Combinatorial bio-conjugation of gemcitabine and curcumin enables dual drug delivery with synergistic anticancer efficacy and reduced toxicity

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1. Synthesis and spectral characterization of GEM-PEG-CUR

The synthesis scheme is shown in Figure S1.

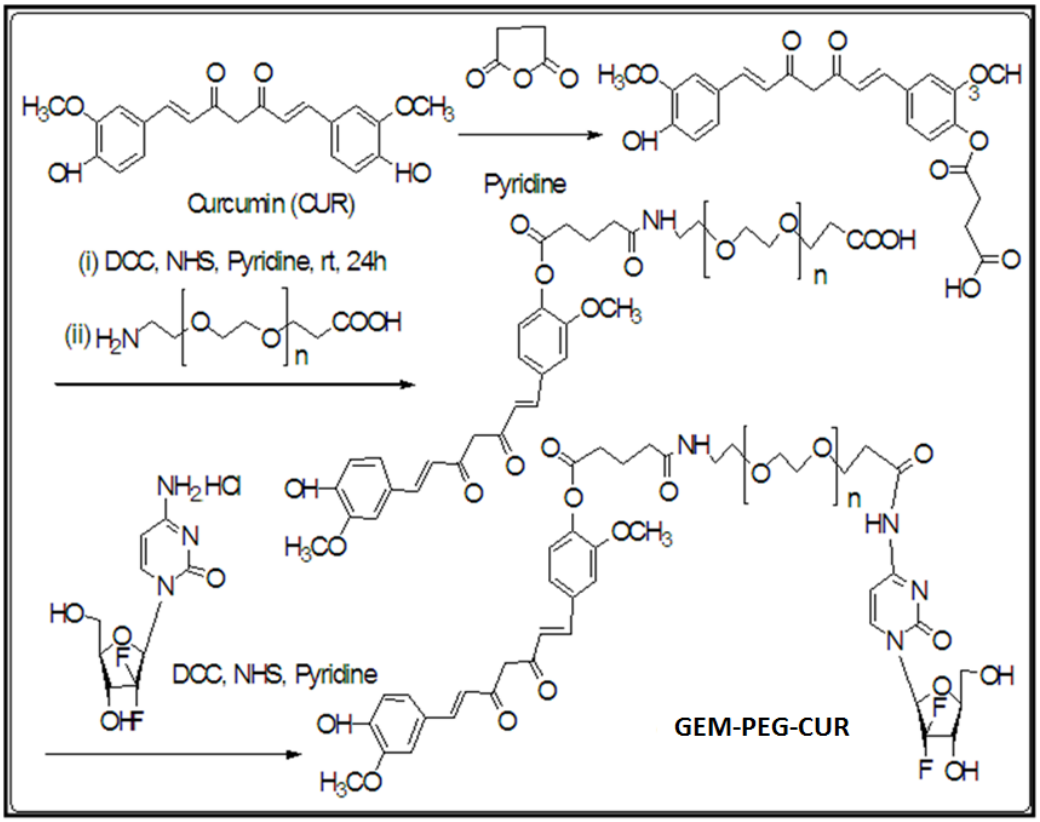

Figure S1: A schematic representation depicting the synthesis of CUR-PEG-GEM conjugate

1.1. Synthesis of curcumin monosuccinate (CUR-COOH)

Curcumin (368 mg, 0.1 mmol) was dissolved in anhydrous benzene and refluxed with succinic anhydride (15 mg, 1.5 mmol) in presence of pyridine (1 ml). After 24 h, the reaction mixture was allowed to cool and precipitated byproducts were filtered out. Purification of the curcumin monosuccinate was achieved using column chromatography technique. Briefly, resultant mixture was adsorbed on 100-200 mesh size silica gel and eluted by hexane - ethyl acetate solvent system. Solvents were concentrated on rotary evaporator and further dried in vacuum under reduced pressure.

State of aggregation: Reddish Orange solid; **Yield:** 75.6%

FTIR (ν_{max}, KBr pellets cm^{-1}): 3503(s, -OH), 2942(Ph-H, C-H), 1627(C=C, C=O), 1601(Ph, C=C), 1510(C=O), 1281 (enol C-O), 1026 (C-O-C).

^{1}H NMR (δ, DMSO-d_6, ppm): 9.8-9.7 (s, PhO**H,** 1H), 7.6-7.5(d, PhC**H**=CH**,** 2H), 7.4-7.3 (d PhC6**H**,6'**H** 2H), 7.2-7.1**(**d, PhC10**H,** 10'**H, 2**H), 6.9-6.8 (d, PhC9'**H,9'H** 2H), 6.8-6.7((d, CH-C**H,** 2H)), 6.1-6.0 (s, C**H**-CO, 1H), 3.9-3.8(OC**H**$_3$).

Mass: 468 (M+)

1.2. PEGylation of curcumin: Synthesis of CUR-PEG-COOH

For synthesis of CUR-PEG derivative, the monosuccinate derivative was transformed to its activated ester using standard protocol of carbodiimide activation and PEGylated as usual. Briefly, CUR-COOH (40 mg, 0.085 mmol) was dissolved in anhydrous DCM and activated with stoichiometric molar equivalent of DCC/NHS in presence of pyridine. After 24h, the reaction mixture was filtered and poured dropwise into ice cold ether to precipitate the NHS ester of CUR-COOH. This activated ester (40 mg, 0.071 mmol) was redissolved in DCM and reacted with NH_2-PEG-COOH (248 mg, 0.071 mmol dissolved in 1 ml of DCM) for 24 h at room temperature. Thereafter, the reaction mixture was filtered and added dropwise into ice-cold ether to precipitate CUR-PEG derivative.

State of aggregation: Yellow solid; **Yield**: 65.8%

FTIR (ν_{max}, KBr pellets cm^{-1}): 3525(s, -OH), 2882(Ph-H, C-H), 1624(s, C=C, C=O), 1510(C=O), 1278(enol C-O), 1026(C-O-C), 1102(br, C-C, PEG)

^{1}H NMR (δ, DMSO-d6, ppm): 8.8-8.7 (s, CH_2CH_2 N**H**CO, 1H, s, PhO**H,** 1H), 8.4-8.3 (d,PhC10,1H), 8.3-8.2**(**d, PhC9**H,** 1H), 7.8-7.7(d, PhC**H**=CH**,** 2H), 7.6-7.5(d PhC6**H**, 2H), **),**

7.4-7.3**(**d, PhC10**H,** 1H), **), 7.3-7.2(**d, PhC10'**H,** 1H), 6.9-6.8(d, CH-C**H,** 2H), 6.8-6.7(d, PhC9'**H,** 1H), 6.6-6.5(s, C**H**-CO, 1H), 4.1-4.0(OCH_3), 3.8-3.2 (b, OCH_2CH_2O).

Mass (MALDI-TOF, ES+): 3957 (M+)

1.3. Conjugation of GEM: Synthesis of GEM-PEG-CUR

CUR-PEG-COOH (100 mg, 0.025 mmol) was activated and added to three fold molar excess of GEM (22.78 mg, 0.075 mmol) dissolved in pyridine. After 24h, the reaction mixture was added drop-wise into ice-cold ether to precipitate the crude GEM-PEG-CUR conjugate. The crude product was dissolved in DCM to precipitate out unreacted GEM. The reaction mixture was filtered and the filtrate was reprecipitated with ether to isolate unreacted CUR-PEG-COOH.

State of aggregation: Yellow solid; **Yield:** 71.4%

UV vis (λ_{max}, nm): 414.5, 255.5

FTIR (ν_{max}, KBr pellets cm^{-1}): 3395(-NH), 2889(Ph-H, C-H), 1624(C=C, C=O), 1536(C=O), 1283(enol, C-O), 1109(br, C-C, PEG)

^{1}H NMR (δ, DMSO-d6, ppm): 9.0-8.9 (b, CH_2CH_2 N**H**CO), 8.8-8.7 (s, PhOH + d, PhC10**,** 1H), 8.6-8.5(d, PhC9**H,** 1H), 8.2-8.1 (b, CHN**H**CO) 8.0-7.9 (d, dFdC-C2**H,** 1H) 7.8-7.7(d, PhC**H**=CH**,** 2H), 7.4-7.3(d PhC6**H**, 2H), 7.3-7.2(d PhC6'**H**, 2H), **6.6-6.5(**d, PhC10'**H,** 1H), 6.5-6.4(d, CH-C**H,** 2H, PhC9'**H,** 1H), 6.1-6.0 dFdC-C6**H,** 1H), 4.3-4.2 (dFdC-C5**H,** 2H), 4.1-3.7 (dFdC-C3**H,** 1H, dFdC-C4**H,** 1H, O**C**H_3, 6H, OCH_2CH_2O).

^{13}C NMR (δ, DMSO-d6, ppm): 210-200 (R-**C**O-R), 190-180 (R-**C**O-X), 130-125 (Ar-**C**), 110-100 (R-**C**H=**C**H-R'), 75-70 (-O**C**H2-**C**H2-O, PEG), 42-40 (**C**H_2 NH-), 30-25 (t-C**C**H_2**C**H_2CO), 20-15 (-**C**H_3).

Mass (MALDI-TOF, ES+): 4215 (m/z)

For UV analysis, 1 mg of conjugate was dissolved in 1 ml of deionized water and UV spectra was recorded in the range of 200-600 nm using Shimadzu UV 1800. The FTIR spectra for GEM-PEG-CUR and intermediates were obtained using a Perkin Elmer, FTIR spectrometer, USA. Briefly, the samples were pressed into KBr pellets before recording their IR absorption spectra. The spectra were detected in KBr disks over a range of 4400-400 cm^{-1}. The chemical structure of GEM-PEG-CURwas authenticated via proton (^{1}H) and carbon (^{13}C) NMR analysis. Briefly, GEM-PEG-CURwas dissolved in DMSO-d_6 (99.9 atom % deuterium-enriched, Sigma -Aldrich Inc., USA) with 0.1 % TMS serving as an internal reference. The NMR experiments were performed using a Bruker BioSpin (Fallanden, Switzerland) Avance -III 400 MHz NMR spectrometer (9.4 T, 54 nm vertical -bore magnet) equipped with a 5 mm BBFO - Plus multinuclear probehead with Z - Gradient, operating at a proton frequency of 400.13 MHz. The spectroscopic task was controlled by a HP xw- 4600 workstation, and the spectral plotting was obtained using Bruker's TOPSPIN 2.1. The molecular mass of GEM-PEG-CUR was determined through matrix assisted laser desorption ionization (MALDI) mass spectroscopy using a Bruker Maldi-TOF instrument. For MALDI-TOF analysis, 0.5 mg of each conjugate was dissolved into 1 ml of deionized water and mass spectra was processed.

2. Result and discussion

2.1. Synthesis and spectral characterization of target conjugate

As a preliminary step to the synthesis of GEM-PEG-CUR conjugate, a hemisuccinate derivative of CUR was synthesized by ring-opening linker elongation of the phenolic hydroxyl (OH) group with succinic anhydride. In order to ensure monosuccinylation, CUR was reacted with only 1.5 molar equivalents of succinic anhydride. The success of succinylation was confirmed by the

appearance of a broad band at 1697 cm^{-1}, which was absent in the FTIR spectrum of pristine CUR (Figure S2).

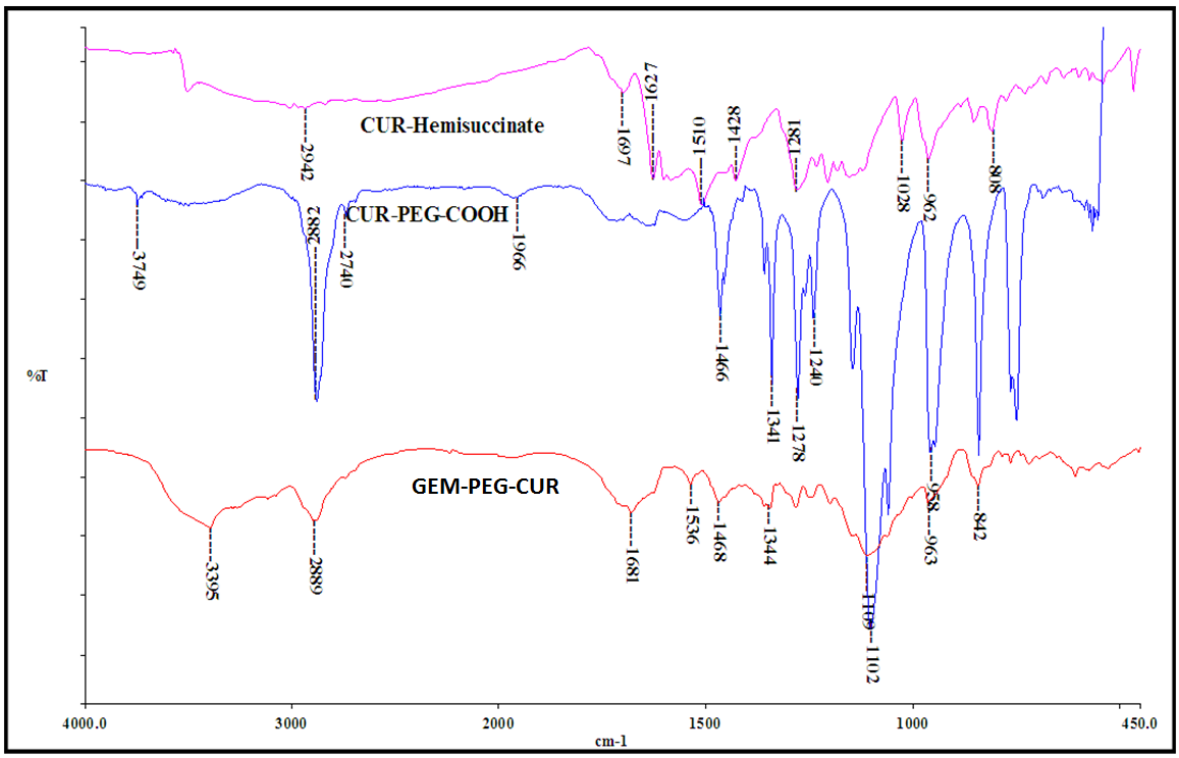

Figure S2: FTIR Spectra of CUR-Hemisuccinate, CUR-PEG-COOH and CUR-PEG-GEM

PEGylation of CUR-succinate with NH_2-PEG-COOH was confirmed by the appearance of an intense band centered at 1102 cm^{-1}, attributed to –O-C- and C-N stretching vibrations of PEG. In addition to increased C-H stretching vibrations at 2882 cm^{-1}, the appearance of new peak at 3749 cm^{-1} was evident. The observed changes may be attributed to the existence of free –OH stretching vibrations (ν COOH) of the terminal carboxyl functionality of heterobifunctional PEG. Finally, CUR-PEG-COOH was smoothly transformed to GEM-PEG-CUR by simple, carbodiimide promoted amidation between GEM and carboxylated CUR-PEG derivative. An inherent broadness was noted in the FTIR spectrum of GEM-PEG-CUR conjugate, particularly

in the range of 1400-1800 cm^{-1} which may be attributed to the superimposition of multiple amide I, amide II, carboxyl, amine and anhydride stretching/bending vibrations in the same range.

In order to confirm the structure of the final product, proton (^{1}H) and carbon (^{13}C) NMR analysis were performed (Figure S3). The ^{1}H NMR spectra of GEM-PEG-CUR conjugate compared to free CUR and CUR-PEG-COOH have been presented in Figure S3(a). The peak assignments have been shown in Figure S3(c) itself.

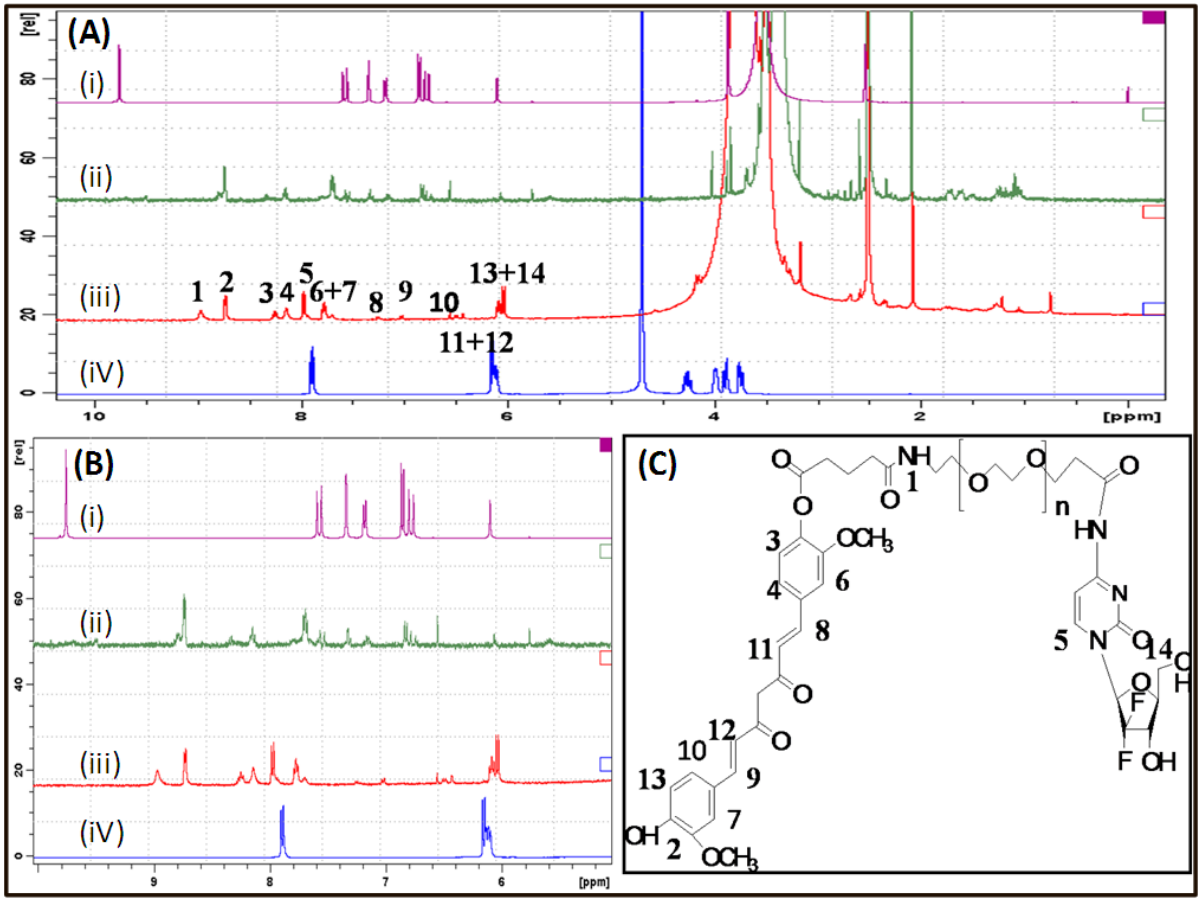

Figure S3: ^{1}H NMR spectra of (A) CUR-Hemisuccinate (i) CUR-PEG-COOH (ii) GEM-PEG-CUR (iii) and free GEM (iv) (B) Expanded view of aromatic region (C) Structural assignment of GEM-PEG-CUR

The ^{1}H NMR spectrum of free CUR presents characteristic peaks of the aryl protons in the range of δ 6.7-7.6 ppm. The characteristic chemical shift due to C1 proton was observed at δ 6.1 ppm. After PEGylation, characteristic peaks of PEG (–O-CH_2-CH_2) protons appeared as a broad signal between δ 3.8-3.2 ppm. The presence of PEG in the final conjugate was also confirmed by ^{13}C NMR analysis in which characteristic peak of the polyoxyethylene (–O-**C**H_2-**C**H_2) carbons were documented between δ 60-65 ppm (Figure S4).

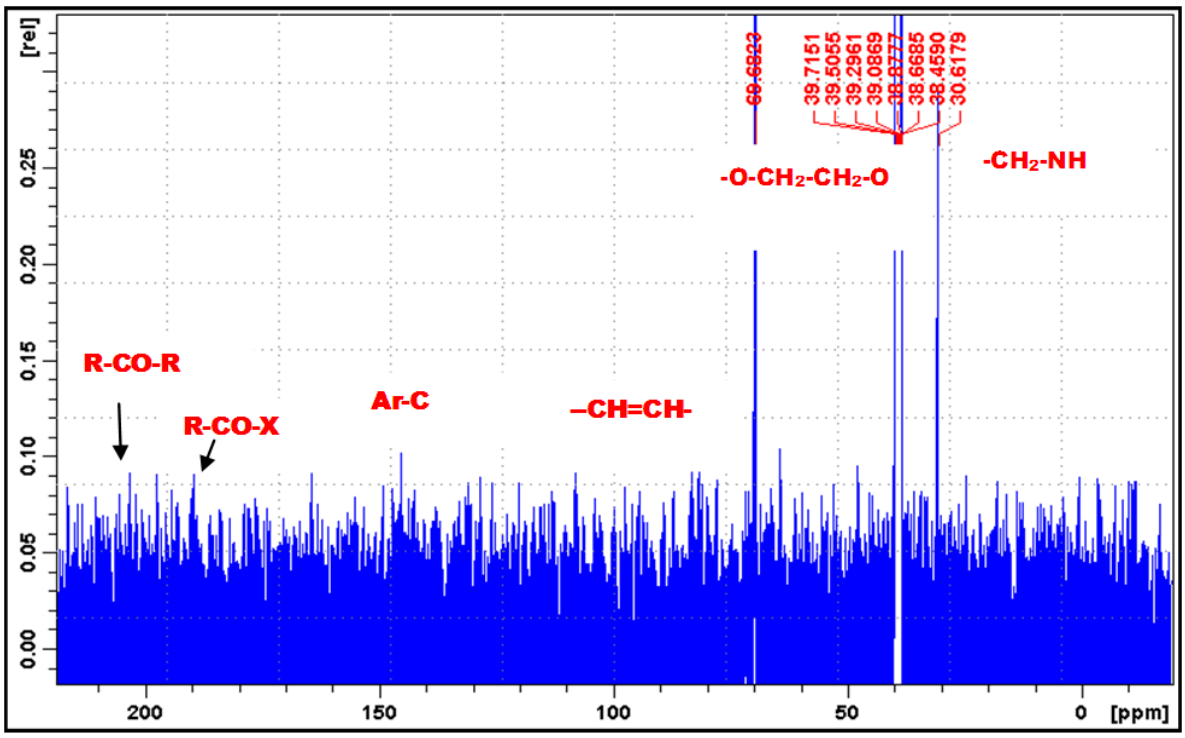

Figure S4: ^{13}C NMR of GEM-PEG-CUR conjugate

Although certain changes with regard to chemical shift position was observed, the proton NMR of the final conjugate showed characteristic proton signals of both CUR and GEM confirming the successful conjugation of GEM with CUR-PEG.

Final confirmation regarding structure came from MALDI-TOF mass spectroscopy (Figure S5).

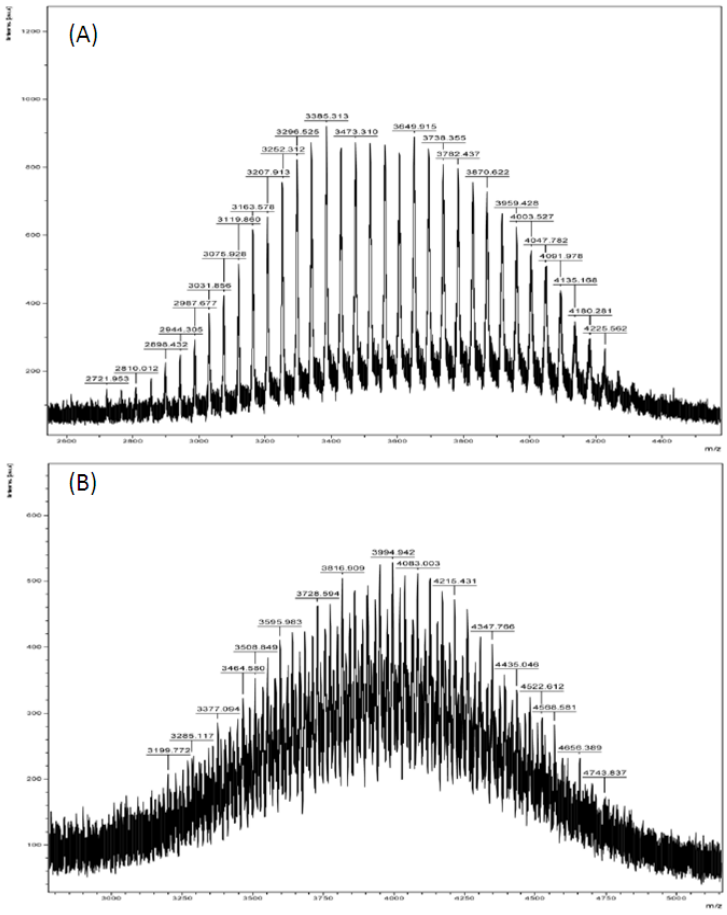

Figure S5: MALDI-TOF-Mass Spectra of (a) CUR-PEG-COOH (b) GEM-PEG-CUR

In case of CUR-PEG, the centre of the bell was observed at m/z 3957, which was close to molecular ion peak of the conjugate. Following GEM conjugation, the centre of the bell shifted to m/z 4215, which is close to the molecular ion peak of GEM-PEG-CUR conjugate.