

Supporting Information

Smart heparin-based bioconjugates synthesized by a combination of ATRP and click chemistry

NMR characterization of bemiparin derivative

Heparin consists of a linear backbone containing, alternating 1,4-linked uronic acid (α -L-iduronic acid [I] or β -D-glucuronic acid [G]) and α -D-glucosamine (A) residues (Figure 1). The units contain differently substituted amino sugar residues, some are N-acetylated (A_{NAc}) instead of N-sulfated (A_{NS}) or amined (A_N), and occasionally some of the iduronic acid residues are nonsulfated (I).

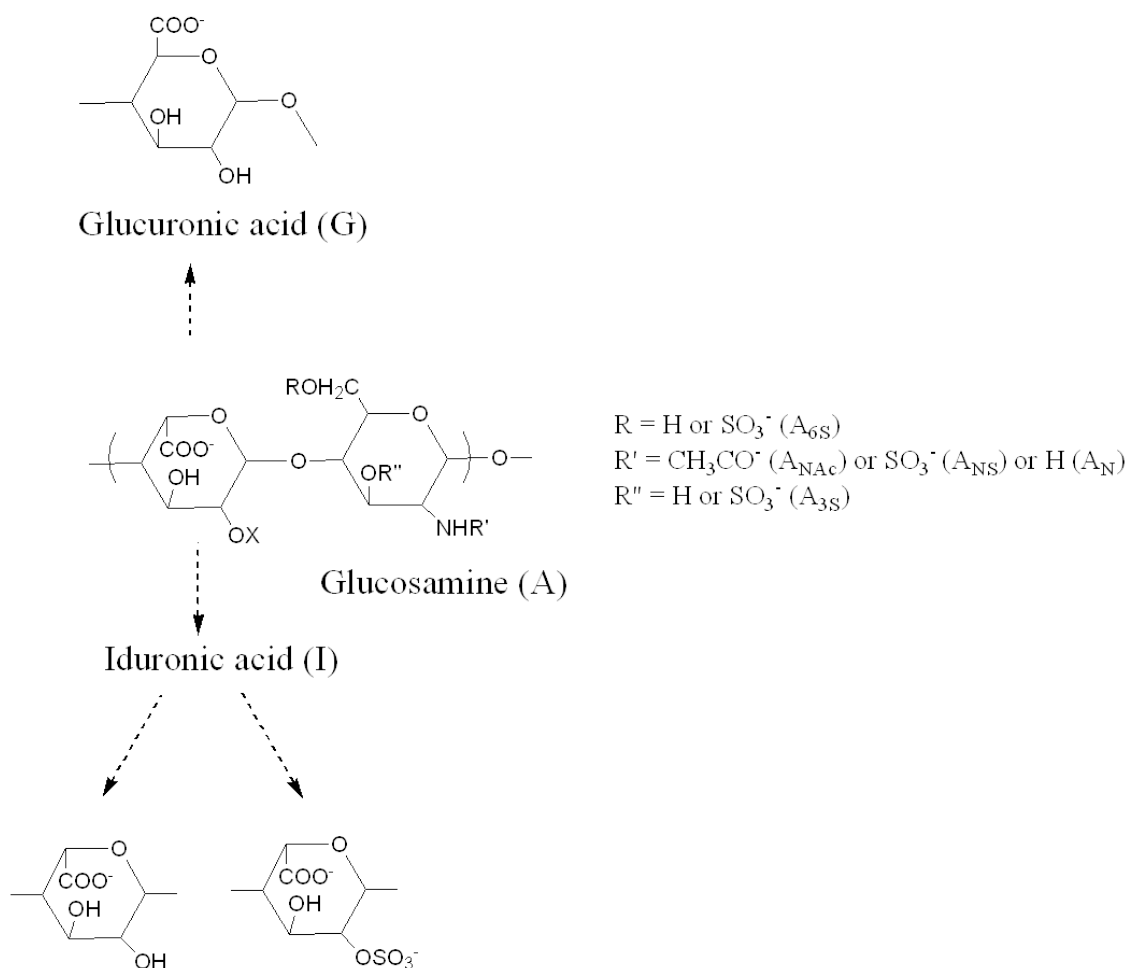


Figure 1. Scheme of the most common substitutions of heparin glucosamine and uronic acid residues.

The ^1H NMR spectra (Figure 2) displays the assigned signals of bemiparin before alkynyl functionalization. The content of A_{NS} was calculated from the signal at 3.25 to 3.40 ppm because the anomeric resonance of A_{NS} shows up as two peaks, the first at 6.10 ppm corresponding to A_{NS} linked to G and the second at 5.58 ppm corresponding to A_{NS} linked to I (Figure 1). The amount of A_{NAC} was calculated by integration of the methyl signal of the N-acetyl group at 1.90 ppm and the sum of the integration signals of $A_{\text{NAC}} + A_{3\text{S}}$ at 1.90 and 5.82 ppm respectively (Equation II). Each bemiparin molecule (3600 Da) contains 10 disaccharide units, 2 of them are A_{NAC} (equation III).

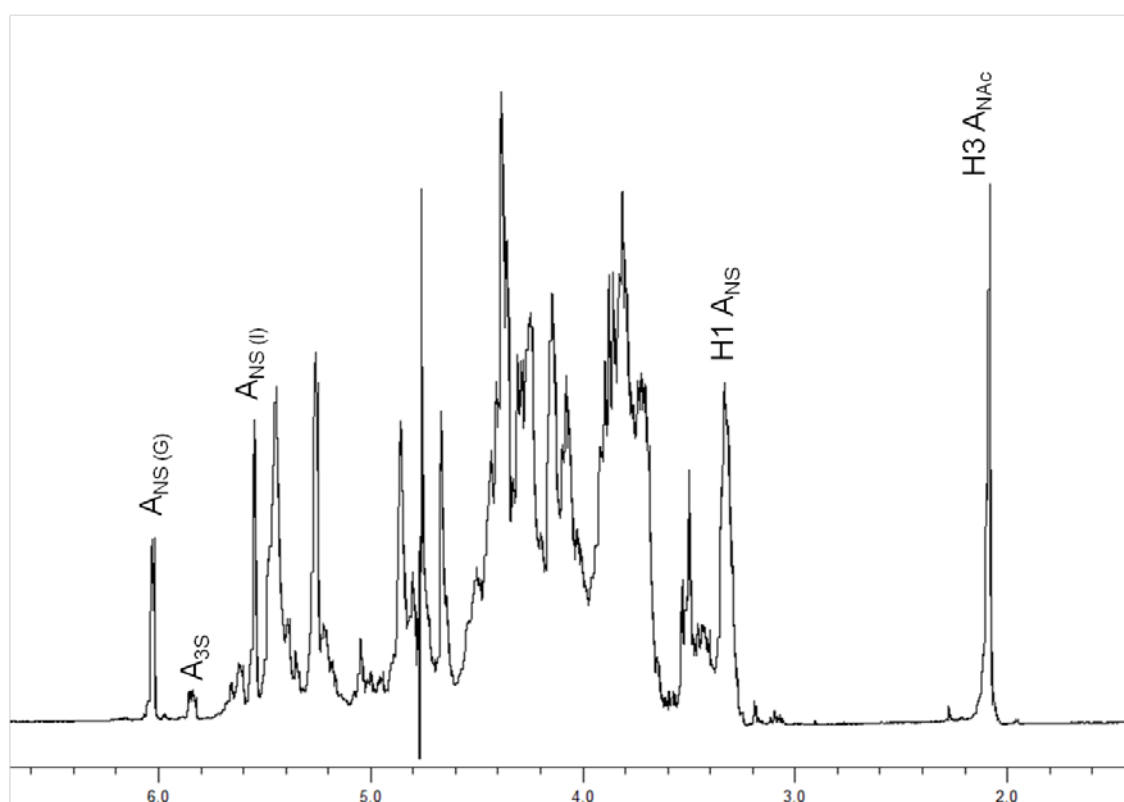


Figure 2. ^1H -NMR spectrum of bemiparin. The spectrum was recorded at 500 MHz on a Varian XL-500 spectrometer, at room temperature from deuterium oxide solution of 15 mg/ml.

Calculation of the percentage of substitution for glucosamine C_2 residue of bemiparin²⁹:

$$A_{\text{T}} = A_{\text{NAC}}/3 + A_{\text{NS}} \quad (\text{Equation I})$$

$$\% \text{NAC} = (A_{\text{NAC}}/3) / A_{\text{T}} \times 100 \quad (\text{Equation II})$$

$$N^{\circ} \text{ NAc groups} = \% \text{ NAc} \times \text{disaccharide units per molecule} \quad (\text{Equation III})$$

$$N^{\circ} \text{ carboxylic groups per molecule} = M_w \text{ disaccharide} / M_w \text{ bemiparin} \quad (\text{Equation IV})$$

^1H and HMBC-NMR characterization of beparin-alkyne derivative

The ratio between the integration intensities of a characteristic resonance signal of acetamide group of bemiparin (NHCOCH_3 , 1.9 ppm) and the signal from the alkyne group at 2.54 ppm or propargylamine (Figure 3), was used to calculate the degree of alkyne functionalization, equation V. The degree of alkyne functionalization was calculated to be 55%.

$$N^{\circ} \text{ alkyne groups} = N^{\circ} \text{ NAc groups} \times A_{\text{alkyne}} / A_{\text{NAc}} \quad (\text{Equation V})$$

$$\% d_{\text{alkyne}} = n^{\circ} \text{ alkyne groups} \times 100 / n^{\circ} \text{ carboxylic groups} \quad (\text{Equation VI})$$

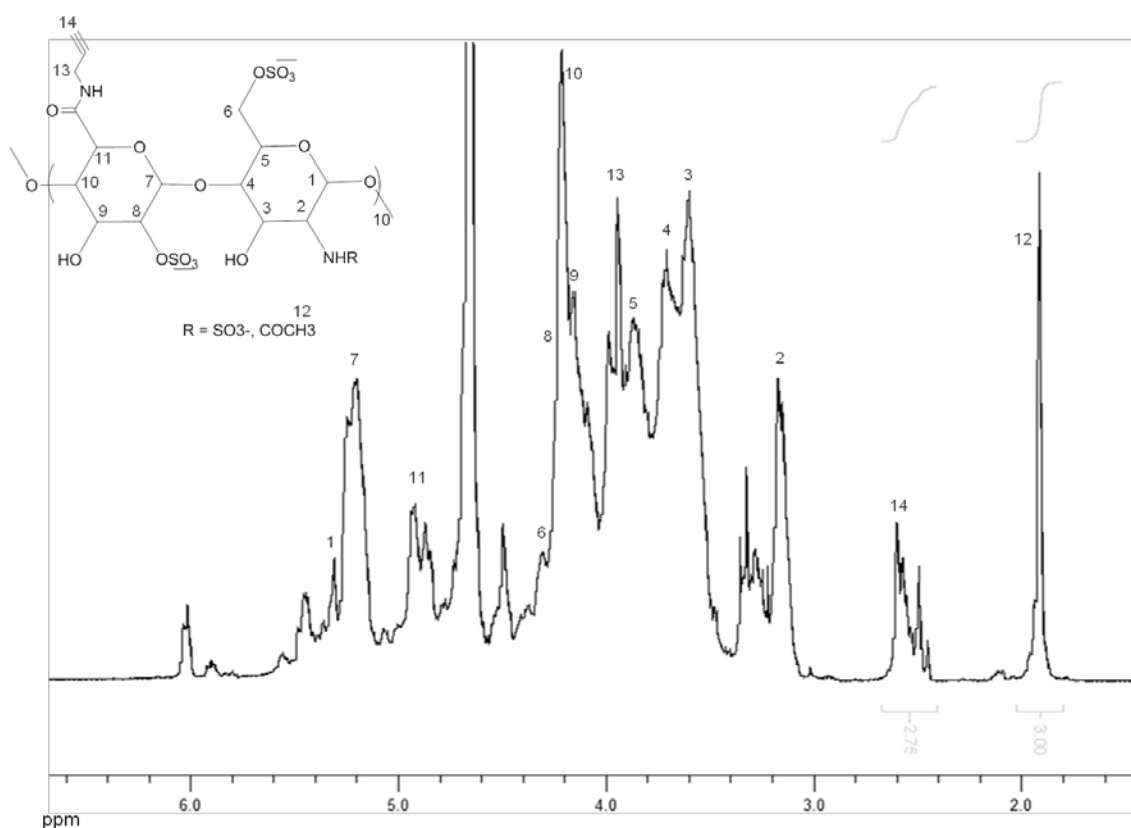
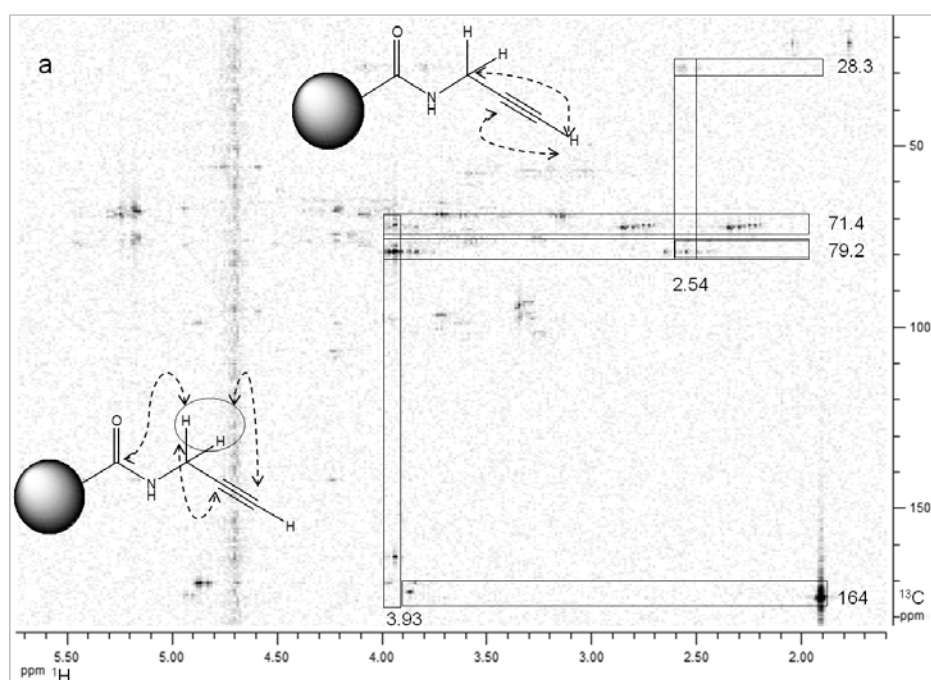


Figure 3. ^1H NMR spectra of bemiparin-alkyne functionalized using DTMM after dialysis for 48h against water.

An HBMNMR experiment was carried out to confirm the covalent attachment of the propargylamine and therefore the degree of amidation. The HBMNMR spectrum (Figure 4) of the alkyne-functionalized bemiparin prepared using DMTMM confirmed the presence of propargylamine covalently attached to bemiparin molecule. The appearance of a signal at 2.54 ppm, which can be assigned to an alkyne proton, can be correlated with the signals at 79.2 and 28.3 ppm belonging to methine and methylene carbons of propargylamine respectively. Furthermore, the methylene protons of propargylamine signal at 3.93 ppm were correlated with the signals at 71.4, 79.2 and 164 ppm attributable to the methylene and methine carbons of propargylamine and the carboxylic carbon of bemiparin respectively. This last correlation indicated that amidation had occurred. However, when EDC and NHS were used to prepare the alkyne-functionalized bemiparin, no correlation between the methylene protons of propargylamine and the carboxylic carbon of bemiparin was observed. The signal at 2.8 ppm disappeared from the ^1H NMR spectra of both products after dialysis against water at pH 10 (pK_a of propargylamine = 9.8) for 48h, which also indicates that the signal at 2.8 ppm arises from the ionic attachment of propargylamine to bemiparin and at a pH > 9.8 the amine of propargylamine is no longer protonated hence the ionic attachment disappeared.



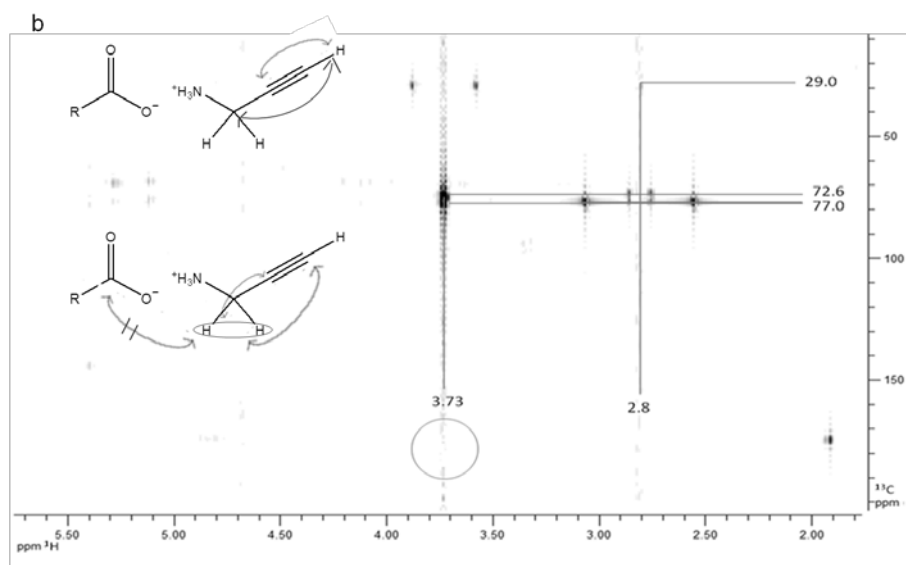


Figure 4. Two-dimensional ^1H - ^{13}C correlation spectrum (HMBC) of bemiarpin-alkyne functionalized using DMTMM (a) or EDC/NHS (b).

SEC results of N_3 -pDMAEMA and N_3 -p(MEO₂MA-co-OEOMA₃₀₀)

SEC results show monomodal molecular weight distributions and polydispersities indexes lower than 1.4 for both α -azide polymers (Figure 5).

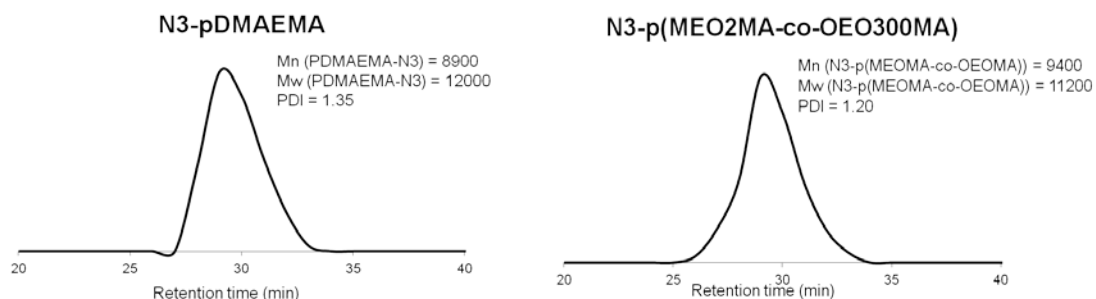


Figure 5. GPC trace of N_3 -PDMAEMA and N_3 -P(MEO₂MA-co-OEOMA₃₀₀) measured in DMF GPC with PMMA Standards.

TGA analysis.

Quantitative evaluation of the composition of bemiparin-PDMAEMA and bemiparin-P(MEO₂MA-co-OEOMA₃₀₀) bioconjugates were determined by taking into account the total weight loss percentage and the percent residue left after thermal degradation. The final molar composition was calculated taking into

account the fractional weight percentages and the molecular weight (M_w) of the products (Equation VII). The amount of bemiparin in the bioconjugates was found to be 22 wt % in the PDMAEMA based bioconjugate and 26 wt % in the P(MEO₂MA-co-OEOMA₃₀₀) copolymer, giving a molar composition of 1:1 for bemiparin-PDMAEMA system and 1:1 for bemiparin-P(MEO₂MA-co-OEOMA₃₀₀).

Equation VII:

$$\text{Bemiparin molar fraction} = [(M_w \text{ bioconjugate} \times \%wt_{\text{bemiparin}}) / M_w \text{ bemiparin} \times 100]$$

$$\text{Polymer molar fraction} = [(M_w \text{ bioconjugate} \times \%wt_{\text{N3-polymer}}) / M_w \text{ N3-polymer} \times 100]$$

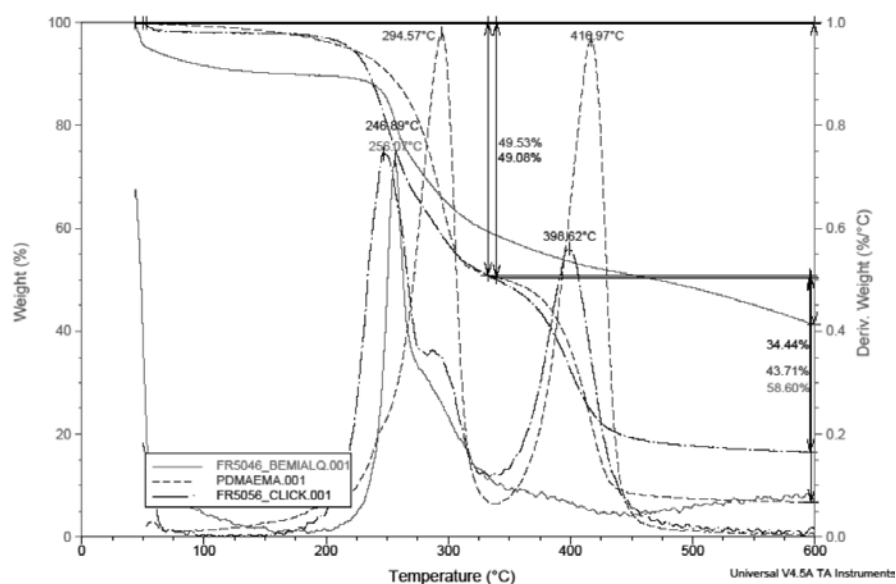


Figure 6. TGA degradation curves and first derivate curves of bemiparin-alkyne (solid silver line), N₃-PDMAEMA (dotted grey line) and click bioconjugate bemiparin-PDMAEMA (dash-dot black line).

$$\% \text{ residue bemiparin} = 50.56 \quad \% \text{ residue N}_3\text{-PDMAEMA} = 6.76 \quad \% \text{ residue click} = 16.48$$

$$50.56x + 6.76y = 16.48 \quad x + y = 1$$

$$X = 0.23 \quad Y = 0.77$$

$$\% \text{ wt bemiparin} = 23 \quad \% \text{ wt N}_3\text{-PDMAEMA} = 77$$

$$15000 \times 0.23 = 3300/3500 = 0.94 \quad 15000 \times 0.77 = 11700/12000 = 0.98$$

1:1 = bemiparin:PDMAEMA

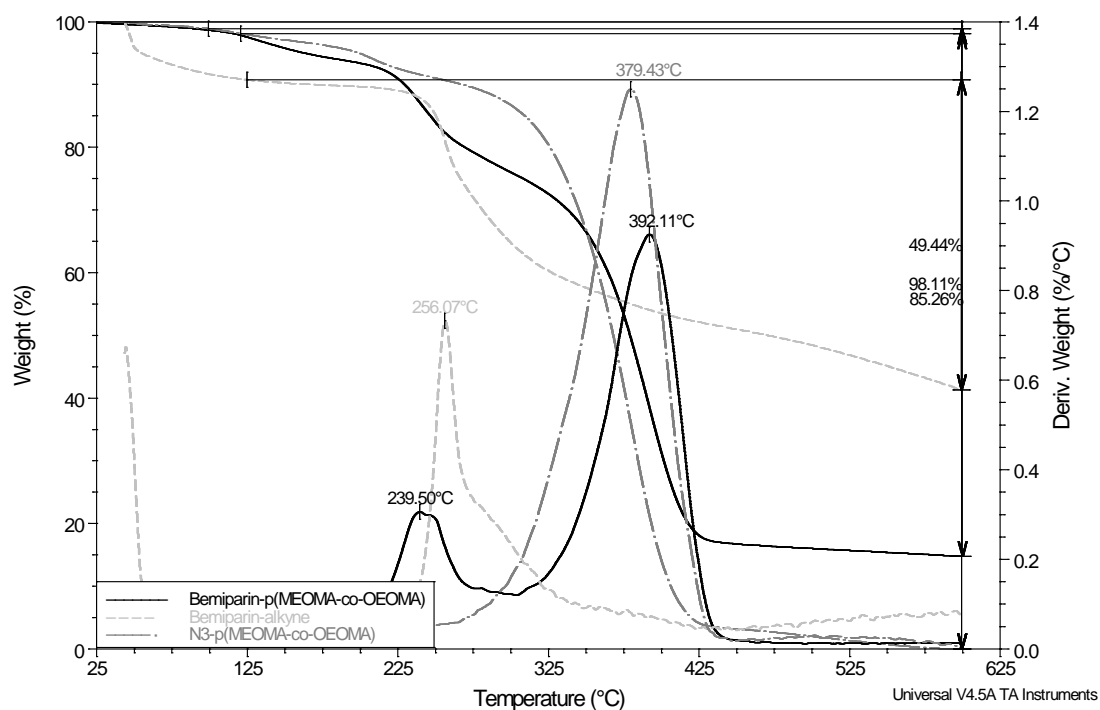


Figure 7. TGA degradation curves and first derivate curves of bemiparin-alkyne (solid black line), N_3 -P(MEO₂MA-co-OEOMA₃₀₀) (dash grey line) and click bioconjugate bemiparin-PDMAEMA (dash-dot silver line).

$$\% \text{ residue bemiparin} = 50.56$$

$$\% \text{ residue } N_3\text{-P(MOE}O_2\text{MA-co-OEOMA}_{300}) = 1.94$$

$$\% \text{ residue click} = 14.74$$

$$50.56x + 1.94y = 14.74$$

$$x + y = 1$$

$$X = 0.26$$

$$Y = 0.74$$

$$\% X = 26$$

$$\% Y = 74$$

$$14800 \times 0.26 = 3848 / 3500 = 1.01$$

$$14800 \times 0.74 = 10952 / 11200 = 0.98$$

$$1:1 = \text{Bemiparin:P(MOE}O_2\text{MA-co-OEOMA}_{300})$$