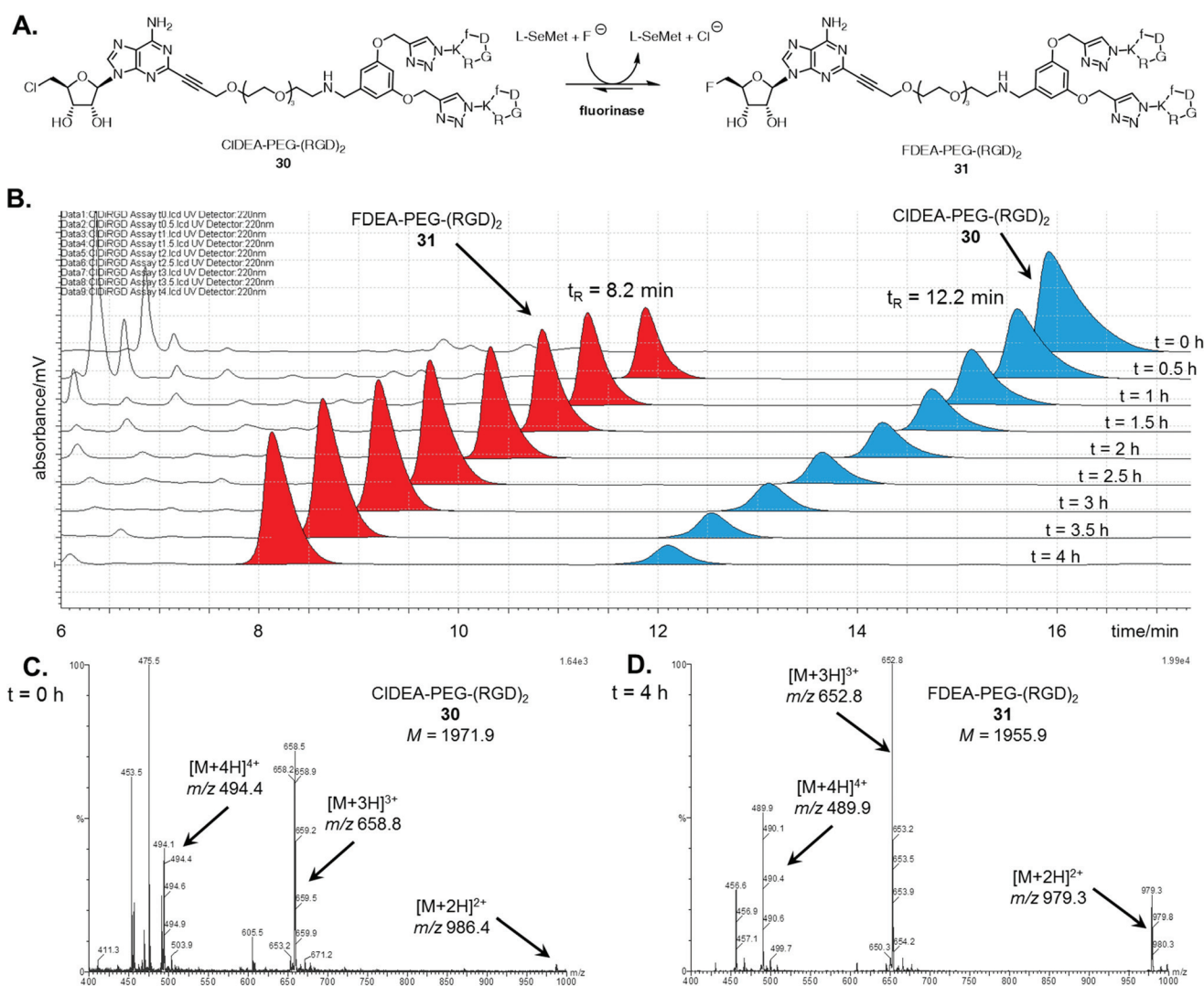


explored. 5'-Chloro-5'-deoxy-2-iodoadenosine **18** was added to an excess of either alkyne **15** or **16** in dry, degassed DMF, in the presence of Pd(PPh<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub> (10 mol%), CuI, and triethylamine. TLC and LC-MS was used to monitoring the formation of coupled products **21** and **25** (Scheme 4).

Coupled di-alkyne **21** and tetra-**25** were subject to column chromatography before fluoride-based deprotection of the silyl groups using 3HF·Et<sub>3</sub>N in methanol. The products **22** and **26** were each passed through a plug of silica gel, then through a C<sub>18</sub> reverse phase cartridge, before being purified by semi-preparative HPLC to give analytically pure samples of CIDEA-PEG-(C≡CH)<sub>2</sub> **22** and CIDEA-PEG-(C≡CH)<sub>4</sub> **26** for CuAAC reactions. Following a similar protocol, FDEA-PEG-(C≡CH)<sub>2</sub> **24** and FDEA-PEG-(C≡CH)<sub>4</sub> **28** were prepared and purified by semi-preparative HPLC.

It became a focus to 'click' the multimeric alkynes to the cRGD peptides in aqueous CuAAC reactions with c(RGDfK[N<sub>3</sub>]) **29** as illustrated in Scheme 5A. The reactions were conducted with CuSO<sub>4</sub>·TBTA (TBTA = tris(benzyltriazolylmethyl)amine<sup>44</sup>) and sodium ascorbate as the catalyst, and were monitored by HPLC. The triazole products were trapped on a C<sub>18</sub> cartridge, and eluted with MeCN/water mixture and purified by semi-preparative HPLC. This gave samples of CIDEA-PEG-(RGD)<sub>2</sub> **30** (1.8 mg, 78% yield) and FDEA-PEG-(RGD)<sub>2</sub> **31** (0.67 mg, 27% yield), the integrity of which was supported by MALDI-TOF MS analyses (see ESI†). The fluorinated analogue, FDEA-PEG-(RGD)<sub>2</sub> **31**, was required as a standard for assessing affinity to targets.

CIDEA-PEG-(C≡CH)<sub>4</sub> **26** was found to be poorly soluble in water and the CuAAC reaction with c(RGDfK[N<sub>3</sub>]) **29**



**Fig. 1** A. Fluorinase catalyzed transhalogenation of CIDEA-PEG-(RGD)<sub>2</sub> **30** to FDEA-PEG-(RGD)<sub>2</sub> **31**. B. HPLC time course (220 nm) of incubation of CIDEA-PEG-(RGD)<sub>2</sub> **30**, blue, (*t<sub>R</sub>* = 12.2 min) with the fluorinase, showing samples taken every 0.5 h over 4 h. A new peak was evident at *t<sub>R</sub>* = 8.2 min, red, identified as FDEA-PEG-(RGD)<sub>2</sub> **31**. Summed mass spectrum (*m/z* = 400–1000) of the LC-MS peaks corresponding to the tetrameric species from a sample taken at C. *t* = 0 h, showing peaks corresponding to multiply charged species of CIDEA-PEG-(RGD)<sub>2</sub> **30**, and at D. *t* = 4 h, showing peaks corresponding to multiply charged species of FDEA-PEG-(RGD)<sub>2</sub> **31**.



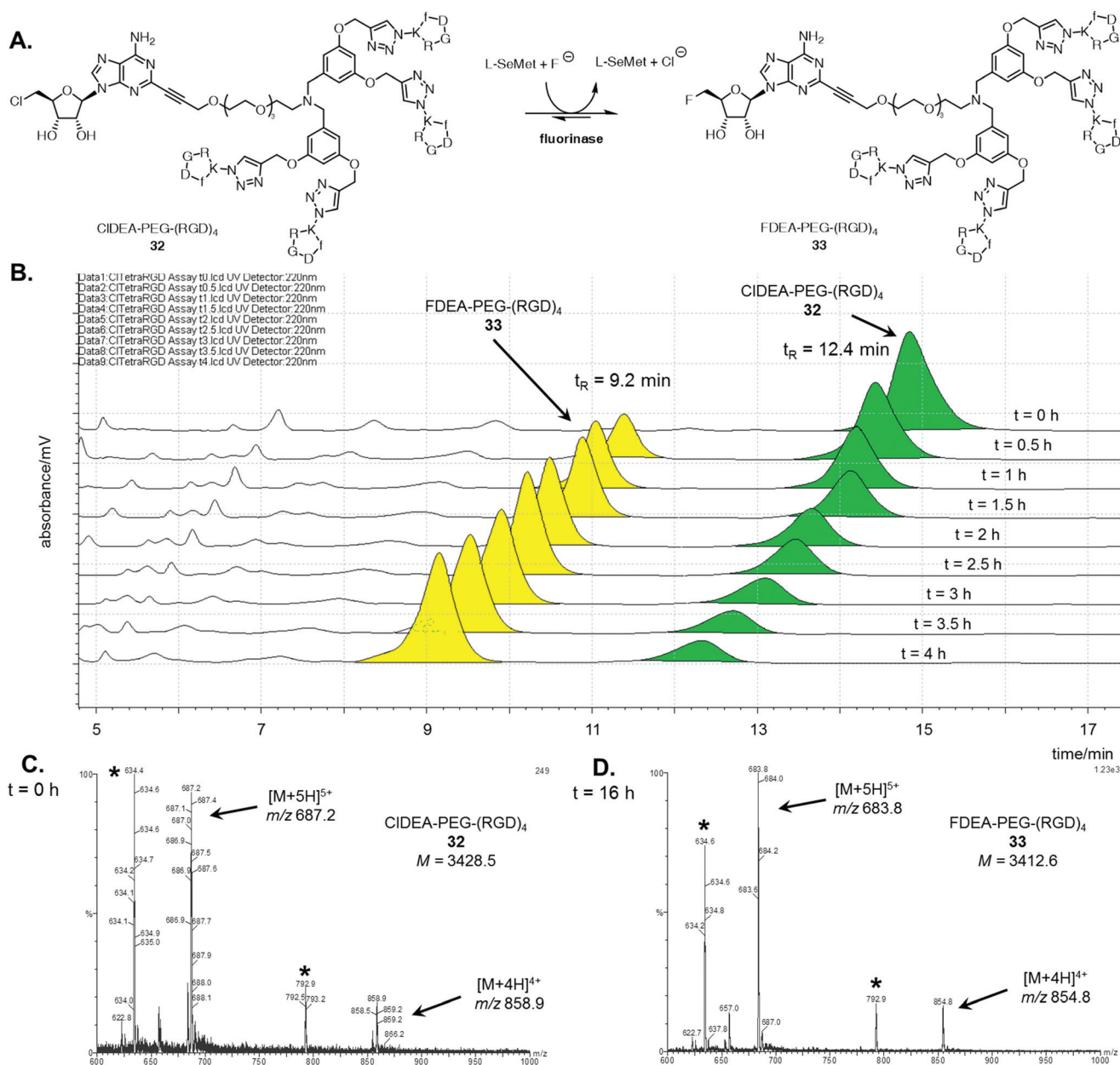


(Scheme 5B) was sluggish, but the use of 50 : 50 DMSO : water as a reaction solvent led to complete conversion within 70 minutes. The reaction product was trapped on a C<sub>18</sub> cartridge, and was washed with water to remove residual DMSO, and then the product was eluted with MeCN : water mixtures. Purification by semi-preparative HPLC gave CIDEA-PEG-(RGD)<sub>4</sub> **32** (1.4 mg, 70% yield), the integrity of which was confirmed by MALDI-TOF MS (see ESI†). The tetra-acetylene, FDEA-PEG-(C≡CH)<sub>4</sub> **26** was similarly reacted in a mixture of DMSO :

water, with c(RGDfK[N<sub>3</sub>]) **29**. HPLC purification gave the desired reference compound FDEA-PEG-(RGD)<sub>4</sub> **33** (0.66 mg, 27% yield) with a *m/z* = 3412.6 ([M + H]<sup>+</sup>) as determined by MALDI-TOF MS (see ESI†).

## 2.2. Evaluation of multimeric RGDs as substrates for the fluorinase

With the multimeric cRGD fluorinase substrates in hand, enzyme catalysed transhalogenation reactions were explored.



**Fig. 2** A. Fluorinase catalysed transhalogenation of CIDEA-PEG-(RGD)<sub>4</sub> **32** to FDEA-PEG-(RGD)<sub>4</sub> **33**. B. HPLC time course (220 nm) of incubation of CIDEA-PEG-(RGD)<sub>4</sub> **32**, green, (*t<sub>R</sub>* = 12.4 min) with the fluorinase, showing samples taken every 0.5 h over 4 h. A new peak was evident at *t<sub>R</sub>* = 9.2 min, yellow, identified as FDEA-PEG-(RGD)<sub>4</sub> **33**. Summed mass spectrum (*m/z* = 600–1000) of the LC-MS peak corresponding to the tetrameric species of samples taken at B. *t* = 0 h, showing peaks corresponding to multiply charged species of CIDEA-PEG-(RGD)<sub>4</sub> **32** and at C. *t* = 16 h, showing peaks corresponding to multiply charged species of FDEA-PEG-(RGD)<sub>4</sub> **33**. Identical fragmentation peaks (denoted with an asterisk (\*)) appear in both spectra, thought to arise from fragmentation of the adenine (C-2)–(C≡CR) bond.



ClDEA-PEG-(RGD)<sub>2</sub> **30** (40 μM) was added to a mixture of L-SeMet (75 μM) and potassium fluoride (50 mM) in a 1 mL volume to explore the transformation illustrated in Fig. 1A. The reaction was initiated by addition of the fluorinase (1 mg mL<sup>-1</sup>).

Aliquots of the reaction were removed for HPLC assay over a 4 h period in order to assess conversion. The HPLC traces in Fig. 1B show the time-dependent appearance of the product at *t*<sub>R</sub> = 8.2 min, a peak which was identified as FDEA-PEG-(RGD)<sub>2</sub> **31** by LC-MS comparison with the synthetic reference previously prepared. The mass spectra of the HPLC peaks corresponding to the multimeric 5'-chloro substrates and 5'-fluoro products are shown in Fig. 1C and D respectively. The peaks for the product contain multiply charged ions of **31** ([M + 2H]<sup>2+</sup>, [M + 3H]<sup>3+</sup>, and [M + 4H]<sup>4+</sup> respectively), confirming its identity as FDEA-PEG-(RGD)<sub>2</sub> **31**.

A series of control experiments, where the reaction was conducted in the absence of either enzyme or L-SeMet, did not result in any conversion of **30** to **31**, confirming an enzyme catalysed process. In the absence of fluoride, the consumption of **30** was observed, however, no fluorinated product was produced. This result is consistent with the generation of a SeSAM intermediate, which cannot be converted to product **31** in the absence of fluoride.

A similar analysis was conducted for the enzymatic reaction with ClDEA-PEG-(RGD)<sub>4</sub> **32**, as illustrated in Fig. 2A. ClDEA-PEG-(RGD)<sub>4</sub> **32** (20 μM) was added to a mixture of L-SeMet (75 μM) and potassium fluoride (50 mM), in a total volume of 1 mL. The reaction was initiated by the addition of the fluorinase (1 mg mL<sup>-1</sup>) and monitored over 4 h. The resultant HPLC profile (Fig. 2B) indicated that **32** was consumed, while a new peak, at *t*<sub>R</sub> = 9.2 min, steadily increased throughout the assay. The retention time of this new peak was identical to that of synthetic FDEA-PEG-(RGD)<sub>4</sub> **33**, and its identity was further confirmed by LC-MS.

Mass spectra the tetrameric substrate and product are shown in Fig. 2C and D respectively. In the sample analysed at *t* = 0 h (Fig. 2C), peaks corresponding to multiply charged species of ClDEA-PEG-(RGD)<sub>4</sub> **32** are observed at *m/z* = 858.9 ([M + 4H]<sup>4+</sup>) and 687.2 ([M + 5H]<sup>5+</sup>). In the sample analysed after an extended 16 h reaction (Fig. 2D), peaks at *m/z* = 854.8 ([M + 4H]<sup>4+</sup>) and 683.8 ([M + 5H]<sup>5+</sup>) are clearly observed. The presence of these ions is consistent with the formation of FDEA-PEG-(RGD)<sub>4</sub> **33** as the product of enzymatic transhalogenation.

Tolerance of the fluorinase to these large multimeric peptides suggests that the C-2 position of a chlorinated nucleoside represents a site for the attachment of a diverse range of peptide cargos for use in enzymatic fluorination. The constructs explored here consist of 10 and 20 amino acids, and they were efficiently fluorinated under neutral aqueous conditions. With the knowledge that the multimeric constructs were suitable substrates for fluorinase catalysed transhalogenation, it was of interest to evaluate the effect the multiple RGD motifs on binding affinity to α<sub>v</sub>β<sub>3</sub> integrin.

### 2.3. Binding affinities of RGD multimers to immobilised α<sub>v</sub>β<sub>3</sub> integrin

The binding affinities of the fluorinated multimers **31** and **33** to α<sub>v</sub>β<sub>3</sub> integrin were determined by their ability to displace a biotin-labelled cRGD peptide from immobilised α<sub>v</sub>β<sub>3</sub> integrin in an ELISA assay.<sup>45</sup> The data are shown in Table 1 including the IC<sub>50</sub> values of some reference peptides.<sup>5,6</sup> The ELISA data indicate that for this series the multimers have a progressively higher integrin affinity (lower IC<sub>50</sub>) as the number of cRGD ligands increases *e.g.*, monomeric **8** (IC<sub>50</sub> = 74 nM), dimeric **31** (IC<sub>50</sub> = 60 nM) and tetrameric **33** (IC<sub>50</sub> = 24 nM). The IC<sub>50</sub> values measured for the multimeric constructs are also lower than the parent azido-peptide **29** (IC<sub>50</sub> = 90 nM) and the multimeric cRGD compounds have significantly lower IC<sub>50</sub> values compared with reference compounds containing linear RGD-containing peptides.

Going from one to two cRGD motifs resulted in a marginal increase in binding affinity however progression to a tetramer showed a significant decrease in IC<sub>50</sub> and gave a compound with the highest affinity of those tested. The linker between the two or four cRGD units is not so extended and steric constraints will likely prevent simultaneous multivalent binding of cRGDs to more than one surface site. Instead the increased affinity is most likely due to an increased effective molarity at the surface.<sup>46</sup> Some multimeric constructs reported in the literature have IC<sub>50</sub> values in the 1–100 nM range,<sup>24</sup> similar to this study although a direct comparison of different assays and dissimilar peptide motifs make accurate comparison difficult.

### 2.4. Enzymatic radiolabelling of multimers with fluorine-18

The ability of the fluorinase to employ these large peptide assemblies of 10 or 20 amino acids as substrates opened up prospects of labelling these peptides with [<sup>18</sup>F]fluoride for PET. Radiolabelling trials of ClDEA-PEG-(RGD)<sub>2</sub> **30** (0.04 mM) was initially investigated using similar reaction conditions to the assays described with [<sup>19</sup>F]fluoride, but with an aqueous [<sup>18</sup>F]fluoride solution generated on the cyclotron. The enzyme

**Table 1** IC<sub>50</sub> values of selected RGD-containing peptides measured as the ability to compete with c(RGDfK[PEG-PEG-biotin]) for binding to immobilised α<sub>v</sub>β<sub>3</sub> integrin. Results are the average ± standard error (s.e.) from three independent experiments, each performed in triplicate. Q is the normalised affinity of the peptides, referenced to GRGDSPK

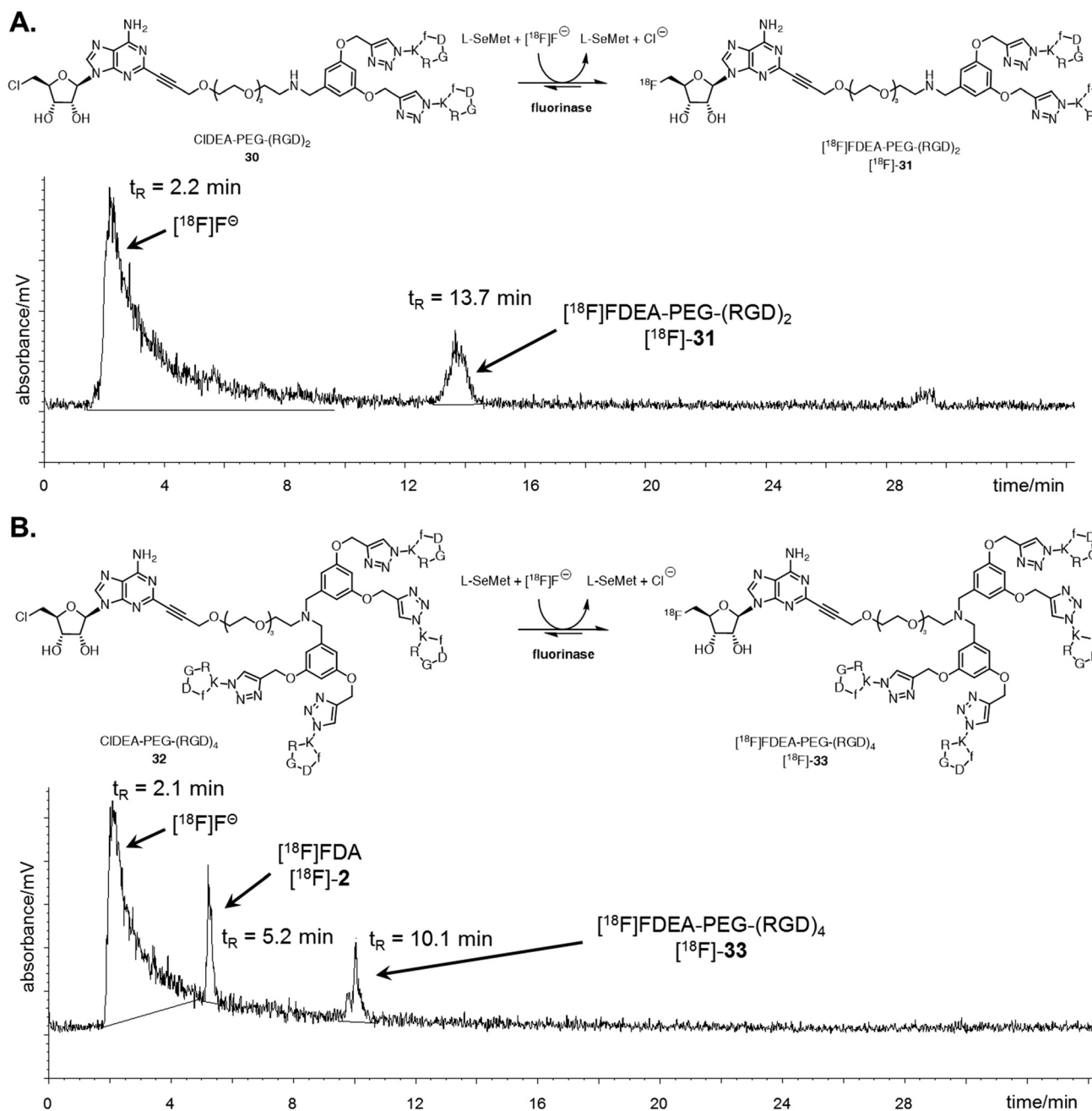
Compound	IC <sub>50</sub> ± s.e./nM	Q
RGD <sup>a</sup>	8560 ± 2240	4.019
GRGDSPK <sup>a</sup>	2130 ± 410	1.000
c(RGDfK[N <sub>3</sub> ]) <b>29</b> <sup>a</sup>	90 ± 10	0.042
FDEA-cRGD <sup>a</sup>	330 ± 30	0.155
FDEA-TEG-cRGD <b>8</b> <sup>a</sup>	74 ± 16	0.033
FDEA-PEG-(cRGD) <sub>2</sub> <b>31</b>	60 ± 13	0.027
FDEA-PEG-(cRGD) <sub>4</sub> <b>33</b>	24 ± 18	0.011

<sup>a</sup> Affinities for these constructs were previously reported, and were measured using an identical assay.



concentration was increased from  $1 \text{ mg mL}^{-1}$  ( $29 \text{ }\mu\text{M}$ ) to  $10 \text{ mg mL}^{-1}$  ( $290 \text{ }\mu\text{M}$ ), as it had previously been shown that high enzyme concentrations improves conversions in radio-labelling experiments.<sup>47</sup> Notably [ $^{18}\text{F}$ ]fluoride ion concentrations are necessarily very low in the pico-molar range. Radiochemical incorporations (RCI) were found to be low under these conditions, and only traces of the product, [ $^{18}\text{F}$ ]FDEA-PEG-(RGD)<sub>2</sub>

[ $^{18}\text{F}$ ]-31 were observed by radio-HPLC. Optimisation trials were carried out with ClIDA 4 to [ $^{18}\text{F}$ ]FDA [ $^{18}\text{F}$ ]-2 as a substrate, and the data suggest that an important factor for improving radio-chemical incorporations is substrate concentration. A RCI of greater than 90% was observed when ClIDA 4 was incubated at a concentration of  $0.6 \text{ mM}$ , compared with a 10% RCI at  $0.04 \text{ mM}$  (see ESI†).



**Fig. 3** A. Reaction scheme and resultant isocratic HPLC radioactivity trace of an experiment where ClIDEA-PEG-(RGD)<sub>2</sub> **30** ( $0.3 \text{ mM}$ ) was freeze dried with the fluorinase ( $20 \text{ mg mL}^{-1}$ ) prior to reaction. [ $^{18}\text{F}$ ]FDEA-PEG-(RGD)<sub>2</sub> [ $^{18}\text{F}$ ]-**31** was observed at  $t_R = 13.7 \text{ min}$ , showing 10% RCI. B. Reaction scheme and resultant gradient HPLC radioactivity trace of an experiment where ClIDEA-PEG-(RGD)<sub>4</sub> **32** ( $0.3 \text{ mM}$ ) was freeze dried with the fluorinase ( $20 \text{ mg mL}^{-1}$ ) prior to reaction. [ $^{18}\text{F}$ ]FDEA-PEG-(RGD)<sub>4</sub> [ $^{18}\text{F}$ ]-**33** was observed at  $t_R = 10.1 \text{ min}$ , showing 5% RCI. An additional peak was also observed at  $t_R = 5.2 \text{ min}$ , identified as [ $^{18}\text{F}$ ]FDA [ $^{18}\text{F}$ ]-**2**, produced due to the presence of residual SAM 1 co-purified with the fluorinase.<sup>48</sup> The peak is also present for ClIDEA-PEG-(RGD)<sub>2</sub> **30** labelling experiments, but is not observed in A due to the HPLC conditions utilised (see ESI† for further discussion).





By contrast, ClDEA-PEG-(RGD)<sub>2</sub> **30** and ClDEA-PEG-(RGD)<sub>4</sub> **32** were found to be poorly soluble in water, and concentrations of 0.6 mM were not achievable in buffer. The upper concentration limit for the peptides was found to be ~0.3 mM. ClDEA-PEG-(RGD)<sub>2</sub> **30** and ClDEA-PEG-(RGD)<sub>4</sub> **32** were incubated in separate experiments with L-SeMet (0.08 mM) and [<sup>18</sup>F]fluoride in water (138 μL–160 μL, 15.2–35.4 MBq). Some precipitation of the substrate was observed upon addition of aqueous [<sup>18</sup>F]fluoride. The reaction mixtures were incubated at 37 °C for 30 minutes, and then the enzyme was heat denatured and the mixture centrifuged. A sample of the clarified supernatant from each reaction was analysed by HPLC, and the resultant HPLC radio-chromatograms are shown in Fig. 3.

Radio-HPLC of the two reaction mixtures (Fig. 3A and B) revealed the presence of the respective [<sup>18</sup>F]-products, along with a broad peak (*t*<sub>R</sub> = 2.2 min), identified as [<sup>18</sup>F]fluoride.<sup>49</sup> The peak at *t*<sub>R</sub> = 13.7 min in Fig. 3A was identified as [<sup>18</sup>F]-**31** by spiking and comparison of the retention time to that of a [<sup>19</sup>F]-reference sample. Similarly the peak at *t*<sub>R</sub> = 10.1 min in Fig. 3B was identified as [<sup>18</sup>F]-**33** relative to a [<sup>19</sup>F]-reference. [<sup>18</sup>F]-FDA [<sup>18</sup>F]-**2** was also observed in this experiment, which must arise as a consequence of low levels of L-AdoMet (SAM) bound to the enzyme and residual from the purification process. This outcome only arose in the reactions of low conversion. Reactions were also conducted with the addition of DMSO 1% (v/v) as a co-solvent, however this did not improve the radiochemical conversions and extending the reaction time or increasing the concentration of L-SeMet had no significant effect. Therefore despite the efficient conversions observed in the “cold” experiments at high [<sup>19</sup>F]fluoride ion concentrations, low [<sup>18</sup>F]fluoride concentrations require to be compensated for by high substrate concentrations, and this limited the efficiency of the radiolabelled experiments.

### 3. Conclusions

Enzymatic transhalogenations using the fluorinase was explored with relatively complex di- and tetra-cRGD constructs. This exploited the previously identified substrate tolerance at C-2 of the adenine base in the substrate motif. The multimeric assemblies, ClDEA-PEG-(RGD)<sub>2</sub> **30** and ClDEA-PEG-(RGD)<sub>4</sub> **32**, were assessed as fluorinase substrates and both were found to undergo efficient enzyme-catalysed transhalogenations. The multimers also showed higher affinities to immobilised α<sub>v</sub>β<sub>3</sub> integrins, which opened up the prospect of using them as cancer imaging agents in [<sup>18</sup>F]-radiolabelled form. This proved only partially successful due to the relatively poor solubility of the multimeric constructs and low [<sup>18</sup>F]-fluoride concentrations. It follows that the development of this approach to larger peptides or proteins will depend on using more highly soluble peptide substrates in buffer. The peptides used here are ‘click’ derived with low solubility, however the approach should prove more useful with natural peptides and antibodies which have evolved high water solubility.

## Acknowledgements

We thank EPSRC and the Scottish Imaging Network (SINAPSE) for grants. DO'H thanks the Royal Society for a Wolfson Research Merit Award and ST is grateful to the John and Kathleen Watson Scholarship for financial support. We are grateful to Dr Catherine Botting and Dr Sally Shirran of the St Andrews Mass Spectrometry Service for MALDI-MS acquisitions. We also thank Dr Sally Pimlott of the University of Glasgow for the use of radiochemistry facilities.

## References

- 1 D. O'Hagan, C. Schaffrath, S. L. Cobb and J. T. G. Hamilton, *Nature*, 2002, **416**, 279.
- 2 H. Deng, L. Ma, N. Bandaranayaka, Z. Qin, G. Mann, K. Kyeremeh, Y. Yu, T. Shepherd, J. H. Naismith and D. O'Hagan, *ChemBioChem*, 2014, **15**, 364–368.
- 3 M. Onega, J. Domarkas, H. Deng, L. F. Schweiger, T. A. D. Smith, A. E. Welch, C. Plisson, A. D. Gee and D. O'Hagan, *Chem. Commun.*, 2010, **46**, 139–141.
- 4 S. Dall'Angelo, N. Bandaranayaka, A. D. Windhorst, D. J. Vugts, D. van der Born, M. Onega, L. F. Schweiger, M. Zanda and D. O'Hagan, *Nucl. Med. Biol.*, 2013, **40**, 464–470.
- 5 S. Thompson, Q. Zhang, M. Onega, S. McMahon, I. Fleming, S. Ashworth, J. H. Naismith, J. Passchier and D. O'Hagan, *Angew. Chem., Int. Ed.*, 2014, **53**, 8913–8918.
- 6 S. Thompson, M. Onega, S. Ashworth, I. N. Fleming, J. Passchier and D. O'Hagan, *Chem. Commun.*, 2015, **51**, 13542–13545.
- 7 H. Deng, S. L. Cobb, A. R. McEwan, R. P. McGlinchey, J. H. Naismith, D. O'Hagan, D. A. Robinson and J. B. Spencer, *Angew. Chem., Int. Ed.*, 2006, **45**, 759–762.
- 8 P. W. Miller, N. J. Long, R. Vilar and A. D. Gee, *Angew. Chem., Int. Ed.*, 2008, **47**, 8998–9033.
- 9 S. Richter and F. Wuest, *Molecules*, 2014, **19**, 20536–20556.
- 10 P. W. Miller, N. J. Long, R. Vilar and A. D. Gee, *Angew. Chem., Int. Ed.*, 2008, **47**, 8998–9033.
- 11 R. Ting, M. J. Adam, T. J. Ruth and D. M. Perrin, *J. Am. Chem. Soc.*, 2005, **127**, 13094–13095.
- 12 C. A. D'Souza, W. J. McBride, R. M. Sharkey, L. J. Todaro and D. M. Goldenberg, *Bioconjugate Chem.*, 2011, **22**, 1793–1803.
- 13 R. Schirrmacher, G. Bradtmöller, E. Schirrmacher, O. Thews, J. Tillmanns, T. Siessmeier, H. G. Buchholz, P. Bartenstein, B. Wängler, C. M. Niemeyer and K. Jurkschat, *Angew. Chem., Int. Ed.*, 2006, **45**, 6047–6050.
- 14 W. Wan, N. Guo, D. Pan, C. Yu, Y. Weng, S. Luo, H. Ding, Y. Xu, L. Wang, L. Lang, Q. Xie, M. Yang and X. Chen, *J. Nucl. Med.*, 2013, **54**, 691–698.
- 15 C. Yu, D. Pan, B. Mi, Y. Xu, L. Lang, G. Niu, M. Yang, W. Wan and X. Chen, *Eur. J. Nucl. Med. Mol. Imaging*, 2015, **42**, 2021–2028.



- 16 A. Goel, J. Baranowska-Kortylewicz, S. H. Hinrichs, J. Wisecarver, G. Pavlinkova, S. Augustine, D. Colcher, B. J. Booth and S. K. Batra, *J. Nucl. Med.*, 2001, **42**, 1519–1527.
- 17 S. Liu, D. S. Edwards, M. C. Ziegler, A. R. Harris, S. J. Hemingway and J. A. Barrett, *Bioconjugate Chem.*, 2001, **12**, 624–629.
- 18 M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794.
- 19 S. Liu, *Mol. Pharm.*, 2006, **3**, 472–487.
- 20 C. Fasting, C. A. Schalley, M. Weber, O. Seitz, S. Hecht, B. Kokschi, J. Dornedde, C. Graf, E. W. Knapp and R. Haag, *Angew. Chem., Int. Ed.*, 2012, **51**, 10472–10498.
- 21 C. J. Avraamides, B. Garmy-Susini and J. A. Varner, *Nat. Rev. Cancer*, 2008, **8**, 604–617.
- 22 R. Haubner, R. Gratias, B. Diefenbach and S. Goodman, *J. Am. Chem. Soc.*, 1996, **118**, 7461–7472.
- 23 S. Liu, *Bioconjugate Chem.*, 2009, **20**, 2199–2213.
- 24 S. Liu, *Bioconjugate Chem.*, 2015, **26**, 1413–1438.
- 25 G. Thumshirn, U. Hersel, S. L. Goodman and H. Kessler, *Chem. – Eur. J.*, 2003, **9**, 2717–2725.
- 26 T. Poethko, M. Schottelius, G. Thumshirn, U. Hersel, M. Herz, G. Henriksen, H. Kessler, M. Schwaiger and H.-J. Wester, *J. Nucl. Med.*, 2004, **45**, 892–902.
- 27 C. Hultsch, M. Berndt, R. Bergmann and F. Wuest, *Appl. Radiat. Isot.*, 2007, **65**, 818–826.
- 28 S. Liu, Z. Liu, K. Chen, Y. Yan, P. Watzlowik, H. J. Wester, F. T. Chin and X. Chen, *Mol. Imaging Biol.*, 2010, **12**, 530–538.
- 29 W. Cheng, Z. Wu, S. Liang, H. Fu, S. Wu, Y. Tang, Z. Ye and H. Wang, *PLoS One*, 2014, **9**, e100521.
- 30 J. Guo, N. Guo, L. Lang, D. O. Kiesewetter, Q. Xie, Q. Li, H. S. Eden, G. Niu and X. Chen, *J. Nucl. Med.*, 2014, **55**, 154–160.
- 31 W. J. McBride, C. A. D'Souza, H. Karacay, R. M. Sharkey and D. M. Goldenberg, *Bioconjugate Chem.*, 2012, **23**, 538–547.
- 32 J. A. F. Joosten, N. T. H. Tholen, F. Ait El Maate, A. J. Brouwer, G. W. van Esse, D. T. S. Rijkers, R. M. J. Liskamp and R. J. Pieters, *Eur. J. Org. Chem.*, 2005, 3182–3185.
- 33 C. Wängler, S. Maschauer, O. Prante, M. Schäfer, R. Schirrmacher, P. Bartenstein, M. Eisenhut and B. Wängler, *ChemBioChem*, 2010, **11**, 2168–2181.
- 34 I. Dijkgraaf, A. Y. Rijnders, A. Soede, A. C. Dechesne, G. W. van Esse, A. J. Brouwer, F. H. M. Corstens, O. C. Boerman, D. T. S. Rijkers and R. M. J. Liskamp, *Org. Biomol. Chem.*, 2007, **5**, 935–944.
- 35 H. Li, H. Zhou, S. Krieger, J. J. Parry, J. J. Whittenberg, A. V. Desai, B. E. Rogers, P. J. a. Kenis and D. E. Reichert, *Bioconjugate Chem.*, 2014, **25**, 761–772.
- 36 D. B. Dess and J. C. Martin, *J. Org. Chem.*, 1983, **48**, 4155–4156.
- 37 H. Staudinger and J. Meyer, *Helv. Chim. Acta*, 1919, **2**, 635–646.
- 38 A. F. Abdel-Magid, K. G. Carson, B. D. Harris, C. A. Maryanoff and R. D. Shah, *J. Org. Chem.*, 1996, **61**, 3849–3862.
- 39 M. J. Robins, F. Hansske, S. F. Wnuk and T. Kanai, *Can. J. Chem.*, 1991, **69**, 1468–1474.
- 40 V. Iaroshenko, D. Sevenard, A. Kotljarov, D. Volochnyuk, A. Tolmachev and V. Sosnovskikh, *Synthesis*, 2009, 731–740.
- 41 K. Sonogashira, Y. Tohda and N. Hagihara, *Tetrahedron Lett.*, 1975, **16**, 4467–4470.
- 42 R. Chinchilla and C. Nájera, *Chem. Soc. Rev.*, 2011, **40**, 5084–5121.
- 43 M. Schilz and H. Plenio, *J. Org. Chem.*, 2012, **77**, 2798–2807.
- 44 T. R. Chan, R. Hilgraf, K. B. Sharpless and V. V. Fokin, *Org. Lett.*, 2004, **6**, 2853–2855.
- 45 M. Piras, I. Fleming, W. Harrison and M. Zanda, *Synlett*, 2012, 2899–2902.
- 46 A. J. Beer, H. Kessler, H.-J. Wester and M. Schwaiger, *Theranostics*, 2011, **1**, 48–57.
- 47 H. Deng, S. L. Cobb, A. D. Gee, A. Lockhart, L. Martarello, R. P. McGlinchey, D. O'Hagan and M. Onega, *Chem. Commun.*, 2006, 652–654.
- 48 C. Dong, F. Huang, H. Deng, C. Schaffrath, J. B. Spencer, D. O'Hagan and J. H. Naismith, *Nature*, 2004, **427**, 561–565.
- 49 D. Ory, J. Van den Brande, T. de Groot, K. Serdons, M. Bex, L. Declercq, F. Cleeren, M. Ooms, K. Van Laere, A. Verbruggen and G. Bormans, *J. Pharm. Biomed. Anal.*, 2015, **111**, 209–214.

