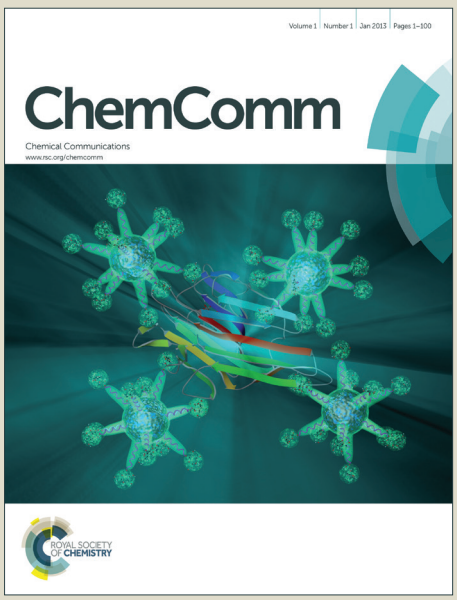
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

COMMUNICATION

Synthesis and Assessment of a Maleimide Functionalized BF_2 Azadipyrromethene Near-Infrared Fluorochrome

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Dan Wu,[a] Shane Cheung,[a] Marc Devocelle,[a] Lijun Zhang,[b] Zhi-Long Chen[b] Donal F. O'Shea*[a,c]†

The first water soluble maleimide bearing NIR BF_2-azadipyrromethene (NIR-AZA) fluorochrome has been synthesised which is capable of rapid thiol conjugations in water with peptides such as glutathione, the cell penetrating peptide (CPP) C(β-A)SKKKKTKV-NH_2 and a thiol substituted *c*RGD. NIR fluorescence imaging showed rapid cellular delivery of the CPP conjugate and effective *in vivo* tumour localization for the *c*RGD conjugate.

The recent rapidly growing interest in use of near infrared (NIR) fluorescence imaging with light > 700 nm can be attributed to the inherent ability to utilize the same fluorophore for both cellular and *in vivo* experimental research with potential existing for translation to clinical use in fluorescence-guided surgery.[1-3] The lower light toxicity of NIR wavelengths provides significant advantage for use in prolonged *in vitro* live cellular imaging. Additionally, as *in vivo* endogenous absorbance is minimal in this spectral region this permits more efficient transmittance through body tissue.[4]

A NIR fluorophore alone is often insufficient for imaging applications, with a bio-conjugatable fluorochrome required such that selective imaging either at a subcellular or whole organism level can be achieved. In general, fluorochrome conjugations are most often carried out by reaction with amino acid residues, such as the amino group of lysine or thiol group of cystine.[5] As lysine residues are more prevalent than cysteine in biomolecules, their conjugation via activated ester coupling can lead to undesired multiple conjugations occurring. The use of the maleimide functional group with thiol Michael addition as the mode of covalent linking is an attractive alternative. It offers several advantages including rapid room temperature reaction in aqueous media and the potential of selectively linking single fluorophores to the biomolecule, due to lower abundance of cysteine residues. To date, the number of NIR-fluorophores is relatively limited with the availability of NIR-fluorochromes being restricted to a small selection.[6] Cyanine dyes such as the clinically approved ICG **1** and Cy5.5 **2** are currently the most commonly utilized probes within the NIR wavelength region, but their chemical properties and photostabilities are often inadequate (Fig. 1).[7,8] In the case of the clinically approved ICG **1** its lack of stability prohibits it use as a fluorochrome but the maleimide fluorochrome of non-clinically approved Cy5.5 is known.[9] As such, a distinct need exists for new classes of NIR emitting fluorochromes, and thus, intensive efforts are currently being put into their development.[6]

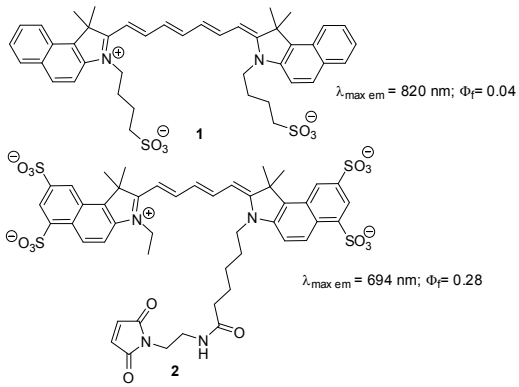


Fig. 1 Structures of ICG **1** and Cy5.5 fluorochrome **2**.

The NIR BF_2-azadipyrromethene (NIR-AZA) are a class of NIR-fluorophore which show considerable promise as *in vitro* and *in vivo* probes. For example, derivative **3** has an absorption λ max at 696 nm and emission at 727 nm in aqueous formulated solutions and most importantly has excellent photostability (Fig. 2).[10-12] This structural scaffold has formed the fluorescent core for several biologically responsive probes, nanoparticles and other materials based applications.[13-19] Yet their use as NIR fluorochromes is very limited with only bio-conjugations via amine/activated ester and azide/alkene reacting pairs having been described.[20,21] In spite of these reports significant challenges for synthesising water soluble NIR-AZA derivatives have been recently highlighted which is an essential requirement for fluorophore bio-conjugation.[22]

[a] *Department of Pharmaceutical and Medicinal Chemistry Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland E-mail: donalfoshea@rcsi.ie*
[b] *Department of Pharmaceutical Science & Technology, College of Chemistry and Biology, Donghua University, Shanghai 201620, China.*
[c] *School of Chemistry and Chemical Biology University College Dublin, Belfield, Dublin 4, Ireland*
† Current address: Royal College of Surgeons in Ireland.
Electronic Supplementary Information (ESI) available: [UV-Vis spectra, NMR spectra, conjugation conversion plots, *in vivo* imaging]. See DOI:10.1039/

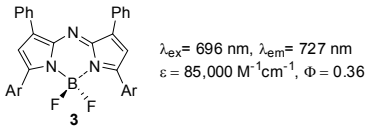


Fig. 2 NIR-AZA fluorophore (Ar = 4-MeOC$_6$H$_4$).

The goal of this work was the synthesis, bio-conjugation and preliminary *in vitro* and *in vivo* assessment of a maleimide substituted NIR-AZA fluorochrome. The designed fluorochrome **4** required the sequential functionalization of the fluorophore with both an aqueous solubilizing alkyl-sulfonate group and tethered maleimide conjugation group as shown in Fig. 3.

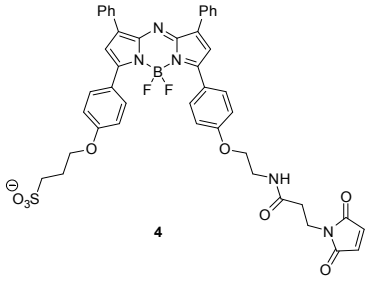
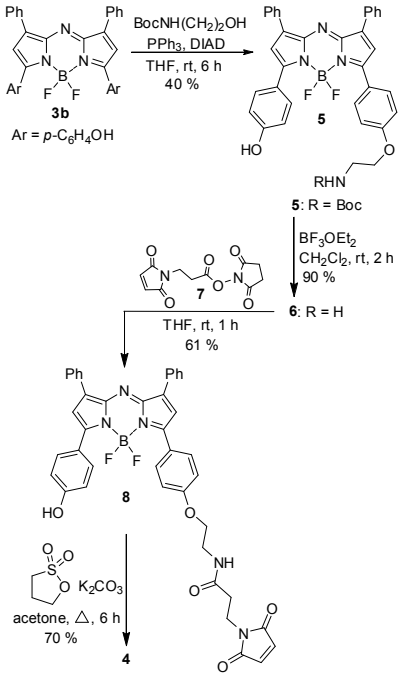


Fig. 3 Maleimide bearing NIR-AZA fluorochrome.

The synthetic route commenced with bisphenol azadipyrromethene **3b** which could be made using a previously reported route in three steps.[23] Mono functionalization of the equivalent phenols of **3b** was achieved by utilizing Mitsunobu coupling with *N*-Boc protected ethanolamine to produce **5**. Subsequent Boc-deprotection of **5** was achieved with BF_3OEt_2 in CH_2Cl_2 at rt for 2 h to give **6** in a 90% yield. Introduction of the maleimide group was achieved by amine coupling with *N*-succinimidyl 3-maleimidopropionate **7** in dry THF, providing **8** in 61% yield following purification. The final synthetic step functionalized **8** with the water solubilizing sulfonate group by reflux with 1,3-propanesultone in dry acetone for 6 h. During the reaction the NIR-fluorochrome product precipitated and following isolation was obtained in a 70% yield. NMR and MS analysis was consistent with the expected product structure.



Scheme 1 Synthetic route to NIR-AZA fluorochrome **4**.

The amino acid cystine and the tripeptide glutathione were chosen as test substrates to explore and develop the conjugation conditions, with reactions monitored by reverse phase HPLC over time. We were pleased to find that rt conversion of **4** to both its cysteine and glutathione conjugate was very effective in PBS at pH 7.2 reaching completion within 30 mins (Fig. 4, see SI for cysteine plots). Formation of conjugation products **9a**, **b** was confirmed by ^{1}H NMR and MS analysis following chromatographic purification on a Sephadex size exclusion column. As pH plays an important role in thiol conjugation selectivity, the conversion profiles of the cysteine and glutathione conjugations were further investigated at pH 6.5 and 5.6. Little difference was found at pH 6.5 and a marginally slower conjugation at 5.6 with completion reached between 1-2 h (Fig. 4 and see SI for cysteine plots). These promising result are indicative that selective Michael addition of thiols over amines could be achievable in conjugations of biomolecules containing both cystine and lysine residues.

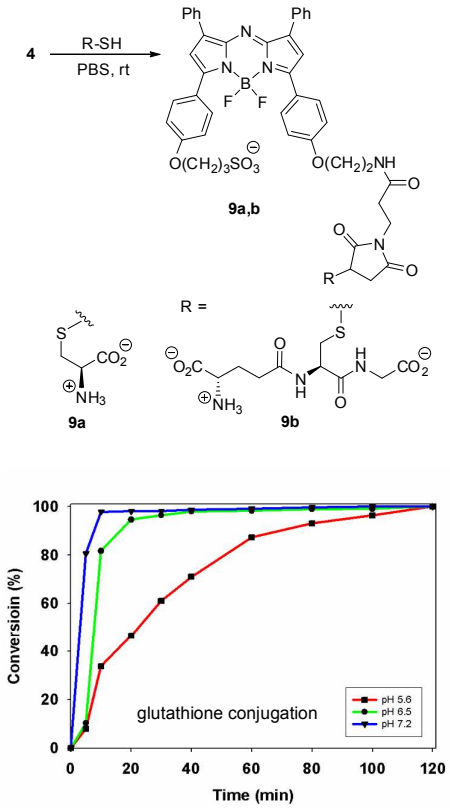


Fig. 4 Conjugation of **4** with cystine and glutathione.

Cell penetrating peptides (CPPs) are peptides with typically 10-30 amino acids, which facilitate delivery into cells and acts as tools for investigation of fundamental cellular processes.[24] The peptides are highly cationic typically containing a majority amount of lysine and arginine residues. The known CPP sequence SKKKKTKV-NH_2, derived from the transcription factor TFIIE-β, was chosen as a test cellular delivery vector for **4**.[25] Following solid phase peptide synthesis of this peptide a β-alanine spacer and terminal cysteine were added (for thiol conjugation) to give the final amino acid sequence of C(β-A)SKKKKTKV-NH_2. To explore potential for *in vivo* solid tumour imaging an RGD peptide sequence was selected as a known targeting motif for the $\alpha_v\beta_3$ integrin heterodimeric cell surface receptors which are overexpressed in tumour cells.[26] Specifically, the protected thiol functionalized cyclo[RGDfK(Ac-SCH_2CO)] was selected for conjugation as it has been proven to be an excellent tumour targeting agent.[27] Following our developed bioconjugation protocol C(β-A)SKKKKTKV-NH_2 and thiol deprotected cyclo[RGDfK($HSCH_2CO$)] were reacted with **4** in PBS at pH 6.5 for 2 h at rt. Conjugates **9c** and **9d** were purified with a sephadex G-25 column and conjugation confirmed with mass spectrometry (Fig. 5).

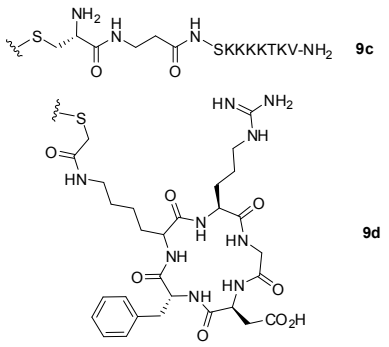
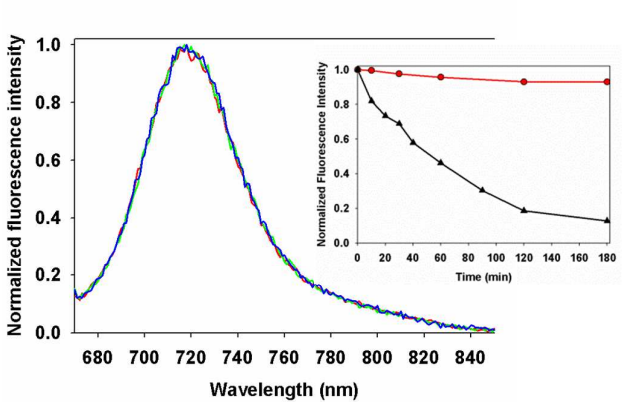


Fig. 5 CPP and cyclic RGD conjugates of **4**.

The spectroscopic properties of the fluorochrome **4** and conjugates **9a**, **b** were examined in water/MeOH (1:1) to determine if conjugation caused any significant differences in photophysical properties (Table 1). The fluorochrome **4** showed sharp absorption and emission bands with maxima at 691 nm and 716 nm respectively (Table 1, blue line) and a fluorescence quantum yield of 0.28. Peptide conjugates **9a**, **b** showed similar properties with little variance in the λ_{max} of absorption and emission (± 5 nm) though with decreased, but still acceptable, fluorescence quantum yield of 0.19 (SI). Comparative photobleaching experiments for ICG **1** and **9a** showed over 90% loss of fluorescence for **1** with less than 10% loss for **9a** after 2 h irradiation, confirming its superiour stability.

To establish the ability of CPP conjugate **9c** to internalize in cells, HeLa Kyoto cells were incubated with **9c** for 2 h and 16 h, followed by nuclei staining with Hoechst 33342 and imaging with widefield microscopy (Fig. 6). Imaging results showed that **9c** was effectively internalized within 2 h, with the fluorescence signal predominately localized in the perinuclear region and remained highly fluorescent after 16 h. Examination of twelve optical focal planes through cells following the 2 h incubation showed localization confined to distinct spot-like regions in the cytosol, with some clustering in the peri-nuclear region.

Table 1 Emission spectra of **4** and conjugates **9a**, **b**[a]. Comparative photobleaching of **9a** and ICG **1**.[b]



Entry	Comp.	λ_{max} abs nm	ε M^{-1} cm^{-1}	λ_{max} em nm	Φ_f[b]
1	**4**	691	82,000	716	0.28
2	**9a**	686	73,000	713	0.19
3	**9b**	686	71,000	714	0.19

[a] in MeOH/H_2O (1:1), conc =5×10^{-6} M. [b] In MeOH, with compound **3** (Φ = 0.36) used as a standard.[6c] Red traces **4**. Green traces **9a**. Blue traces **9b**. [b] 1×10^{-6} M DMEM solutions of **1** (black line) and **9a** (red line) irradiated with 150 W fibre optic delivered light of 620(±30) nm at 25 °C. (See SI for UV-Vis spectra).

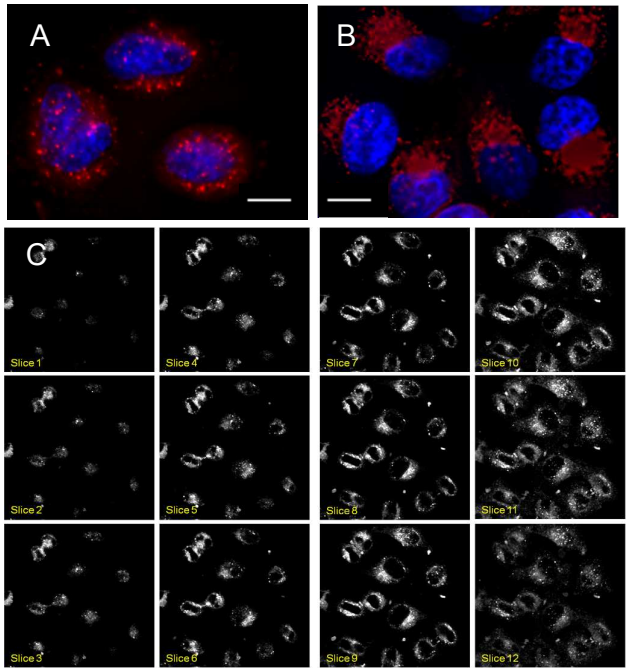


Fig. 6 Top: Images following (A) 2 h and (b) 16 h incubation of HeLa cells with 5 μM **9c,** Scale bar 10 μm. Bottom: (C) Z-Stack images following 2 hour incubation of **9c** with HeLa cells. Excitation at 635 nm with 649 nm long pass emission filter. Hoechst 33342 used to stain nuclei.

A distinct advantage of NIR fluorophores is their ability to directly transfer from *in vitro* to *in vivo* imaging due to transparency of biological tissue at these longer wavelengths. To test the *in vivo* imaging performance of cycloRGD conjugate **9d**, the human esophageal cancer cell line Eca-109 was utilized to grow subcutaneous tumours and the ability of **9d** to tumour localize over time was determined.[28,29] Following an i.v. tail vein injection of **9d** (1 mg/Kg), images were acquired at regular intervals over the course of 24 h. It was encouraging to observe that tumour localization was evident after 1 h with clear tumour discrimination still maintained at 4 h post administration (Fig. 7). Additional fluorescence was also observed from the liver and other tissues, but at 24 h the fluorescence was significantly diminished with almost complete clearance by 48 h (SI).

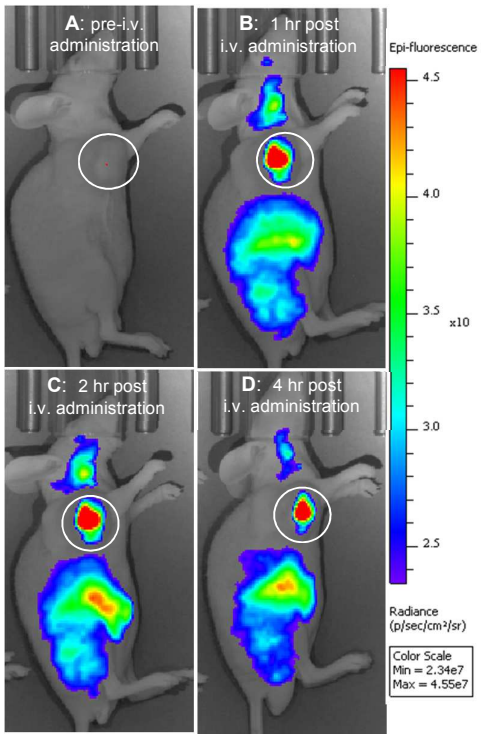


Fig. 7. *In vivo* imaging of **9d** using a Eca-109 subcutaneous tumour model. with intensity scale (excit. at 630 nm, emiss. at 700 nm). Circle indicates tumour region of interest.

Conclusions

The first water soluble maleimide bearing BF_2-azadipyrromethene NIR fluorochrome has been synthesized and shown to have excellent NIR photophysical characteristics. The maleimide group reacts efficiently with bio-molecules containing thiol groups permitting aqueous conjugation with a CPP and *c*RGD peptide. Preliminary *in vitro* and *in vivo* imaging assessments gave positive results for cellular and tumour imaging, indicating a high potential for use as a deliverable NIR-probe. Future studies will include the conjugation of tumour targeting motifs to further broaden the possibilities of *in vivo* imaging targets, with applications directed towards fluorescence guided surgery.

DOS gratefully acknowledges Science Foundation Ireland grant number 11/PI/1071(T) for financial support. WD thanks the China Scholarship Council for a Ph.D. fellowship. Competing financial interest; a patent application has been filed on azadipyrromethene based NIR fluorophores (PCT/EP2010/065991) in which DOS has a financial interest.

Notes and references

1. K. M. Dean and A. E. Palmer, *Nat. Chem. Biol.,* 2014, **10**, 512-523.
2. M. de Jong, J. Essers and W. M. van Weerden, *Nat. Rev. Cancer,* 2014, **14**, 481-493.
3. A. L. Vahrmeijer, M. Hutteman, J. R. van der Vorst, C. J. H. van de Velde and J. V. Frangioni, *Nat. Rev. Clin. Oncol.,* 2013, **10**, 507-518.
4. Z. Guo, S. Park, J. Yoon and I. Shin, *Chem. Soc. Rev.*, 2014, **43**, 16-29.
5. J. Kalia and R. T. Raines, Curr. Org. Chem., 2010, **14**, 138–147.
6. V. Pansare, S. Hejazi, W. Faenza and R. K. Prud'homme, Chem. Mater., 2012, **24**, 812–827.
7. V. Saxena, M. Sadoqi and J. Shao, *J. Photochem. Photobiol. B,* 2004, **74**, 29-38.
8. V. Saxena, M. Sadoqi and J. Shao, *J. Pharm. Sci.,* 2003, **92**, 2090-2097.
9. W. Pham, Y. Choi, R. Weissleder and C.-H. Tung, *Bioconjugate Chem.,* 2004, **15**, 1403-1407.
10. A. Gorman, J. Killoran, C. O'Shea, T. Kenna, W. M. Gallagher and D. F. O'Shea, *J. Am. Chem. Soc.,* 2004, **126**, 10619-10631.
11. M. J. Hall, L. T. Allen and D. F. O'Shea, *Org. Biomol. Chem.,* 2006, **4**, 776-780.
12. P. Batat, M. Cantuel, G. Jonusauskas, L. Scarpantonio, A. Palma, D. F. O'Shea and N. D. McClenaghan, *J. Phys. Chem. A.,* 2011, **115**, 14034-9.
13. S. Shimizu, T. Iino, A. Saeki, S. Seki and N. Kobayashi, *Chem. Eur. J.,* 2015, **21**, 2893.
14. H. Liu, H. Lu, Z. Zhou, S. Shimizu, Z. Li, N. Kobayashi and Z. Shen, *Chem. Commun.,* 2015, **51**, 1713.
15. V. Bandi, F. P. D'Souza, H. B. Gobeze and F. D'Souza, *Chem. Eur. J.*, 2015, **21**, 2669.
16. B. Zou, H. Liu, J. Mack, S. Wang, J. Tian, H. Lu, Z. Li and Z. Shen, *RSC Adv.*, 2014, **4**, 53864.
17. Y. Wang, L. Chen, R. M. El-Shishtawy, S. G. Aziz and K. Muellen, *Chem. Commun*., 2014, **50**, 11540.
18. A. Palma, L. A. Alvarez, D. Scholz, D. O. Frimannsson, M. Grossi, S. J. Quinn and D. F. O'Shea, *J. Am. Chem. Soc*., 2011, **133**, 19618.
19. D. Wu and D. F. O'Shea, *Org. Lett.*, 2013, **15**, 3392.
20. M. Tasior and D. F. O'Shea, *Bioconjugate Chem*., 2010, **21**, 1130.
21. D. Wu, S. Cheung, R. Daly, H. Burke, E. M. Scanlan and D. F. O'Shea, *Eur. J. Org. Chem*., 2014, 6841-6845.
22. A. Kamkaew and K. Burgess, *Chem. Commun.,* 2015, **51**, 10664-10667.
23. J. Murtagh, D. O. Frimannsson and D. F. O'Shea, *Org. Lett.,* 2009, **11**, 5386-5389.
24. S. M. Farkhania, A. Valizadeha, H. Karamib, S. Mohammadia, N. Sohrabic and F. Badrzadehd, *Peptides,* 2014, **57**, 78-94.
25. A. D. Ragin, A. Rashida, R. A. Morgan and J. Chmielewski, *Chem. Biol.,* 2002, **8**, 943-948.
26. U. K. Marelli, F. Rechenmacher, T. R. Ali Sobahi, C. Mas-Moruno and H. Kessler, *Frontiers Oncology*, 2013, **3**, 1-12.
27. R. M. Schiffelers, G. A. Koning, T. L. M. ten Hagen, M. H. A. M. Fens, A. J. Schraa, A. P. C. A. Janssen, R. J. Kok, G. Molema and G. Storm, *J. Controlled Release,* 2003, **91**, 115–122.
28. J-K. He, X.-S Wu, Y. Wang, R. Han, Z.-H. Qin and Y. Xie, *Acta Pharmacol. Sin.,* 2013, **34**, 295-300.
29. J. Xie, Y. Li, Y. Cao, C. Xu, M. Xia, M. Qin, J. Wei and W. Wang, *Biomater. Sci.*, 2013, **1**, 1216-1222.