Covalent cell surface functionalization of human fetal osteoblasts for tissue engineering

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Abstract

The chemical functionalization of cell-surface proteins of human primary fetal bone cells with hydrophilic bioorthogonal intermediates was investigated. Toward this goal, chemical pathways were developed for click reaction-mediated coupling of alkyne derivatives with cellular azido-expressing proteins. The incorporation via a tetraethylene glycol linker of a dipeptide and a reporter biotin allowed the proof of concept for the introduction of cell-specific peptide ligands and to follow the reaction in living cells. Tuning the conditions of the click reaction resulted in chemical functionalization of living human fetal osteoblasts with excellent cell survival.

Key words: human osteoblasts – fetal cells – cell functionalization – click reaction – multivalent linker – cell survival

Introduction

During the last decade, bioorthogonal chemical reactions have been developed to probe biomolecules in living systems.[1-5] The chemical motifs involved in these reactions include peptide sequence with specific side chain combinations, carbonyl moieties for condensation with aminooxy or hydrazides probes, and azides for Staudinger ligation with triarylphosphines and cycloadditions to alkynes.[6,7] The biocompatibility of these reactions provides efficient tools for the modification of proteins, the labeling of enzymes, the metabolic labeling of cell-surface glycans and lipids within their native cellular environment and thus allows to study the biological processes in which these biomolecules are involved. Further development of highly reactive bioorthogonal reagents, avoiding the use of metal promoters,[8-14] extended the application of these probing methodologies to living cells and animals.

A promising approach in tissue engineering is to seed implants with the cells of interest prior to implantation. Such an approach may request chemical functionalization of the cell surface. Human fetal cells have an interesting potential for therapeutic use in tissue engineering and regeneration, including bone tissue engineering, due to their rapid growth rate and their ability to differentiate *in vitro* into mature osteoblasts as well as their histocompatibility,[15-17] eliminating the need for anti-graft rejection medication of the patients.

For further development of bone implants, we aimed to develop means to chemically functionalize human fetal osteoblasts toward future application of these modified cells in implant biomaterials. As small peptides[18,19] can promote cell adhesion and proliferation, we have focused on the development of hydrophilic ligands containing amino acid residues. Here, the use of copper-mediated and copper-free catalyzed azide-alkyne [3+2] dipolar cycloaddition was investigated for the covalent functionalization of cell-surface proteins of human primary fetal osteoblasts. Chemical ligands containing amino acid residues, a terminal

alkyne for functionalization of cell surface proteins through copper-mediated and copper-free [3+2] dipolar cycloadditions and a biotin label for probing cell functionalization were designed and synthesized. Then their potential to covalently functionalize human fetal osteoblasts was evaluated. The efficiency of the coupling reaction was determined using the reporter biotin conjugate, as previously used in a different cellular model,[20,21] and the long-term survival of the functionalized cells following the click reactions was optimized.

Experimental Procedures

General methods

Commercial reagents (Fluka, Aldrich, Bachem) were used without purification. Anhydrous solvents were obtained by filtration. Liquid/solid flash chromatography (FC): columns of silica gel (0.040-0.63 mm, Merck No.9385 silica gel 60, 240-400 mesh). Eluent: mixture of light petroleum ether (PE) and ethyl acetate (EtOAc) or mixture of dichloromethane (DCM) and methanol (MeOH). TLC for reaction monitoring: Merck silica gel 60 F_{254} plates; detection by UV light; Pancaldi reagent, $KMnO_4$, ninhydrine or phosphomolybdic acid. IR spectra: Perkin-Elmer-1420 spectrometer. ^{1}H NMR spectra: Bruker-ARX-400 spectrometer (400 MHz); δ(H) in ppm relative to the solvent's residual ^{1}H signal [$CHCl_3$, δ(H) 7.27, MeOD, δ(H) 3.31] as internal reference; ^{13}C NMR spectra: same instrument as above (100.6 MHz); δ(C) in ppm relative to solvent's C-signal [$CDCl_3$, δ(C) 77.1, MeOD, δ(C) 49.0] as internal reference. GC-MS spectrometer (Nermag R-10-10C, chemical ionization (NH3) mode m/z); MALTI-TOF spectrometer (Axima-CFR+, Kratos, Manchester); ESI-Q spectrometer (Finnigan SSQ 710C, Thermoquest, UK); ESI-QT spectrometer (Ultima spectrometer, Micromass, Manchester).

Syntheses

***Tert*-butyl N^2-[9A fluoren-9-ylmethoxy)carbonyl]-N-3,6,9,12-tetraoxa-pentadec-14-yn-1-yl-L-α-glutaminate (5).** Fmoc-glu-(O*t*Bu)-OH (360 mg, 0.9 mmol, 1 equiv) and PyBOP (540 mg, 1.0 mmol, 1.2 equiv) were dissolved in DCM (8 mL). DIPEA[a] (0.3 mL, 1.7 mmol, 2 equiv) was added, followed by a solution of **3**[22] (200 mg, 0.9 mmol, 1 equiv) in dichloromethane (4 mL) at 0 °C. After 15 min, the reaction mixture was warmed to 25 °C and stirred for 12 h. The solvent was removed under reduced pressure and the resulting mixture was dissolved in EtOAc. This solution was washed with saturated NH_4Cl, H_2O, 5 % $NaHCO_3$

and finally with saturated NaCl. The organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The product was purified by flash column chromatography (EtOAc/petroleum ether 2:1 then 3:1) (332 mg, 60 %). IR (film): 3285, 2870, 1715, 1665, 1520, 1450, 1390, 1350, 1245, 1150, 1085, 1045, 950, 840, 760, 740, 645, 620, 555, 540, 515, 500 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ=7.76 (d, ^3J= 7.5 Hz, 1H, H_{arom}), 7.60 (d, ^3J= 7.4 Hz, 1H, H_{arom}), 7.40 (t, ^3J= 7.4 Hz, 1H, H_{arom}), 7.32 (t, ^3J= 7.4 Hz, 1H, H_{arom}), 6.75 (s, 1H, NH), 5.83 (s, 1H, NH), 4.41 (m, 2H, H-1''), 4.21 (m, 4H, H_2-13, H-9, H-2), 3.69-3.45 (m, 16H, $(CH_2\text{-}O)_8$), 2.36 (m, 2H, H-4, H_2-3), 2.01 (m, 3H, H-4, H_2-3, H-15), 1.46 (s, 9H, $(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ=172.7, 171.9, 143.8, 141.3, 127.7, 127.1, 125.1, 119.9, 80.7, 74.7, 70.2, 70.1, 70.0, 69.9, 68.6, 67.0, 58.3, 54.4, 47.2, 39.3, 31.6, 28.1. HRMS (ESI): (*m*/*z*): calcd for $C_{35}H_{46}N_2O_9$+H: 639.3282, found: 639.3292. $[\alpha]_D^{25}$ = - 22 (*c*=0.085, CH_2Cl_2).

***Tert*-butyl N^2-[4-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl-pentanoyl]-N-(3,6,9,12-tetraoxapentadec-14-yne-1-yl)-L-α-glutaminyl-L-phenylalaninate (9).** N^2-[9A fluoren-9-ylmethoxy)carbonyl]-N-3,6,9,12-tetraoxa-pentadec-14-yn-1-yl-L-α-glutaminate **5** (536 mg, 0.84 mmol, 1 equiv) was dissolved into a solution of 4 M HCl in dioxane (3.20 mL) at 25 °C. After stirring for 1h the solvent was removed and HCl was coevaporated in presence of acetonitrile to afford acid **6** as a white solid (420 mg, 86 %). N^2-[9A fluoren-9-ylmethoxy)carbonyl]-N-3, 6, 9, 12-tetraoxa-pentadec-14-yn-1-yl-L-α-glutaminate **6** (100 mg, 0.17 mmol, 1 equiv) and PyBOP (107 mg, 0.21 mmol, 1.2 equiv) were dissolved in dry DCM (1.6 mL). Triethylamine (88 µL 0.51 mmol, 3 equiv) was added at -5 °C. A solution of L-Phe-O*t*Bu (44 mg, 0.17 mmol, 1 equiv) in DCM (1.2 mL) was added dropwise. The reaction mixture was stirred until total conversion of the starting material (monitored by TLC). The solvent was removed under reduced pressure and EtOAc was added. The organic phase was washed with saturated NH_4Cl, H_2O and finally aqueous 5 % $NaHCO_3$. The resulting mixture

was dried over anhydrous $MgSO_4$, filtered and concentrated. The product **7** was purified by flash column chromatography (CH_2Cl_2/MeOH: 95:5) to afford a colorless oil (100 mg, yield 75 %). *Tert*-butyl N^2-[9A fluoren-9-ylmethoxy)carbonyl]-N-(3,6,9,12-tetraoxa-pentadec-14-yn-1-yl)-L-α-glutaminyl-L-phenylalaninate **7** (100 mg, 0.13 mmol, 1 equiv) was dissolved into a solution of 20 % piperidine in DMF (2.5 mL) at 25 °C. After stirring for 1h the solvent was evaporated and piperidine was removed by coevaporation with toluene. The product was purified by flash column chromatography (CH_2Cl_2/MeOH: 95:5) to afford a colorless oil (17 mg, 18 %). *Tert*-butyl N-(3,6,9,12-tetraoxa-pentadec-14-yn-1-yl)-L-α-glutaminyl-L-phenylalaninate (17 mg, 0.03 mmol, 1 equiv) and biotin-OSu (10 mg, 0.03 mol, 1 equiv) were dissolved in DMF (0.3 mL) at 25 °C. Triethylamine (6.3 µL, 0.05 mmol, 1.5 equiv) was added. The reaction mixture was stirred for 12 h at 25 °C. The solvent was removed and the product was purified by HPLC (XTerra Prep RP C18, (19x150 mm Waters)) to afford **9** as a colorless oil (7 mg, 30 %). IR (film): 3275, 2925, 2860, 2405, 1645, 1535, 1455, 1365, 1255, 1200, 1150, 1095, 1030, 840, 700, 605, 580, 570, 520 cm^{-1}. ^1H NMR (400 MHz, MeOD): δ=7.25 (m, 5H, H_{arom}), 4.54 (dd, ^2J= 8.2, ^3J= 6.7 Hz, H-6a), 4.49 (dd, 1H, ^2J= 7.8, ^3J= 4.8 Hz, H-2, H-2'), 4.30 (dd, 1H, ^2J= 7.8, ^3J= 4.4 Hz, H-2, H-2'), 4.25 (dd, 1H, ^2J= 8.2, ^3J= 6.4 Hz, H-3a), 4.18 (d, 2H, ^4J= 2.4 Hz, H-13), 3.67-3.34 (m, 16H, $(CH_2\text{-}O)_8$, 3.20 (m, 1H, H-4'''), 3.07 (dd, 1H, ^2J= 13.8, ^3J= 6.7 Hz, H_2-3), 2.98 (dd, 1H, ^2J= 13.8, ^3J= 5.5 Hz, H_2-3), 2.92 (dd, 1H ^2J= 12.7, ^3J= 5.0 Hz, H_2-6'''), 2.85 (t, 1H, ^3J= 2.4 Hz, H-15), 2.70 (d, 1H, ^3J= 12.7 Hz, H_2-6'''), 2.27 (m, 4H, $(CH_2)_2$), 199-1.44 (m, 8H, $(CH_2)_4$), 1.42 (s, 9H, $(CH_3)_3$). ^{13}C NMR (100 MHz, MeOD): δ=176.0, 174.8, 173.9, 172.7, 166.2, 138.2, 130.4, 129.4, 127.9, 83.0, 76.0, 71.5, 71.5, 71.3, 71.2, 70.4, 70.1, 63.2, 61.6, 59.0, 56.9, 55.9, 54.2, 41.0, 40.4, 38.6, 36.4, 32.8, 29.6, 29.4, 29.0, 28.2, 26.6. HRMS (ESI): (*m*/*z*): calcd for $C_{39}H_{59}N_5O_{10}S$+H: 790.4061, found: 790.4073. $[\alpha]_D^{25}$ = - 55 (*c*=0.04, CH_2Cl_2).

***Tert*-butyl N^2-[5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-pentanoyl]-N-(3,6,9,12-tetraoxapentadec-14-yne-1-yl)-L-α-glutaminyl-L-prolinate (10).** N^2-[9A fluoren-9-ylmethoxy)carbonyl]-N-3,6,9,12-tetraoxa-pentadec-14-yn-1-yl-L-α-glutaminate **5** (100 mg, 0.17 mmol, 1 equiv) and PyBOP (107 mg, 0.21 mmol, 1.2 equiv) were dissolved in dry DCM (1.6 mL). Triethylamine (88 µL, 0.51 mmol, 3 equiv) was added at -5 °C. A solution of L-Pro-O^{t}Bu (36 mg, 0.17 mmol, 1 equiv) in DCM (1.2 mL) was added dropwise. The reaction mixture was stirred until total conversion of the starting material. The solvent was removed under reduced pressure and EtOAc was added. The organic phase was washed with saturated NH_4Cl, H_2O and finally 5 % $NaHCO_3$. The resulting mixture was dried over anhydrous $MgSO_4$, filtered and concentrated. The product was purified by flash column chromatography (CH_2Cl_2/MeOH: 95:5) to afford a colorless oil (81 mg, yield 65 %). *Tert*-butyl N^2-[9A fluoren-9-ylmethoxy)carbonyl]-N-(3,6,9,12-tetraoxa-pentadec-14-yn-1-yl)-L-α-glutaminyl-L-prolinate **8** (81 mg, 0.11 mmol, 1 equiv) was dissolved into a solution of 20 % piperidine in DMF (2.2 mL) at 25 °C. After stirring for 1h the solvent was evaporated and piperidine was removed by coevaporation with toluene. The product was purified by flash column chromatography (CH_2Cl_2/MeOH: 95:5 then 9:1) to afford a colorless oil (34 mg, 60 %). *Tert*-butyl N-(3,6,9,12-tetraoxa-pentadec-14-yn-1-yl)-L-α-glutaminyl-L-prolinate (34 mg, 0.07 mmol, 1 equiv) and biotin-OSu (23 mg, 0.07 mol, 1 equiv) were dissolved in DMF (0.7 mL) at 25 °C. Triethylamine (13.8 µL, 0.99 mmol, 1.5 equiv) was added. The reaction mixture was stirred at 25 °C for 12 h. The solvent was removed and the product was purified by HPLC (XTerra Prep RP C18, (19x150 mm Waters)) to afford **10** as a colorless oil (12 mg, 25 %). IR (film): 3270, 2870, 2475, 1640, 1535, 1445, 1345, 1245, 1200, 1090, 1035, 940, 840, 650, 605, 595, 530, 520 cm^{-1}. ^{1}H NMR (400 MHz, MeOD): δ =4.48 (m, 1H, H-6a), 4.33 (m, 2H, H-3a, H-2), 4.19 (d, 2H, ^4J= 2.3 Hz, H_2-13), 3.68-3.54 (m, 16H, $(CH_2$-$O)_8$), 3.42 (m, 2H, H_2-5), 3.21 (m, 1H, H-4), 2.93 (dd, 1H, ^2J= 12.7, ^3J= 5.0 Hz, H_2-6) 2.86 (t, 1H, ^3J= 2.4 Hz, H-

15), 2.71 (d, 1H, ^{2}J= 12.7 Hz, H_2-6), 2.48-1.56 (m, 16H, $(CH_2)_8$), 1.47, (s, 9H, $(CH_3)_3$).^{13}C NMR (100 MHz, MeOD): δ =176.1, 173.3, 173.2, 82.7, 76.0, 71.6, 71.6, 71.3, 70.5, 70.1, 63.3, 61.7, 61.1, 59.1, 57.0, 54.3, 48.4, 41.1, 40.6, 40.4, 36.5, 31.6, 30.3, 29.6, 29.4, 29.3, 28.3, 26.7, 25.6. HRMS (ESI): (*m*/*z*): calcd for $C_{35}H_{57}N_5O_{10}S$+H: 740.3904, found: 740.3927. $[\alpha]_D^{25}$ = - 31 (*c*=0.035, CH_2Cl_2).

N-(*Tert*-butoxycarbonyl)glycyl-N-(2-{2-[2-(2-hydroxyethoxy) ethoxy]ethoxy}ethyl)prolinamide (11) Triethylamine (2.3 mL, 16.5 mmol, 3 equiv) and PyBOP (3.2 g, 6.1 mmol, 1.1 equiv) were added to a solution of Boc-Gly-Pro (1.5 g, 5.5 mmol, 1 equiv) in DCM (26 mL). After 15 minutes 2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethanol (1.1 g, 5.5 mmol, 1 equiv) in DCM (26 mL) was added. The reaction mixture was stirred until total conversion of the starting material (monitored by TLC). The solvent was removed under reduced pressure and the product was purified by column chromatography (CH_2Cl_2/MeOH 95:5) to afford a colorless oil (2.34 g, 95 %). IR (film): 3410, 2875, 2495, 1650, 1440, 1365, 1250, 1165, 1120, 980, 840, 630, 540, 505 cm^{-1}. ^{1}H NMR (400 MHz, MeOD): δ=4.44 (dd, ^{2}J= 8.6, ^{3}J= 2.8 Hz, $1H_a$, H-2) 4.40 (dd, ^{2}J= 8.6, ^{3}J= 3.7 Hz, $1H_b$, H-2), 3.91 (m, H_2-2'), 3.68-3.35 (m, 18H, $(CH_2$-$O)_6$, CH_2-OH, H_2-5), 3.22 (q, $N^+H(CH_2CH_3)_3$), 2.35-1.86 (m, 4H, H_2-3, H_2-4), 1.45 (s, 9H, $(CH_3)_3$), 1.31 (t, $N^+H(CH_2CH_3)_3$).^{13}C NMR (100 MHz, MeOD): δ=174.6, 170.5, 158.5, 80.6, 73.6, 71.6, 71.3, 71.2, 71.1, 70.4, 70.3, 62.2, 61.9, 61.4, 47.6, 43.8, 40.4, 40.5, 33.3, 30.7, 28.7, 25.6, 23.40. HRMS (ESI): (*m*/*z*): calcd for $C_{20}H_{37}N_3O_8$+H: 448.2659, found: 448.2658. $[\alpha]_D^{25}$ = - 26 (*c*=0.09, CH_2Cl_2).

***Tert*-butyl N^2-[5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-pentanoyl]-N-(3,6,9,12-tetraoxa-pentadec-14-yn-1-yl)-L-α-glutaminylglycyl-N-(2-{2-[2-(2-hydroxyethoxy) ethoxy]ethoxy}ethyl)-L-prolinamide (12)** N^2-[9A fluoren-9-ylmethoxy)carbonyl]-N-3,6,9,12-tetraoxa-pentadec-14-yn-1-yl-L-α-glutaminate **6** (100 mg,

0.17 mmol, 1 equiv) and PyBOP (107 mg, 0.21 mmol, 1.2 equiv) were dissolved in dry DCM (1.6 mL). Triethylamine (88 µL, 0.51 mmol, 3 equiv) was added at -5 °C. A solution of glycyl-*N*-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)-L-prolinamide **11** (60 mg, 0.17 mmol, 1 equiv) in DCM (1.2 mL) was added dropwise. The reaction mixture was stirred until total conversion of the starting material (monitored by TLC). The solvent was removed under reduced pressure and EtOAc was added. The organic phase was washed with saturated NH_4Cl, H_2O and finally 5 % $NaHCO_3$. The resulting mixture was dried over anhydrous $MgSO_4$, filtered and concentrated. The product was purified by flash column chromatography (CH_2Cl_2/MeOH: 9:1) to afford a colorless oil (20 mg, yield 12 %). *Tert*-butyl N^2-[9A fluoren-9-ylmethoxy)carbonyl]-N-(3,6,9,12-tetraoxa-pentadec-14-yn-1-yl)-L-α-glutaminylglycyl-N-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)-L-prolinamide (20 mg, 0.02 mmol, 1 equiv) was dissolved into a solution of 20 % piperidine in DMF (0.5 mL) at 25 °C. After stirring for 1h the solvent was evaporated and piperidine was removed by coevaporation with toluene. The product was purified by flash column chromatography (CH_2Cl_2/MeOH: 9:1 then CH_3CN/NH_4: 5:1) to afford a colorless oil (13 mg, 86 %). *Tert*-butyl N-(3, 6, 9, 12-tetraoxa-pentadec-14-yn-1-yl)-L-α-glutaminylglycyl-N-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)-L-prolinamide (13 mg, 0.02 mmol, 1 equiv) and biotin-OSu (7 mg, 0.02 mol, 1 equiv) were dissolved in DMF (0.2 mL) at rt. Triethylamine (4.0 µL, 0.03 mmol, 1.5 equiv) was added. The reaction mixture was stirred at 25 °C for 12 h. The solvent was removed and the product was purified by HPLC (XTerra Prep RP C18, (19x150 mm Waters)) (to afford **12** as a colorless oil (6 mg, 33 %). IR (film): 3265, 3075, 2920, 2865, 1780, 1640, 1535, 1445, 1345, 1330, 1245, 1200, 1090, 1030, 940, 830, 645, 600, 545, 540, 525, 510, 500 cm^{-1}.^{1}H NMR (400 MHz, MeOD): δ=4.49 (dd, 1H, ^2J= 7.8, ^3J= 5.1 Hz, H-6a), 4.43 (dd, 1H, ^2J= 8.6, ^3J= 3.7 Hz, H-2, H-2'') 4.33 (m, 2H, H-3a, H-2, H-2''), 4.19 (d, 2H, ^4J= 2.4 Hz, H-13), 4.15 (dd, 1H, H-2'), 3.94 (dd, 1H, ^2J= 17.0, ^3J= 4.6 Hz, H-2'), 3.69-3.35

(m, 34H, $(CH_2\text{-}O)_{16}$, H_2-5), 3.21 (m, 1H, CH), 2.93 (dd, 1H, ^2J= 12.8, ^3J= 5.0 Hz, H_2-6) 2.86 (t, 1H, ^3J= 2.4 Hz, H-15), 2.71 (d, 1H, ^2J= 12.7 Hz, H_2-6), 2.40-1.45 (m, 16H, $(CH_2)_8$).^{13}C NMR (100 MHz, MeOD): δ=176.0, 175.3, 174.0, 171.8, 169.9, 76.0, 73.7, 71.6, 71.55, 71.5, 71.4, 71.3, 71.2, 70.3, 70.1, 70.1, 63.2, 62.2, 61.9, 61.7, 59.0, 57.0, 54.1, 47.8, 42.8, 41.0, 40.6, 40.4, 36.4, 36.3, 32.9, 30.8, 29.6, 29.5, 29.4, 28.9, 26.7, 26.6, 25.7. HRMS (ESI): (*m*/*z*): calcd for $C_{41}H_{69}N_7O_{14}S$+H: 916.4702, found: 916.4717. $[\alpha]_D^{25}$ = - 18 (*c*=0.05, CH_2Cl_2).

[5-[3a, 4a, 6a)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-pentanoyl]-N-(3,6,9,12-tetraoxa-pentadec-14-yn-1-yl)pentanamide (13) Biotin-OSu (0.83 g, 2.4 mmol, 1 equiv) followed by triethylamine (0.5 mL, 3.6 mmol, 1.5 equiv) were added to a solution of 3, 6, 9, 12-tetraoxapentadec-14-yn-1-amine (0.56 g, 2.4 mmol, 1 equiv) in DMF (24 mL). The reaction mixture was stirred overnight at rt. The solvent was removed under reduced pressure and the desired product was purified by column chromatography (CH_2Cl_2/MeOH 95/5) to provide a colorless oil (560 mg, 51 %). IR (film): 3245, 2925, 2865, 1810, 1780, 1700, 1645, 1550, 1460, 1350, 1325, 1305, 1265, 1245, 1210, 1090, 1035, 945, 860, 840, 760, 635, 600, 540, 530, 505 cm^{-1}.^{1}H NMR (400 MHz, MeOD): δ=4.49 (dd, 1H, ^2J= 8.0, ^3J= 5.0 Hz, H-6a), 4.31 (dd, 1H, ^2J= 7.9, ^3J= 4.5 Hz, H-3a), 4.19 (d, 2H, ^4J= 2.5 Hz, H-13), 3.69-3.61 (m, 12H, $(CH_2\text{-}O)_6$, 3.56 (t, 2H, ^3J= 5.3 Hz, CH_2-O), 3.36 (m, 2H, CH_2-O), 3.20 (m, 1H, H-4a), 2.93 (dd, 1H ^2J= 12.8, ^3J= 5.0 Hz, H_2-6), 2.85 (t, 1H, ^3J= 2.4 Hz, H-15), 2.71 (d, 1H, ^3J= 12.7 Hz, H_2-6), 2.23 (t, 2H, ^3J= 7.3 Hz, CH_2), 1.77-1.41 (m, 6H, $(CH_2)_3$).^{13}C NMR (100 MHz, MeOD): δ=176.22, 75.97, 71.55, 71.51, 71.50, 71.34, 71.23, 70.57, 70.11, 63.34, 61.60, 59.04, 56.98, 41.04, 40.38, 36.77, 29.74, 29.48, 26.83, 26.28. HRMS (ESI): (m/z): calcd for $C_{21}H_{35}N_3O_6S$+H: 458.2325, found: 458.2337. $[\alpha]_D^{25}$ = 22 (*c*=0.195, CH_2Cl_2).

***N*-[14-(2,2-difluorocyclooct-3-yn-1-yl)-13-oxo-3,6,9-trioxa-12-azatetradec-1-yl]-5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (16)** The (2,2-difluorocyclooct-3-yn-1-yl)acetic acid (10 mg, 0.05 mmol, 1 equiv) was dissolved with dry dichloromethane

(0.38 mL) in a flame-dried round-bottom flask and put under an argon atmosphere. A solution of amino-PEG-biotin (20.70 mg, 0.05 mmol, 1 equiv) and N-ethyldiisopropylamine (12.69 mg, 0.07 mmol, 1.5 equiv) in dry DCM (0.38 mL) was added to the reaction mixture. The system was stirred at rt for 5 h, and then purified by flash chromatography (9:1 to 8:2 EtOAc/MeOH) to afford a white solid (12.10 mg, 0.02 mmol, 41 %). IR (film) : 3265, 2930, 2870, 2220, 1705, 1645, 1555, 1460, 1385, 1280, 1240, 1130, 1030, 1010, 940, 750, 690, 665, 650, 605 cm^{-1}.^{1}H NMR (400 MHz, MeOD-d_4) : δ= 4.50 (dd, 1H, ^2J = 7.7, ^3J= 5.0 Hz, H-34,), 4.31(dd, 1H, ^2J= 7.7, ^3J= 4.5 Hz, H-30), 3.64 (app. d, 8H, ^3J= 5.6 Hz H_2-15, H_2-16, H_2-18, H_2-19), 3.55 (t, 4H, ^3J= 5.4 Hz, H_2-34, H_2-21), 3.37 (m, 4H, H_2-12, H_2-22), 3.21 (m, 1H, H-29), 2.93 (dd, 1H, ^2J= 12.8, ^3J= 4.9 Hz, H_2-35a), 2.83-2.74 (m, 1H, H-1), 2.71 (d, 1H, ^2J= 12.7 Hz, H_2-35b), 2.51 (dd, 1H, ^2J= 12.7, ^3J= 3.8 Hz, H_2-9a), 2.47-2.31 (m, 2H, H_2-5), 2.22 (app. t, 3H, ^3J= 6.9 Hz, H_2-25, H_2-9b), 2.17-2.08 (m, 2H, H_2-6), 1.86-1.51 (m, 6H, H_2-26, H_2-28, H_2-7), 1.48-1.29 (m, 4H, H_2-27, H_2-8) ppm.^{13}C NMR (100 MHz, MeOD-d_4) :δ=176.1, 173.8, 166.1, 111.7, 111.5, 71.6, 71.27, 71.26, 70.6, 70.5, 63.3, 61.6, 57.0, 54.4, 41.0, 40.4, 40.3, 36.7, 35.9, 33.77, 33.75, 33.3, 33.2, 29.8, 29.5, 28.9, 26.8, 20.6 ppm. HRMS (ESI): (m/z): calcd for $C_{28}H_{44}F_2N_4O_6S$+H: 603.3028, found: 603.3027. $[\alpha]_D^{25}$ = 130 (*c*=0.105, CH_2Cl_2).

Cellular assays

Cells and cell culture reagents

Human fetal osteoblasts were derived by the explant technique from the femoral bone of a 12-weeks old fetus according to a protocol accepted by the Lausanne Hospital and University Ethics Committee and with the mother's oral and written approval (protocol No 51/01, 2008). The cells were used at passage 6 of the initial culture. Cells were routinely grown in DMEM medium containing 4.5 g/l glucose, 10% heat-inactivated fetal calf serum (FCS) and

penicillin/streptomycin (P/S). All cell culture reagents were obtained from Invitrogen, Basel, Switzerland. L-azidohomoalanine (AHA) was purchased from Invitrogen, bovine serum albumin (BSA), copper sulfate and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) from Sigma-Aldrich, Buchs, Switzerland and the WST-1 reagent (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-teterazolium) from Roche Diagnostics, Rotkreuz, Switzerland. The click-reactions were performed in the BD Falcon^{TM} CultureSlides (BD Biosciences, Belgium).

Experimental conditions for the click reactions:

Human fetal osteoblasts (80-90 % confluent) were washed with warm (37°C) PBS and incubated in methionine-free DMEM culture medium containing 4.5 g/l glucose, antibiotics (P/S) and 3% FCS for 30-60 min to deplete methionine reserves. Then they were incubated for 4 h in the presence of AHA (50 µM) in fresh complete culture medium. The culture medium was removed and the cell layer was washed twice with PBS. Then the click reactions were performed and efficacy of the reaction was evaluated by fluorescence microscopy or fresh complete medium was added and culture continued for assessing cell survival using the WST-1 test.

Fluorescence microscopy experiments

Click-reaction (Scheme 5A): Stock solutions of compounds **9**, **10**, **12**, **13** (0.2 mM in PBS), TBTA (1.7 mM in 4:1 ^{t}butanol:DMSO) and $CuSO_4$ (20 mM in water) were prepared. For microscopy experiments, the click reaction was performed in a total volume of 500 µl, containing PBS (410 mL), compounds **9**, **10**, **12**, **13** (20 µM), TBTA (80 µM) and $CuSO_4$ (0.8 mM) sequentially added to the cells. Incubation was performed for 1 h at 37 °C. The reaction mixture was removed and two washings with PBS allowed the complete removal of the different reagents. Streptavidin-Cy3 (Sigma-Aldrich) (1:100) and DAPI (4',6-diamidino-2-phenylindole, Roche Diagnostics) (1 µg/mL in PBS) were then added and incubated with the

cells for 30 min in the dark. After two washings with PBS during 5 min in the dark, the cells were fixed using a 4 % *p*-formaldehyde solution at 4° C. Two additional washings with PBS were performed. The chamber was removed from the slide glass by using the chamber removal device and the evaluation of the click-reaction was performed by fluorescence microscopy and slides photographed under a fluorescence microscope (Zeiss Axioplan 2).

Click-reaction (Scheme 5B): Stock solutions of compounds **12** and **13** (1 mM in PBS), $CuSO_4$ (2 mM in water), THPTA (10 mM in PBS), aminoguanidine (50 mM in PBS) and sodium ascorbate (100 mM) were prepared. The reaction mixture containing compounds **12** or **13** (100 µM), aminoguanidine (1 mM), $CuSO_4$ (50 µM), THPTA (250 µM) and sodium ascorbate (2.5 mM) in PBS was pre-incubated for 1 h at 4 °C The cell culture medium was removed and the cell layer was washed twice with HBSS. For microscopy experiments, the reaction mixture was added to the cells at 4 °C for 5 min. The reaction mixture was removed and two washings with PBS at 37 °C allowed the complete removal of the different reagents. The cells were then fixed using a 4 % *p*-formaldehyde solution for 10 min at room temperature. After two washings with warm PBS, the cells were incubated with PBS containing 1 % BSA for 1 h at room temperature, then the labeling was performed as above.

Click-reaction (Scheme 5C): Stock solution of compound **16** (2.5 mM in PBS:DMF (7:3)) was prepared. For microscopy experiments, the click reaction was performed in a solution containing compound **16** (100 µM) in DMEM containing additional HEPES (20 µM). Incubation was performed for 1 h at 25 °C. The reaction mixture was removed and two washings with PBS at 37 °C allowed the complete removal of the reagents. The cells were then fixed using a 4 % p-formaldehyde solution for 10 min at room temperature. After two washings with warm PBS, the cells were incubated with PBS containing 1 % BSA for 1 h at room temperature and the labeling was performed as above.

Evaluation of the conjugation of the ligands to the cells: The copper-free click reaction with compound **16** (100 µM) and the THPTA/copper-catalyzed click reaction with compounds **12** or **13** (100 µM) were performed on human fetal osteoblasts grown on histological slides and pre-incubated with AHA, and subsequently exposed to fluorescent streptavidin to label biotin according to the described procedures. To evaluate for non-specific binding, human fetal osteoblasts without pre-incubation with AHA were exposed to compounds **16** or **13** (100 µM) in the presence of the copper catalyst then to fluorescent streptavidin. The cell-associated fluorescence was determined by fluorescence microscopy (Zeiss Axioplan 2). For each treatment, a picture was taken under identical exposure conditions and the fluorescence of individual cells (20 cells per treatment) was quantified using Image J software. Results are the mean $\pm$ sd of the mean fluorescence intensity (MFI) per cell.

Alternatively, human fetal osteoblasts grown in 96-wells plates (Costar) and either copper-free or THPTA/copper-catalyzed click reactions were performed with controls and the compound **16** (100 and 200 µM) or compounds **13** (100 and 200 µM) . The cells layers were carefully washed and 100 µl/well of a 1:1000 dilution of HRP (horse radish peroxidase)-cojugated streptavidin (Dako, Baar, Switzerland, 0.83 mg/ml) in PBS containing 1% BSA were added for 30 min at 37°C, then after washing with PBS containing 0.05% Tween, 100 µl/well of a 3,3,5,5'-tetramethylbenzidine (TMB)-H_2O_2 solution were added and the increase of absorbance at 450 nm was recorded for 30 min at 37 °C in a thermostated absorbance multiwall-plate reader (iEMS, LabSystems). Then the liquid was aspirated and the cell layers were lyzed in 20 µl/well of lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 10% v/v glycerol, 1% v/v NP40, 1% w/v CHAPS) then µl/well of PBS-0.1% Triton X-100 were added, and the protein content of the lyzed cells was quantified using the BCA kit (BCA Protein Assay Kit,

Pierce, Rockford, USA) and bovine serum albumin as standard, according to the provider's protocol.

WST-1 Cell Viability Assay

Cytotoxicity of the click reagents: cell cultures were treated in culture medium with various concentrations of the reagents for 1 h at 37 °C. The culture medium was removed and the cells were washed twice with HBSS, then cultures were continued in complete medium for 24 h to 96 h. The culture medium was removed, cells were rinsed with culture medium without phenol red (Invitrogen) and incubated with the WST-1 reagent (Roche Diagnostics), diluted 1:10 in culture medium without phenol red and without FCS for 3 h. Optical density of the supernatants was measured at 450 nm in a multiwell-plate reader (iEMS Reader MF, Labsystems, Bioconcept) against a blank containing culture medium and WST-1.

Copper-catalyzed click reaction: cell cultures were treated with the reaction mixture containing compound **13** (20 µM), $CuSO_4$ (0.8 mM), TBTA (80 µM) in PBS at 37 °C for 1 h. The culture medium was removed and the cells were washed twice with HBSS, then cultures were continued in complete medium. Cell survival was evaluated after 24 h to 96 h as above.

Modified copper-catalyzed click reaction: cell cultures were treated with the reaction mixture containing compound **13** (100 µM), aminoguanidine (1 mM), $CuSO_4$ (50 µM), THPTA (250 µM) and sodium ascorbate (2.5 mM) in PBS at 4 °C for 5 min. Then cell survival was evaluated after 24 h to 96 h as above.

Copper-free click reaction: cell cultures were treated with compound **16** (100 µM) in DMEM containing 4.5 g/l glucose, antibiotics (P/S) and additional HEPES (20 µM) for 1 h at room temperature. Then cell survival was evaluated after 24 h to 96 h as above.

Results

Synthesis of chemical ligands for functionalization of cell proteins

Following the procedure of Svensson,[22] tetraethylene glycol was transformed into mono-azide **2** in 61% yield (Scheme 1). The alkynyl functionality was introduced by alkylation in the presence of propargyl bromide, followed by Staudinger reduction[23,24] to provide the amine **3** in good yield. Coupling with semi-protected L-glutamic acid **4** and subsequent acidic cleavage of the resulting *tert*-butyl ester afforded the intermediate derivative **6**, presenting three orthogonal groups for the successive introduction of amino acids or peptides, a labeling agent and a group for the attachment to cell surface glycoproteins. Further coupling with protected amino acids was performed in the presence of PyBOP, at -5°C, to deliver the intermediates **7** and **8** in good yields (Scheme 2).

Cleavage of the carbamate under basic conditions, followed by conjugation with an activated biotin moiety afforded compounds **9** and **10**, ready to undergo copper catalyzed [3+2] cycloaddition to azido-modified cell surface glycoproteins. A similar route was applied to introduce a Gly-Pro dipeptide. Boc-Gly-Pro was functionalized in 95% yield with an amino-tetraethylene glycol chain to increase hydrophilicity. Further coupling with intermediate **6** at low temperature and derivatization with a biotin label afforded compound **12** as an alkynyl substrate for click-reaction with azido-modified cellular proteins.

The simpler model molecule **13** was also prepared to determine the appropriate conditions for the copper-mediated click reaction. The synthesis was performed starting from amine **3** which was coupled with an activated biotin in the presence of triethylamine (Scheme 3). The desired product **13** was provided in 51 % yield.

To avoid the use of a metal catalyst, a chemical ligand suitable for copper-free click reaction was also synthesized. The difluorinated cyclooctyne (DIFO) moiety developed by Bertozzi and co-workers[11] which has demonstrated a high potential to bind to cell surface proteins via a

copper-free click reaction was introduced in one of our conjugates for the functionalization of human fetal osteoblasts. The DIFO **14** was coupled with the tetraethylene glycol-biotin derivative **15** in the presence of PyBOP at room temperature to provide the model compound **16** (Scheme 4) which can react with azido-modifed cell proteins.

Biological evaluation of the synthesized compounds using click reactions

The cell experiments were designed to determine which experimental coupling conditions represent the best compromise between chemical reactivity and biocompatibility. The functionalization of the cell proteins of human primary fetal osteoblasts was performed under three different experimental conditions: two copper-catalyzed click reactions in the presence of different copper complexes and protective reagents and one copper-free click reaction performed in cell culture medium without fetal calf serum (FCS) (Scheme 5). The biocompatibility of the copper catalyst and of the synthesized compounds was determined using assessment of cell viability with the WST-1 assay up to 96 h after the click reactions.

In order to express azide-modified proteins, human fetal osteoblasts were cultured in the presence of the non-natural amino acid L-azidohomoalanine (AHA) which can replace methionine without cell alterations,[6] thus, allowing the insertion of an azide moiety in cellular proteins. Proliferating primary human fetal osteoblasts were labeled for 4 h with AHA added to methionine-free cell culture medium before performing the click reactions. The model molecule **13** was used first to establish the optimal conditions for the copper-mediated click reaction with cells. Different ratios of copper sulfate to the copper-reducing agent tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) were evaluated at a fixed concentration (10 µM) of **13**. Reported standard conditions use 1 mM copper sulfate and 0.1 mM TBTA at 25°C.[6] Here, the reaction was performed at 37°C in phosphate buffered saline pH 7.4 (PBS) for 1 h in order to decrease the loading of copper catalyst. Cell nuclei labeling was performed

with the fluorescent reporter 4',6-diamidino-2-phenylindole (DAPI). Detection of cell surface ligation of **13**, as evidenced by the binding of fluorescent streptavidin-Cy3 to the reporter biotin moiety of the prepared conjugate, was demonstrated by fluorescence microscopy (Figure 1). The results showed that a ratio of copper to TBTA of 10 to 1 was optimal, confirming published information.[25] Therefore, for further experiments the concentrations used were 0.8 mM copper sulfate and 0.08 mM TBTA. Then we determined the optimal concentration of the alkyne moiety for performing the click reaction with cells using increasing concentrations of **13**. The best results were obtained with a 20 μM concentration of **13** (Figure 1B), whereas in the absence of preincubation of cells with AHA no labeling was detected (Figure 1A). Thus, these conditions were applied for the functionalization of the fetal osteoblast with compounds **9**, **10** and **12**. (Figure 1C-1E). The functionalization of cells, as evidenced by the binding of fluorescent streptavidin to cells, was observed for all compounds **9**, **10**, **12** and **13**.

However, an important and very rapid cytotoxicity was observed under these experimental conditions (Figure 2A). While ligands **9**, **10**, **12** and **13** did not show any cytotoxicity at concentrations up to 100 μM (Figure 2C), the catalytic system TBTA/$CuSO_4$ induced cell death after 1 h exposure at concentrations higher than 0.8 mM in copper (Figure 2B). This significant cytotoxicity of the copper catalytic promoter prompted us to explore alternative conditions for the functionalization of the fetal osteoblasts by click reaction.

Hong and co-workers have recently reported[26] a modified copper-catalyzed click reaction with decreased cytotoxicity that allows the labeling of living cells. In this modification, the use of the water soluble tris-(hydroxypropyltriazolylmethyl)amine (THPTA) promoter in the catalytic system in the presence of sodium ascorbate increases the reaction rate allowing a lower copper concentration and captures the reactive oxygen species (ROS) generated by the metal, thus decreasing cytotoxicity. Aminoguanidine also added to the reaction mixture traps

dehydroascorbate and its decomposition products before they can react with protein side chains.[26] These reaction conditions were applied for the functionalization of fetal osteoblasts with **12** and **13**. Aminoguanidine (1 mM), **12** or **13** (100 µM), copper sulfate (50 µM), THPTA (250 µM) and sodium ascorbate (2.5 mM) were sequentially added to the cells. After 5 min at 4°C, the reagents were removed and labeling of cell nuclei with DAPI and detection of streptavidin-Cy3 binding by fluorescence microscopy were performed. The functionalization of the cell surface was observed for both **12** and **13** (Figure 3B and 3C), whereas in the absence of preincubation of cells with AHA no labeling was detected (Figure 3A). After the reaction, the cells were washed free of the reagents and cultured in complete culture medium and the WST-1 assay was performed after 24, 48, 72 and 96 h (Figure 4A). The results demonstrated a good cell survival since human fetal osteoblasts survived and proliferated for up to four days after the copper-catalyzed click reaction performed with the THPTA ligand in the presence of aminoguanidine.

Finally, the functionalization of the human fetal osteoblasts by copper-free click reaction with compound **16** was evaluated. Compounds presenting activated difluorinated cyclooctyne[11] can react with azide moieties without copper catalyst since the loss of the ring strain (18 kcal/mol) and the decrease of the lowest unoccupied molecular orbital (LUMO) energy by the two fluoride atoms allow the decrease of the activation energy of the click reaction. The copper-free click reaction of compound **16** (100 µM) with human fetal osteoblasts was performed at room temperature for 1 h in culture medium without FCS containing additional HEPES (20 µM) for buffering. Cell nuclei were labeled with DAPI and the cell functionalization was demonstrated by fluorescence microscopy using Cy3-streptavidin (Figure 3E), whereas in the absence of preincubation of cells with AHA no labeling was detected (Figure 3D). An excellent cell survival rate for up to 96 h after the cycloaddition process was evidenced (Figure 4B).

The level of conjugation of compounds **12**, **13, 16** or in the absence of AHA (for non-specific binding) to human fetal osteoblasts was evaluated following click reactions performed under non-cytotoxic conditions using two techniques. The cell-associated fluorescence of individual cells (20 cells per treatment) was quantified under identical exposure conditions using Image J software as the mean fluorescence intensity (MFI) per cell (Figure 5A), showing a slightly increased conjugation rate of compounds **16** under copper-free click-reaction and **12** under copper-catalyzed click-reaction compared to compound **13** under copper-catalyzed click-reaction. Quantification was also performed using HRP-streptavidin and a cellular-ELISA assay. The increase in cell-bound HRP enzymatic activity per well was related to the amount of cell proteins per well (Figure 5B), showing a dose-dependent binding of both compounds **13** and **16**, without noticeable differences between both, suggesting that copper-free and protected copper-mediated click reactions performed with comparable efficacy.

Discussion

Human fetal cells represent an appealing approach for the seeding of implant biomaterials for tissue engineering, including bone engineering, as these cells do not require the use of anti-graft rejection treatment.[16] This study has demonstrated the possibility to functionalize primary human fetal osteoblasts through click chemistry with amino acid-containing chemical ligands able to maintain cell survival and proliferation. While ligands **9**, **10** and **12** were easily conjugated to azido-modified surface proteins of fetal osteoblasts in the presence of a Cu(I) promoter, these reaction conditions induced a drastic loss of cell viability even with reduced reaction time. This major drawback was circumvented by the introduction of a highly reactive alkynyl functionality in the ligands (compound **16**), thus avoiding the use of a copper catalyst in the cycloaddition process. Nevertheless, as the preparation of DIFO containing ligands[11] is more complex, requiring a much higher number of steps compared to a simple alkynyl

containing ligand, we turned our attention to the protection of cells from the Cu(I) catalyst by the aminoguanidine-THPTA combination recently proposed by Hong et al.[26] In our hands, these experimental conditions applied to primary human fetal osteoblasts resulted in both efficient cell functionalization and excellent cell survival. In addition, all chemical ligands prepared to promote further cell adhesion and proliferation showed perfect biocompatibility with the studied cells.

In conclusion, we have shown that biocompatible biotin-containing ligands can be designed and synthesized for conjugation to cellular azido-modified proteins of primary human fetal osteoblasts. Using the click reaction, cell functionalization was probed with fluorescent streptavidin. Three methods of cell functionalization were explored allowing a comparison in term of efficiency and biocompatibility of different experimental conditions. The use of the classical TBTA/$CuSO_4$ catalytic system led to efficient conjugation but suffered from high cytotoxicity which prevented further application for developing implant biomaterials. Protection of the cells from the copper catalyst through the use of the THPTA-ascorbate system in the presence of aminoguanidine led to a good reaction rate while maintaining a high level of cell viability. Similarly, good reaction rates and excellent cell viability were observed for the copper-free click reaction. Incorporation of peptidic components in the ligands was possible maintaining the efficacy and biocompatibility of functionalization and the survival of the primary human fetal osteoblasts. These results open the route for the use of peptide-functionalized human fetal osteoblasts as seeding components for implant biomaterials.

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The authors declare no conflicts of interest.

Supporting Information Availability. Detailed protocols for synthesis and analytical data for compounds **5**, **9**, **10**, **12**, **15** and **16** are provided as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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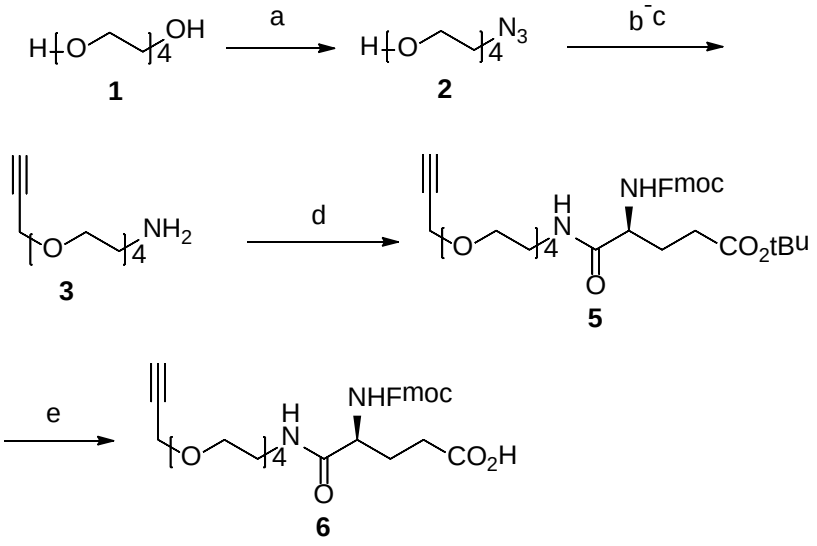
[a]**Abbreviations List**

AHA	L-azidohomoalanine
BSA	bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
DIFO	difluorinated cyclooctyne
DIPEA	N,N-diisopropylethylamine
DMEM	Dulbecco modified Eagle medium
FCS	fetal calf serum
HBSS	Hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horse radish peroxidase
LUMO	lowest unoccupied molecular orbital
PBS	phosphate buffered saline
P/S	penicillin/streptomycin
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
ROS	reactive oxygen species
TBTA	tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
TMB	3,3,5,5'-tetramethylbenzidine
THPTA	tris-(hydroxypropyltriazolylmethyl)amine
WST-1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-teterazolium

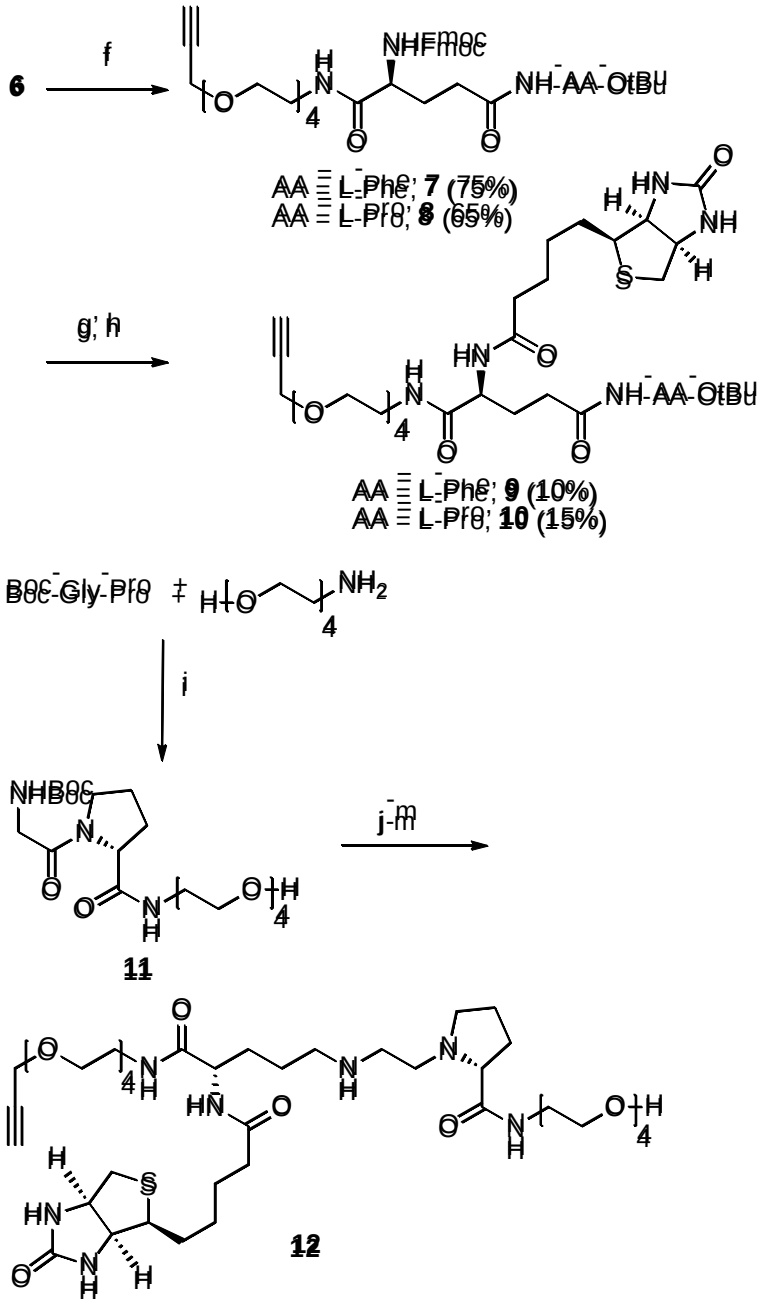
Scheme

Scheme 1: *Synthesis of the glutamic acid derivative containing three orthogonal groups.*



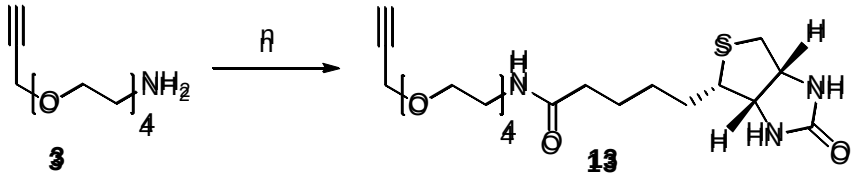
Reagents and conditions: (a) Ref. 18, DMF, 61%; (b) Propargylbromide, NaH, THF; (c) Triphenylphosphine, THF/H_2O, 62 % for 2 steps; (d) **4**, PyBOP, DIPEA, CH_2Cl_2 60 %; (e) 4 M HCl, dioxane, 86 %

Scheme 2: *Synthesis of ligands for cell functionalization*



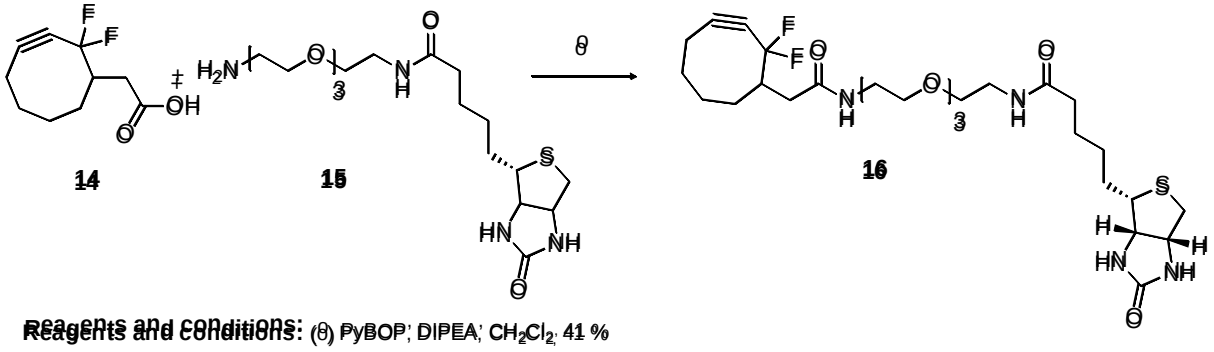
Reagents and conditions: (f) AA-OtBu, PyBOP, NEt_3, CH_2Cl_2; (g) 20 % piperidin, DMF; (h) Biotin-OSu, NEt_3, DMF; (i) PyBOP, NEt_3, CH_2Cl_2, 95 %; (j) 4 M HCl, dioxane; (k) 6, PyBOP, DIPEA, -5 °C, CH_2Cl_2, 12 %; (l) 20 % piperidine, DMF, 86 %; (m) Biotin-OSu, NEt_3, DMF, 33 %

Scheme 3: *Synthesis of the model molecule for the copper click reaction*



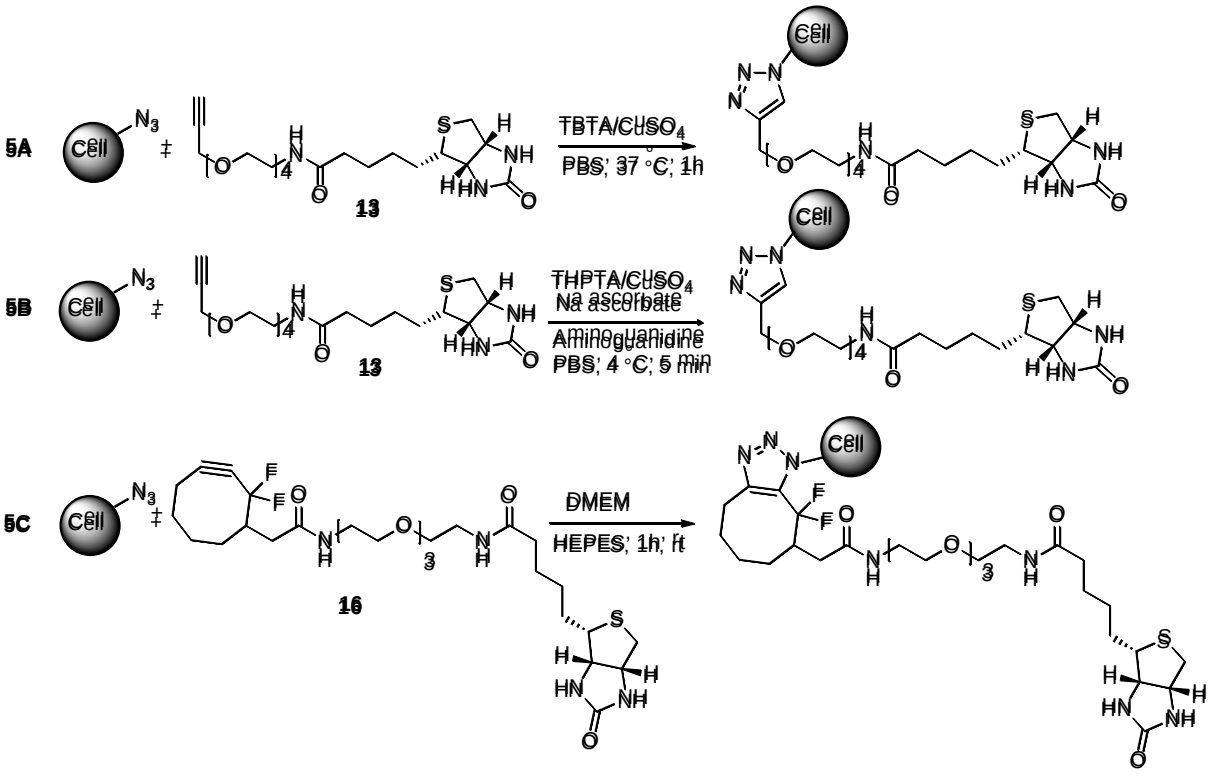
Reagents and conditions: (i) Biotin-OSu, NEt_3, DMF, 51 %

Scheme 4: *Synthesis of the model molecule for the copper-free click reaction*



Reagents and conditions: (θ) PyBOP, DIPEA, CH_2Cl_2, 41 %

Scheme 5. *Experimental conditions for the functionalization of human primary fetal osteoblasts.*



5A: copper-mediated click reaction using tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) as catalyst without copper-protective reagents

5B: copper-mediated click reaction using tris-(hydroxypropyltriazolylmethyl)amine (THPTA) as catalyst with ascorbate and aminoguanine as copper-protective reagents

5C: copper-free click reaction in cell culture medium

Figures and Figure Legends

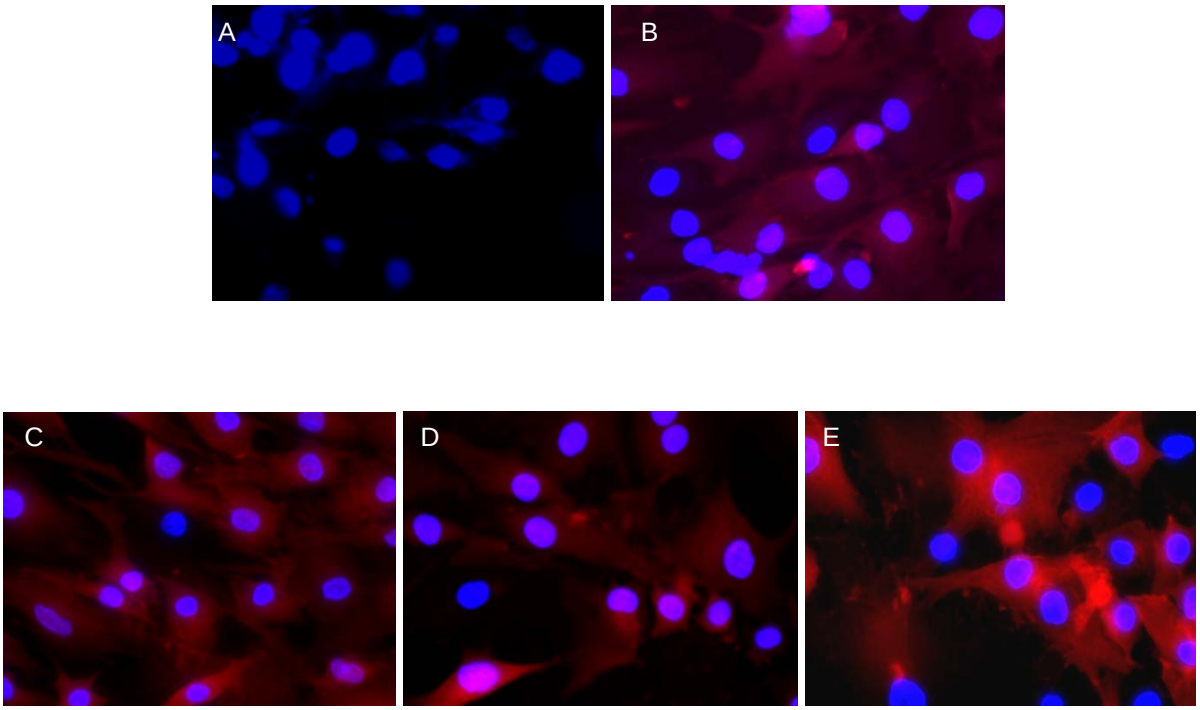


Figure 1: *TBTA/copper-catalyzed click reaction performed on human fetal osteoblasts.*

A. To evaluate for non-specific binding, human fetal osteoblasts without pre-incubation with AHA were exposed to the TBTA/copper catalyst and biotin-containing compound **13**, then to fluorescent streptavidin to label biotin and DAPI to label nuclei. **B-E.** Human fetal osteoblasts were pre-incubated with AHA and subsequently exposed for 1 h at 37 °C to compounds **13** (B), **9** (C), **10** (D) or **12** (E) using TBTA/copper-catalyzed click reaction, then to fluorescent streptavidin to label biotin and DAPI to label cell nuclei. Bound compounds fluoresce red and cell nuclei blue.

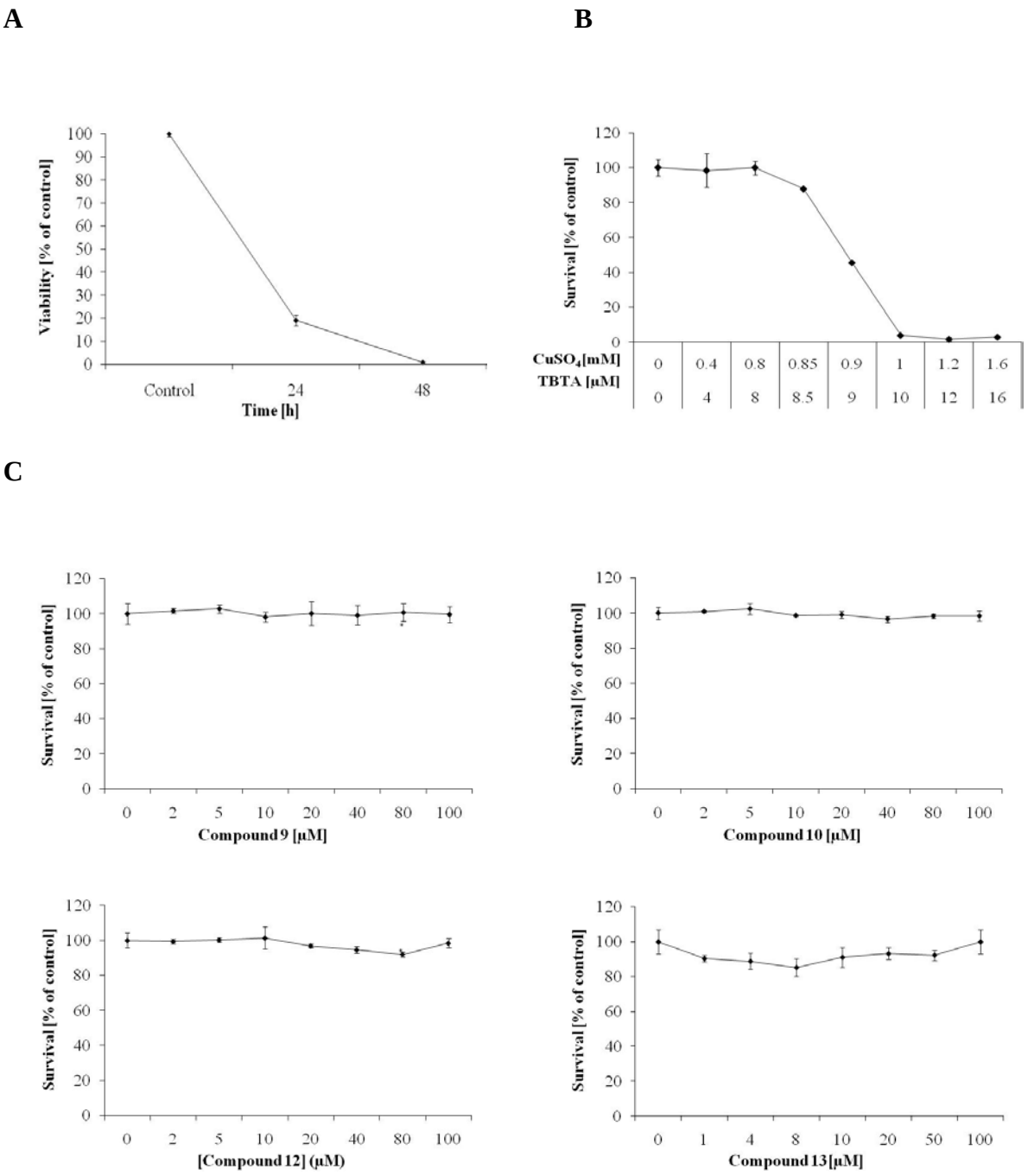


Figure 2: *Evaluation of the biocompatiblity of the TBTA/copper catalyst and of compounds* ***9***, ***10***, ***12*** *and* ***13****.*

A. TBTA/copper-catalyzed click reaction was performed for 1 h at 37 °C between compound **13** and human fetal osteoblasts pre-incubated with AHA, cell culture medium was changed and culture was continued for 24 to 48 h. Then the WST-1 assay was performed. **B.** Human

fetal osteoblasts without pre-incubation with AHA were exposed for 1 h at 37 °C to the TBTA/copper catalytic system at increasing concentrations of the catalysts, then the WST-1 assay was performed. **C.** Human fetal osteoblasts without pre-incubation with AHA were exposed for 1 h at 37 °C to compound **9**, **10**, **12** and **13** in the absence of TBTA/copper catalysts, cell culture medium was changed and culture was continued for 24 h, then the WST-1 was performed. Results are the means $\pm$ sd of triplicate wells of a representative experiment out of 3 independent experiments.

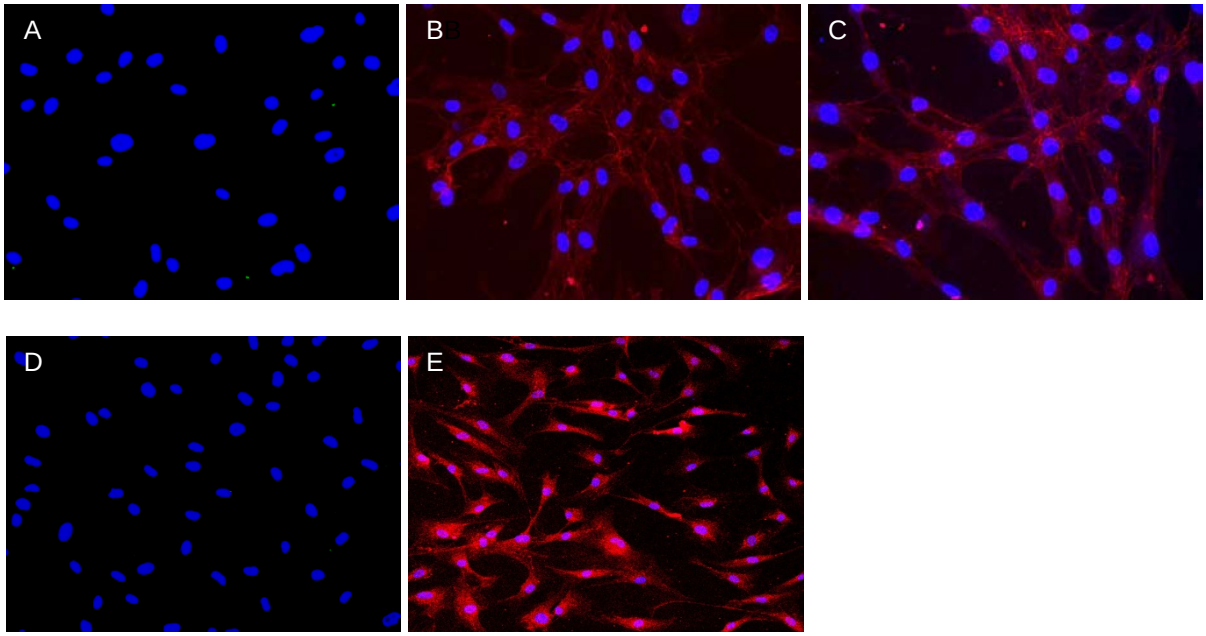


Figure 3: *Modified THPTA/copper-catalyzed and copper-free click reactions performed on human fetal osteoblasts.*

A. To evaluate for non-specific binding, human fetal osteoblasts without pre-incubation with AHA were exposed to biotin-containing compound **13** in the presence of the modified THPTA/copper catalyst, then to fluorescent streptavidin to label biotin and DAPI to label cell nuclei. **B.** and **C.** Human fetal osteoblasts were pre-incubated with AHA and subsequently exposed for 5 min at 4 °C to compounds **13** (B) or **12** (C) using the THPTA/copper-catalyzed click reaction, then exposed to fluorescent streptavidin to label biotin and to DAPI to label cell nuclei. **D.** To evaluate for non-specific binding, human fetal osteoblasts without pre-incubation with AHA were exposed to biotin-containing compound **16,** then to fluorescent streptavidin to label biotin and DAPI to label cell nuclei. **E.** Human fetal osteoblasts were pre-incubated with AHA and subsequently exposed to compound **16** for 1 h at 25 °C using copper-free click reaction, then to fluorescent streptavidin to label biotin and to DAPI to label cell nuclei. Bound compounds fluoresce red and cell nuclei blue.

A **B**

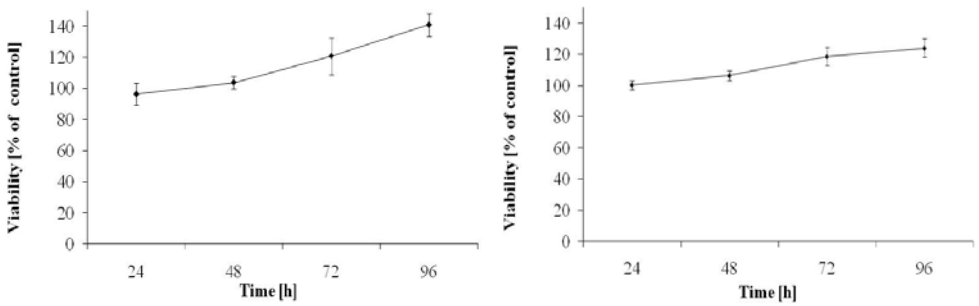
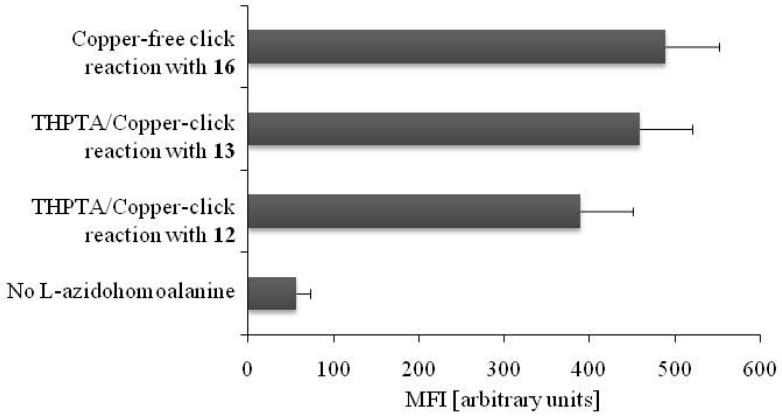


Figure 4: *Evaluation of human fetal osteoblasts viability after modified THPTA/copper-catalyzed and copper-free click reactions.*

A. Modified THPTA/copper-click reaction was performed for 5 min at 4 °C between compound **13** and human fetal osteoblasts pre-incubated with AHA, cell culture medium was changed and culture was continued for 24 to 96 h, then the WST-1 assay was performed. **B.** Copper-free click reaction was performed for 1 h at 25 °C between compound **16** and human fetal osteoblasts pre-incubated with AHA, cell culture medium was changed and culture was continued for 24 to 96 h, then the WST-1 assay was performed. Results are the means ± sd of triplicate wells of a representative experiment out of 3 independent experiments.

A



B

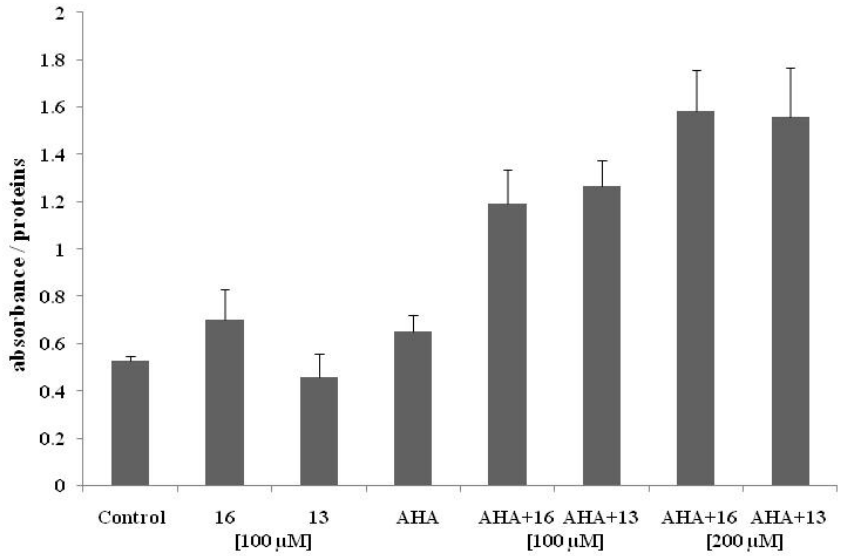


Figure 5: *Evaluation of compounds conjugation to the cells*

Following click reactions performed on human fetal osteoblasts under non-cytotoxic conditions with compound **12 or 13, or 16** or in the absence of AHA or the compounds (for non-specific binding) (**A**): the cell-associated fluorescence was quantified using 100 µM of either compounds **12**, **13** or **16**. Results are expressed as the mean fluorescence intensity (MFI) per cell ± sd; or (**B**) the enzymatic activity of cell-associated HRP-strptavidin was related to the protein content of the cells. Control: nocompound or AHA added to the cells, **16** or **13**: cells exposed to the compounds in the absence of AHA; AHA: cells exposed to AHA in the absence of the compound; AHA+**16**/+**13**: cells exposed to AHA and the compounds. Results are expressed as the ratio of the cell-bound HRP activity to the protein cotent per well.

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