Supporting Information

Title

Dual targeting of folate receptor-expressing glioma tumor-associated macrophages and epithelial cells in brain using carbon nanosphere-cationic folate nanoconjugate

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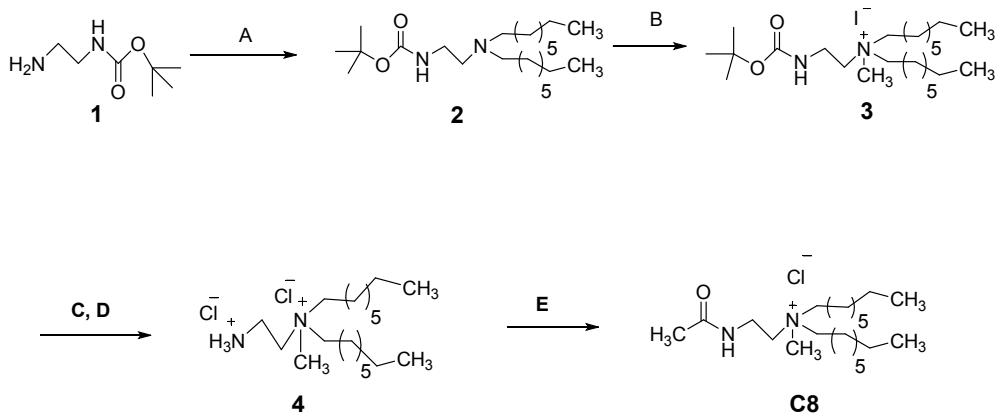
Synthetic Scheme of C8:



Scheme 1: Synthesis of C8: Reagents and conditions; (A) 1-Bromo octane, K_2CO_3 in Ethyl Acetate, 60 °C, 48 h. (B) CH_3I in Dry $CHCl_3$, 12 h; (C) Dry DCM, TFA (2:1; v/v), 0°C, 3h. (D) Amberlyst A-26 for Cl^- ion exchange (E) CH_3COCl, Triethyl amine, Dry CH_2Cl_2.

Synthesis of C8 (*N*-(2-acetamidoethyl)-*N*-methyl-*N*-octyloctan-1-aminium chloride

(Scheme 1):

***Step-1*: Synthesis of tert-butyl (2-(dioctylamino) ethyl) carbonate (Compund-2)**:

Monoboc-protected ethylenediamine (Compound-**1,** 1.8g) was added to Ethyl Acetate (20ml).

To this mixture K_2CO_3 (6.4 g , 4eq) followed by 1-Bromo ocatane was added and stirred at

65 °C for 48 h. Reaction mass was filtered and the ethyl acetate was evaraparated to get crude. Purification is done by column chromatographic separation using 60-120 mesh silica gel and 1% Methanol-Chloroform (v/v) as eluent. The separation yielded compound-**2** as colourless liquid (3g, 70% yield).

^{1}H NMR (300 MHz, $CDCl_3$) :δ/ppm = 0.9 [m, 6H, CH_3-$(CH_2)_7$-]; 1.1−1.6 [bs, 20H, -$(CH_2)_{10}$-; m,9H, CO-O-$C(CH_3)_3$]; 1.5[m, 4H, -CH_2-N-CH_2-$(CH_2)_4$]; 2.3−2.5 [m,4, -CH_2-N-$(CH_2)_2$-$(CH_2)_2$-]; 2.5[t, 2H, -NH-CH2-CH2-N-$(CH_2)_2$-];3.2 [m, 1H, -NH-CH2-CH2-N-$(CH_2)_2$-]; 3.6[t, 2H, -NH-CH_2-CH_2-N-$(CH_2)_2$-];

ESI-MS: m/z = 386 $[M+H]^+$ for $C_{23}H_{48}N_2O_2$

Step-2 **:Synthesis of N-(2-((tert-butoxycarbonyl)amino)ethyl)-N-methyl-N-octyloctan-1-aminium iodide (3):** Compound-**2**, (1.8g) was added to methyl iodide (5ml) and stirred at room temperature for 12h. Methyl iodide was evaporated to get crude. Purification is done by column chromatographic separation using 60-120 mesh silica gel and 2% Methanol-Chloroform (v/v) as eluant. The separation yielded compound-**3** as white solid (2.5g, 80% yield). .

^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm = 0.9 [m, 6H, CH_3-$(CH_2)_7$-]; 1.1-1.6 [bs, 20H, -$(CH_2)_{10}$-; m,9H, CO-O-$C(CH_3)_3$]; 1.5[m, 4H, -CH_2-N-CH_2-$(CH_2)_4$]; 3.2-3.3 [s,3, -CH_2-$N(CH_3)$-$(CH_2)_2$-$(CH_2)_2$-]; 3.4-3.6 [m, 4H, -CH_2-$N(CH_3)$-$(CH_2)_2$-$(CH_2)_2$-]; 3.6-3.8[m, 4H, -NH-CH_2-CH_2-N-$(CH_2)_2$-];

ESI MS: m/z = 400 $[M - I^-]^+$ for $C_{24}H_{51}N_2O_2$

Step-3: **Synthesis ofN1-methyl-N1,N1-dioctylethane-1,2-diaminium chloride (4)**

Compound-**3**, (2g) was dissolved in 2 mL dry $CHCl_3$, and to that 1 mL trifluoroacetic acid (TFA) was added and the mixture was allowed to stir for 3 h at 0^0C. TFA was removed with nitrogen flow and the residue was subjected to chloride ion exchange chromatography over amberlyst A-26 chloride ion exchange resin. The compound4 was obtained after chloride ion exchange (1.2 g, 80% yield) directly continued to next step.

ESI-MS: m/z = 300 $[M - 2Cl]^+$ for $C_{19}H_{43}N_2$.

Step-4: **Synthesis of C8:**

Compound-4 was dissolved in Dry CH_2Cl_2 and to this mixuture Acetyl Chloride was added. followed by the addition of Triethylamine (0.459g, 2eq) andThe reaction was stirred for 2h at 0°C. Then 1N, HCl 20 mL was added and the crude was extracted by $CHCl_3$ dried and purified by using 60-120 mesh silica gel and 2% Methanol-Chloroform (v/v) as eluant.

^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm = 0.9 [m, 6H, CH_3-$(CH_2)_7$-]; 1.1-1.6 [bs, 20H, -$(CH_2)_{10}$]; 1.5[m, 4H, -CH_2-N-CH_2-$(CH_2)_4$]; 2.0 [S, 3H, CH_3CO] ; 3.1-3.2 [S,3H, -CH_2-N(CH_3)-$(CH_2)_2$-$(CH_2)_2$-]; 3.2-3.4 [m, 6H, -CH_2-CH_2-N(CH_3)-$(CH_2)_2$-$(CH_2)_2$-]; 3.6-3.7[m, 2H, -NH-CH_2-CH_2-N(CH_3)-$(CH_2)_2$-];
ESI-MS: m/z = 341.35 $[M+H]^+$ for $C_{21}H_{45}N_2O$

Characterization of CSP-F8-DOX (CFD) through FTIR analysis:

Functional group analysis of DOX containing targeted carbon nanospheres (CFD) was performed by FTIR spectrum. Peaks obtained at 3258, 1695, 1645, 1606, 1510 and 1122 cm^{-1} are corresponding to O-H, C=O, amine groups of pteridine ring and either C=N or C=C of F8 (Figure S1) whereas in case of CSP we found peaks at 1005, 807, 588 belong to C-O-C and polymerized OH groups in CSPs, respectively. Importantly, we found additional peak at 1119.3 $cm^{-1}$for the F8 in CSP-F8. 1670 cm^{-1} in DOX loaded CSP (i.e., CD) could be due to hydrophilic interactions between C=O and other hydrophilic groups such as OH and NH_2. Further, F8 and DOX loaded CSPs exhibited similar peaks.

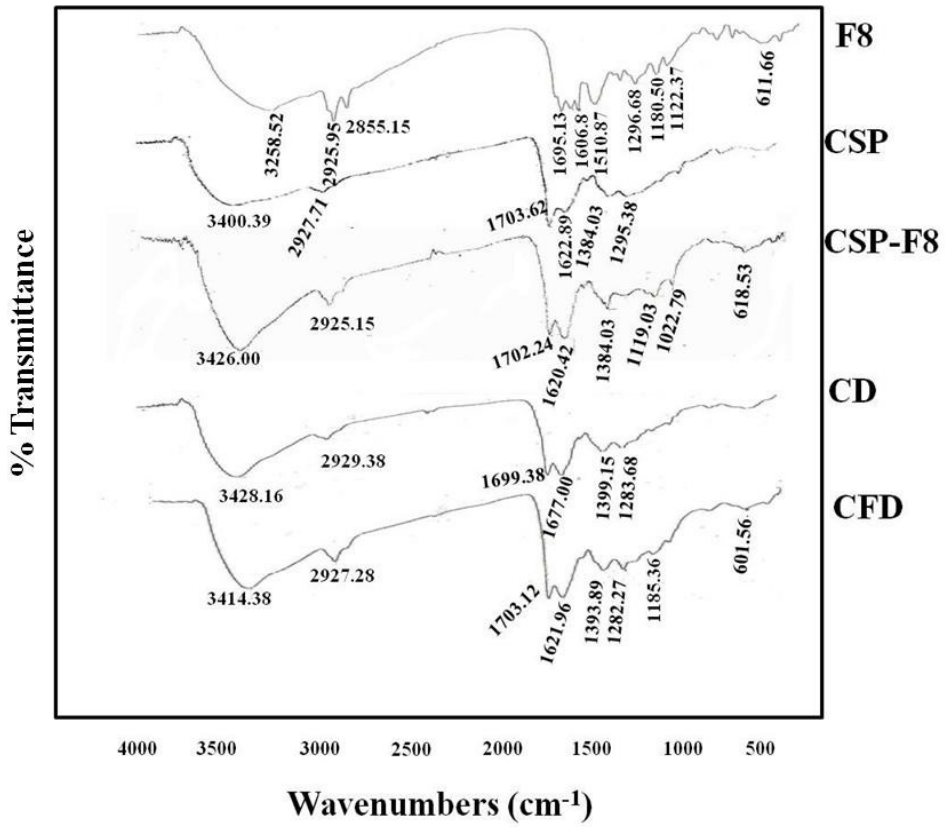


Figure S1. FTIR- Spectrum of different CSPs along with F8

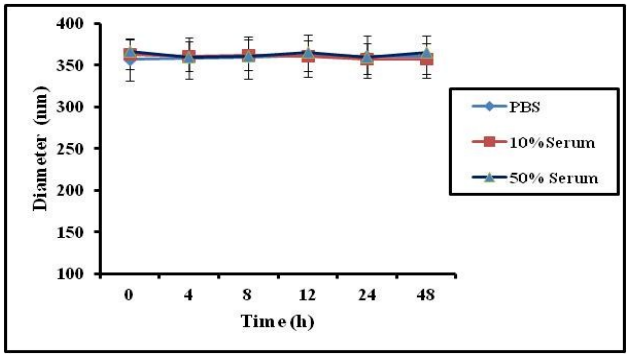


Figure S2. Stability of CFD in different serum concentrations along with PBS

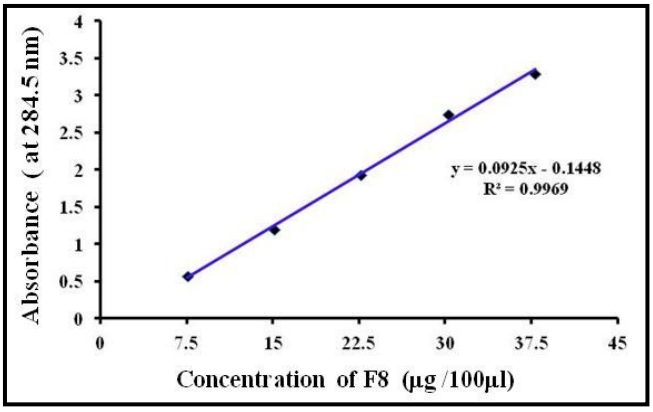


Figure S3. Standard curve for F8

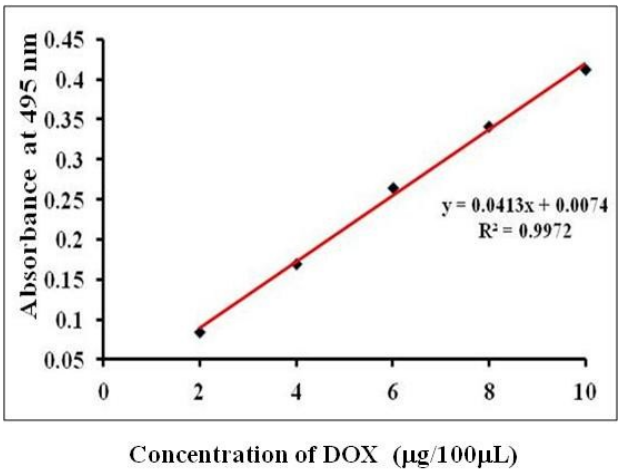


Figure S4. Standard curve for DOX

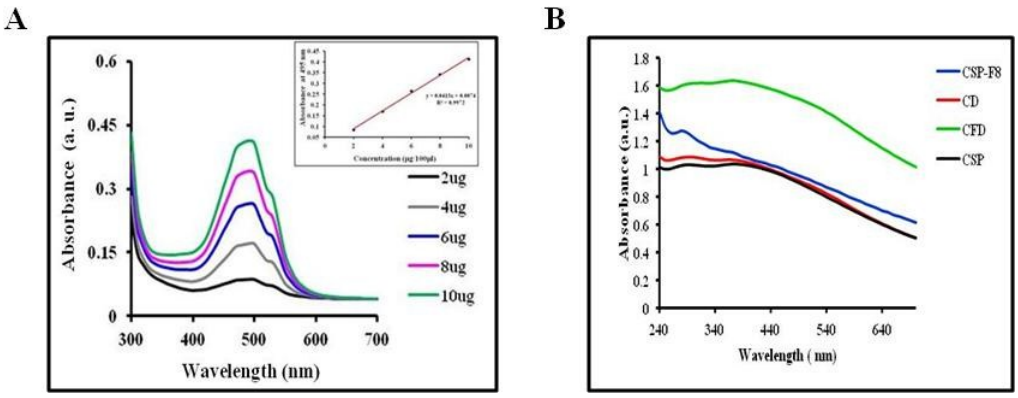
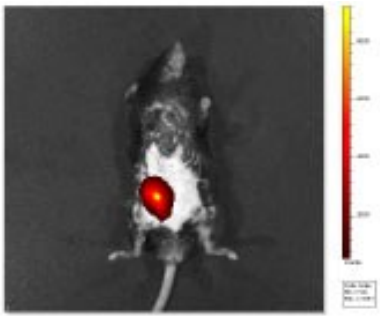
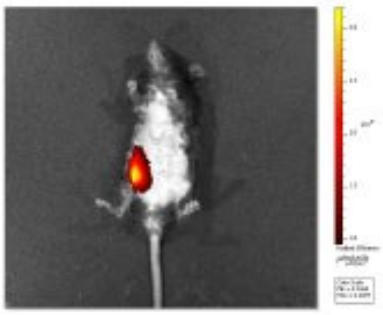


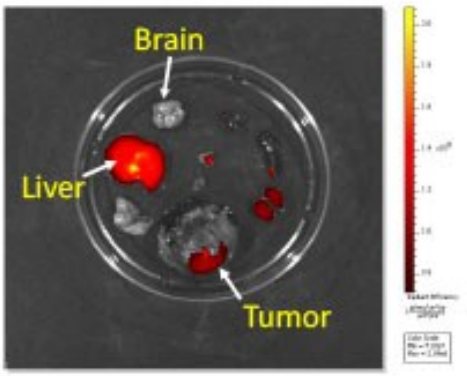
Figure S5. A) Dose dependent absorption spectra of DOX. Absorption starts at 300-700 nm with abs max at 495 nm. Corresponding standard curve for the confirmation of drug loading as a inset. B) UV-VIS spectra of different CSP conjugates.



Free DiR dye, 0 min



Free DiR dye 6 hr



Ex vivo free DiR dye in organs of subcutaneous tumor-bearing mice, 24h

Figure S6: Fate of free DiR dye intraperitoneally injected in mice: No gross change of the fate of free DiR dye after 6h of IP injection. In a tumor bearing mice, after 24h of IP injection free DiR dye invaded adjoining tissues such as liver, tumor mass etc. But, free DiR dye could not gain access to brain even after 24h post-injection.

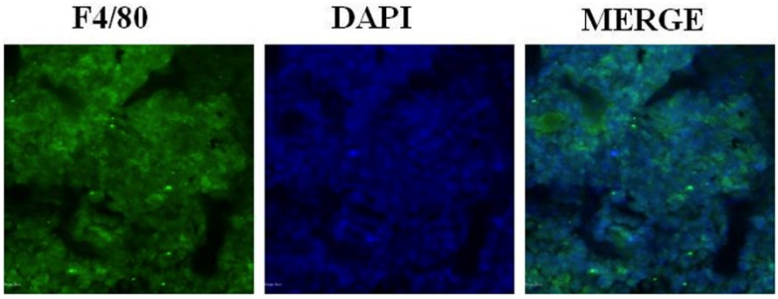


Figure S7. F4/80 staining in tumor region of orthotopic GL261 glioma bearing mice. This indicates the presence of TAM in glioma lesions in brain.

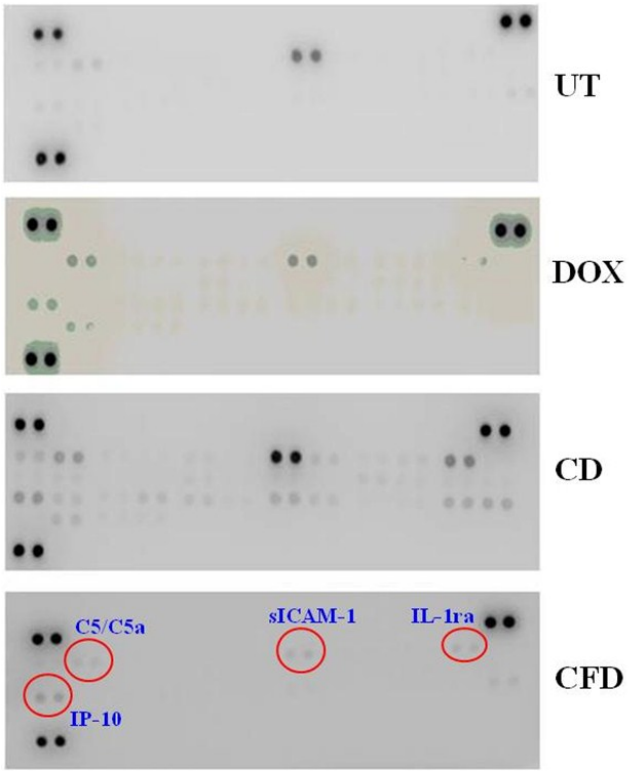


Figure S8. Chemokine expressions in brain tumor lysates developed from orthotopic GL261 glioma tumor bearing mice treated with different groups.

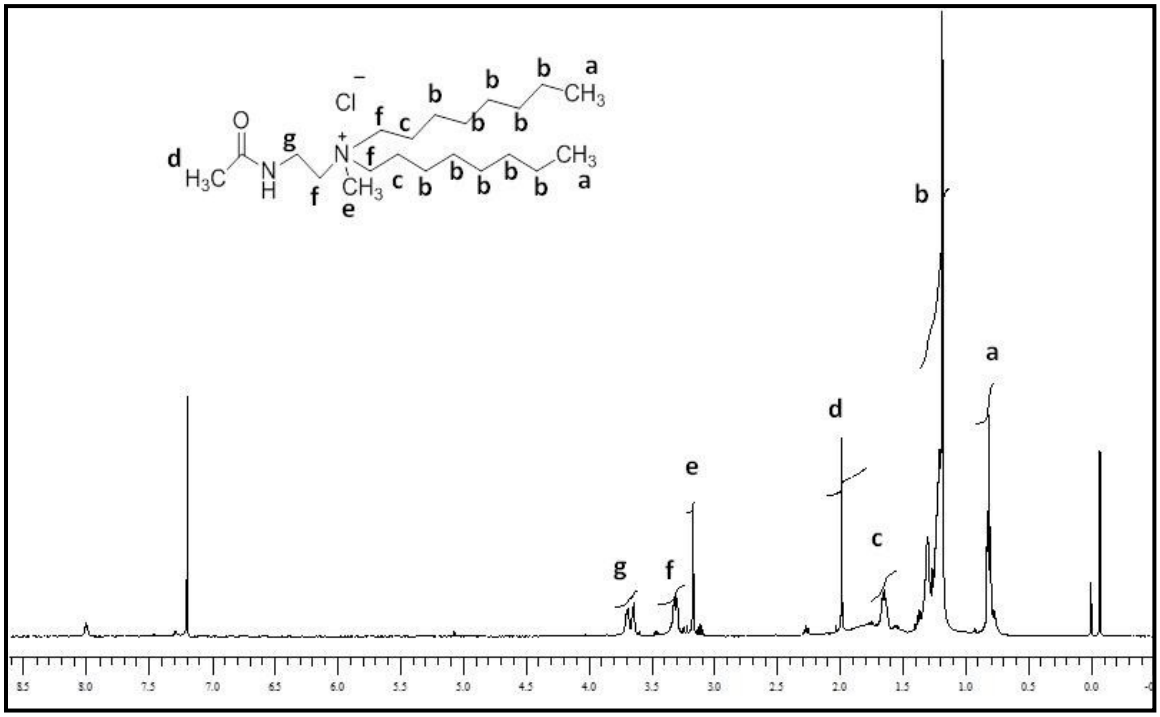


Figure S9: ^{1}H - NMR (300 MHz, $CDCl_3$) Spectra of C8

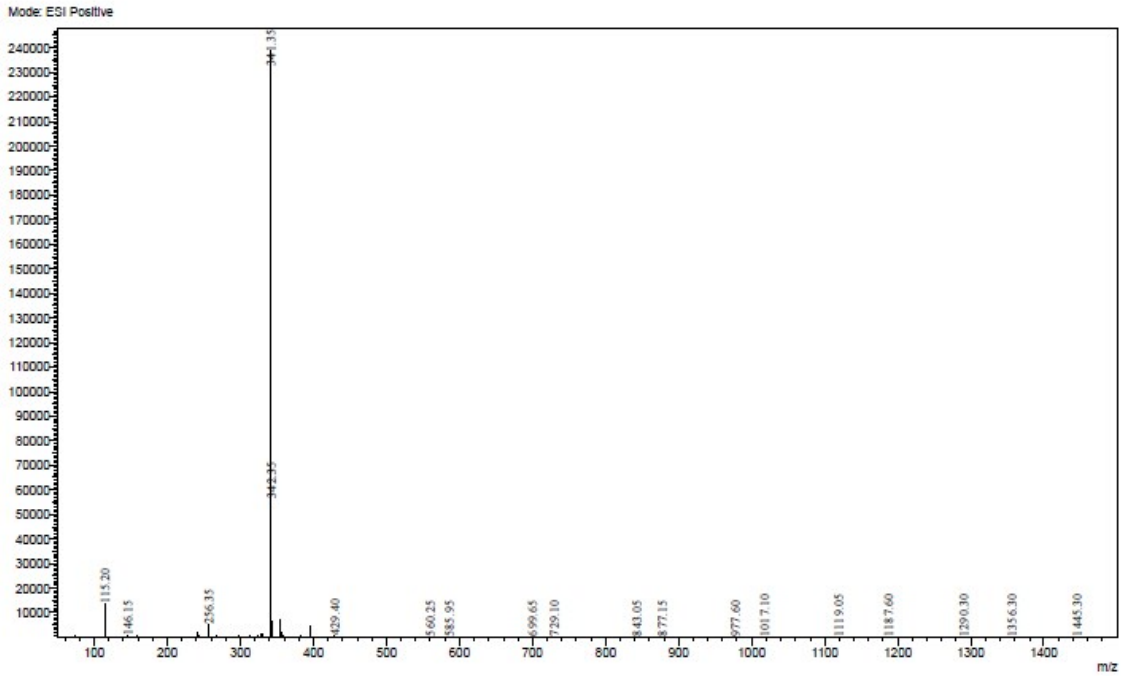


Figure S10: ESI-Mass Spectra of C8

References:

S1. Yuan-Jia Pan, Dian Li, Sha Jin, Chuan Wei, Ke-Yi Wu, Jia Guo and Chang-Chun Wang. Folate-conjugated poly(N-(2-hydroxypropyl)-methacrylamide-co-methacrylic acid) nanohydrogels with pH/redox dual-stimuli response for controlled drug release. Polym. Chem., 2013, 4, 3545–3553

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