Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Reversible cross-linking of hyperbranched polymers: A strategy for the combinatorial decoration of multivalent scaffolds

Michael Barth, Rainer Fischer, Roland Brock, and Jörg Rademann[*]

[*] Prof. Dr. J. Rademann, Medicinal Chemistry, FMP Research Institute for Molecular Pharmacology, Robert-Rössle-Str. 10, 13125 Berlin, Germany. Fax: (int.) 49-30-94793280. E-mail: rademann@fmp-berlin.de. and: Free University Berlin, Institute for Chemistry, Takustr. 3, 14195 Berlin, Germany.

Dipl.-Chem. M. Barth, Eberhard-Karls-University Tübingen, Institute for Organic Chemistry, Auf der Morgenstelle 18, 72076 Tübingen, Germany.

Dipl.-Biochem R. Fischer, Dr. R. Brock,
Eberhard-Karls-University Tübingen, Institute for Cell Biology, Auf der Morgenstelle 15, 72076 Tübingen, Germany

General procedures

Polyethylene imine was obtained from Aldrich. Solvents were purchased in HPLC grade. All the peptide syntheses were carried out in plastic syringes equipped with Teflon® filters. The NMR measurements were made on a Bruker Avance 400 MHz spectrometer. High resolution MAS-NMR was recorded on Bruker ARX 400 MHz. The FT-ATR-IR measurements were performed on a Bruker Vector 22 containing a Harrick split-pea ATR unit. HPLC was conducted on a Waters 600 S.

Synthetic procedures

Synthesis of 4-Hydroxymethylbenzaldehyde **1**

Terephthalic dialdehyde (10.0 g, 0.075 mol) was dissolved in 66 ml of dry tetrahydrofuran (THF). After adding sodium borohydride (0.8 g, 0.021 mol) the suspension was stirred for one hour at RT. The solvent was removed and the residue was taken up in ethyl acetate (EtOAc) (130 ml). The solution was washed with water (2 times 65 ml) and brine and dried over Na_2SO_4. Solvent was removed and the residue was flash-chromatographed with SiO_2 (n-hexane/ethyl acetate 2:1) to get **1** as a white solid (1.9 g, 19 %): ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.79 (s, 2 H; CH_2OH, 7.53 (d, ^2J = 7.9 Hz, 2 H; ArH), 7.87 (d, ^2J = 8.1 Hz, 2 H; ArH), 9.98 (s, 1 H, CHO); ^{13}C NMR (400 MHz, $CDCl_3$) δ = 64.6, 127.0, 130.0, 135.7, 148.0, 192.2.

See literature C. Mak, N. Bampos, S. Darling, M. Montalti, L. Prodi, J. Sanders, *J. Org. Chem.* 2001, *66*, 4476-4486.

Synthesis of di-*iso*propyl-bis[(4-formylbenzyl)oxy]silane **2**

To a solution of **1** (1.124 g, 8.26 mmol) in pyridine (11 ml) di-*iso*propyl-dichlorosilane (0.746 ml, 4.13 mmol) was added and heated for 1 h at 60 °C. The solution was washed with aqueous $KHSO_4$ (22 ml, pH 3) and dichloromethane (44 ml). The organic layer was washed again with water (2 times 22 ml) and dried over $NaSO_4$. After removal of the solvent the residue was flash-chromatographed with SiO_2 (n-hexane/EtOAc 4.25:1) to yield **2** as a white solid (1.03 g, 65 %): ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.14 (m, 14 H; *iso*propyl-H), 4.92 (s, 4 H; Ar-CH_2-O), 7.48 (d, ^2J(H,H) = 8.1 Hz, 4 H, ArH), 7.85 (d, ^2J(H,H) = 8.3 Hz, 4 H, ArH), 9.99 (s, 2 H, CHO); ^{13}C NMR (400 MHz, $CDCl_3$) δ = 12.3, 17.5, 64.3, 126.3, 130.0, 135.6, 148.0, 192.1.

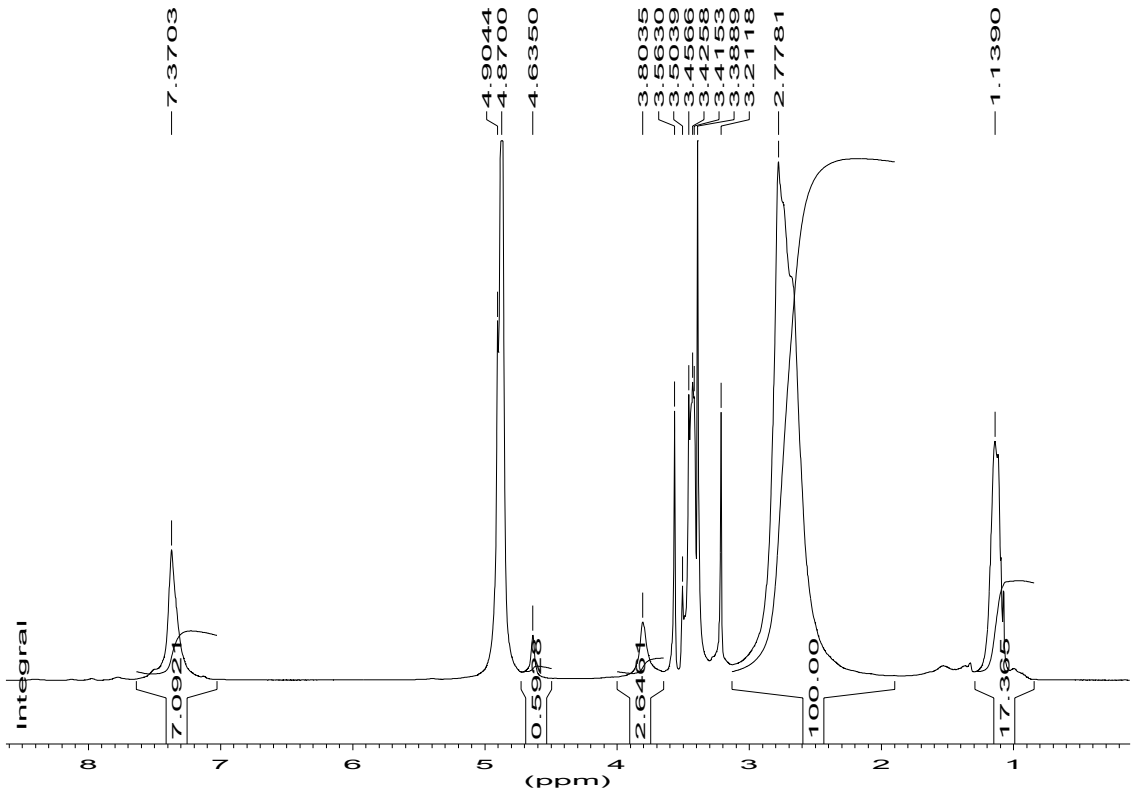

Figure 1: ^{1}H-MAS-suspension NMR of resin **4** (MeOD, rotation frequency 4500 Hz). δ = 0.8-1.3 ppm (m, Si-*iso*propyl, rel. integration 17.3) 2.0-3.1 ppm (m, PEI-CH_2, 100), 3.6-3.9 (bs, *sec*-N-CH_2-aryl, 2.65), 4.5-4.7 (bs, *tert*-N-CH_2-aryl, 0.89), 4.9 ppm (s, Si-O-CH_2-Aryl), 7.0-7.6 (bs, aryl-H, 7.09).

Solvent	Water	Methanol	DMF	THF	DCM
Swelling factor [ml/g]	7.7	8.3	4.6	4.9	6.7

Tab. 1: Swelling factors of resin **4** in ml/g resin.

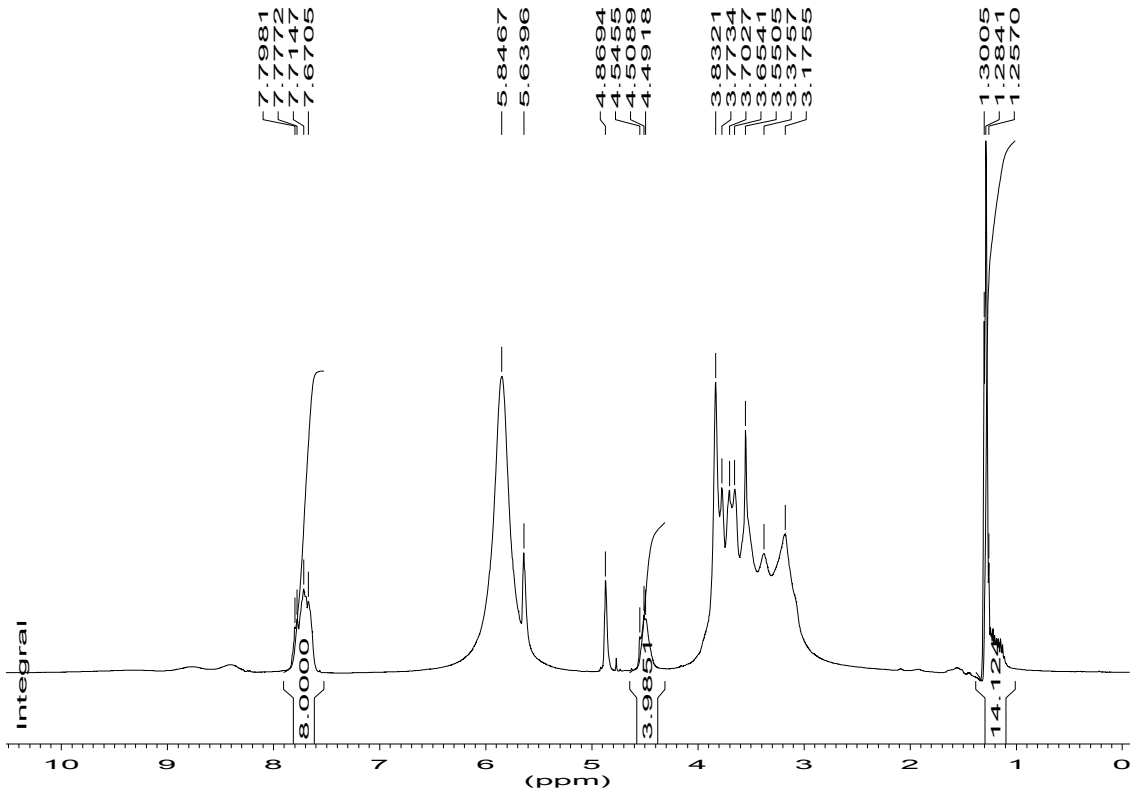

Figure 2: ^{1}H-NMR of decomposed resin **4** after treatment with trifluoroacetic acid (MeOD). δ = 1.0-1.35 ppm (m, Si-*iso*propyl, rel. integration 14.1) 2.6-4.1 ppm (m, PEI-CH_2), 3.6-3.9, 4.3-4.6 ppm (bs, HO-CH_2-aryl-CH_2-N, 4.0), 7.5-7.9 ppm (bs, aryl-H, 8.0). The completeness of the decomposition of resin **4** can be seen from the good resolution of the peak for the *iso*propylgroups of the crosslinker compared to the broadened peaks belonging to the PEI-polymer.

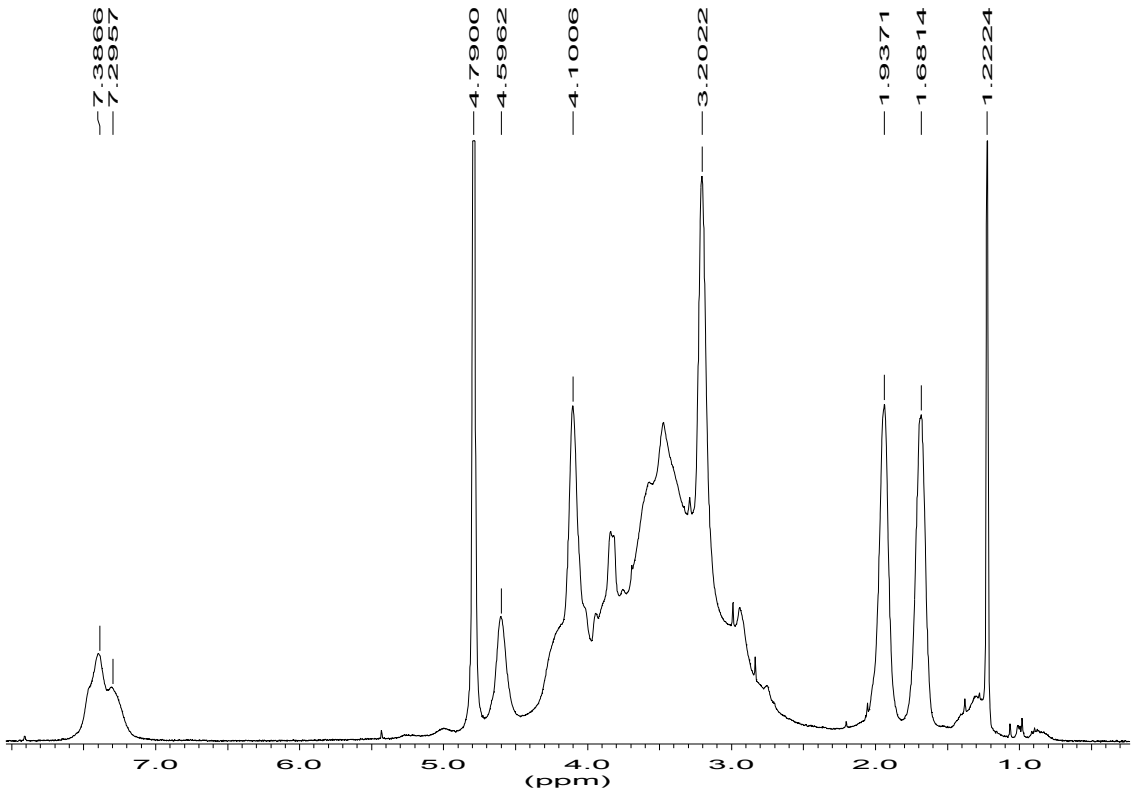

Figure 3: ^{1}H-NMR from decomposed resin **5** with the sequence Arg-Gly after precipitation with cold diethyl ether and lyophilization (*tert*-BuOH/H_2O : 4:1)(D_2O). No signals from the Pbf-protecting group of Arg or the Boc group could be detected. . δ = 1.22 ppm (s, *tert*-BuOH), 1.68 and 1.93 ppm (m, sidechain of Arg), 3.2 (s, Gly), 2.5-4.4 ppm (m, PEI-CH_2), 4.6 ppm (bs, HO-CH_2-aryl-CH_2-N), 7.1-7.7 ppm (m, aryl-H).

Synthesis of resins **6**

4-[(acetyloxy)methyl]benzoic acid (291 mg, 1.5 mmol), tetramethyl-*O*-(benzotriazol-1-yl)-uronium tetrafluoroborate (TBTU) (482 mg, 1.5 mmol) *N*-hydroxybenzotriazole (HOBt) (230 mg, 1.5 mmol) and *N*-di*iso*propylethyl amine (DIPEA) (257 µl, 1.5 mmol) in *N,N*-dimethylformamide (DMF) (6 ml) was coupled to resin **4** (100 mg). After 4 h the resin was washed with DMF and DCM and dried in vacuo. The remaining secondary amines were capped using di-*tert.*-butyl-dicarbonate (655 mg, 3 mmol) and DiPEA (1.03 ml, 6 mmol) in DMF (5 ml) two times for 2 h. The resin was washed as described above. The completeness of the acylation was tested using the Kaiser-test and the Chloranil-test, respectively. After cleaving the acetyl-protecting group with sodium methoxide (0.1 M in methanole) for 30 min, Fmoc-glycine (446 mg, 1.5 mmol) was coupled using 1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT,

444 mg, 1.5 mmol) and *N*-methylimidazole (119 µl, 1.5 mmol) in DCM (6 ml) two times for 3 h. The resin was washed with DCM, DMF, DCM and dried in vacuo. Loading of the resin was determined photospectrometrically by cleaving the Fmoc-group from the resin using 20 % piperidine in DMF.

Synthesis of resin **7**

4-[(acetyloxy)methyl]benzoic acid (16 mg, 0.08 mmol), TBTU (26 mg, 0.08 mmol), HOBt (12 mg, 0.08 mmol) and DiPEA (14 µl, 0.08 mmol) in DMF (4 ml) was coupled to resin **4** (200 mg). After 4 h the resin was washed with DMF and DCM and dried in vacuo. The resin was swollen in methanol and treated with sodium methoxide (0.1 M in methanole) for 30 min. Fmoc-glycine (238 mg, 0.8 mmol) was coupled using first MSNT, (237 mg, 0.8 mmol) and *N*-methylimidazole (63.5 µl, 0.8 mmol) in DCM (4 ml) for 3 h and then TBTU (257 mg, 0.8 mmol), HOBt (123 mg, 0.08 mmol), DiPEA (137 µl, 0.8 mmol) in DMF (4 ml) for 3 h. The resin was washed with DMF and DCM and dried in vacuo. The resin was capped with di-*tert.*-butyl-dicarbonate (1310 mg, 6 mmol) and DiPEA (2.06 ml, 12 mmol) in DMF (5 ml) two times for 2 h. Loading of the resin was determined as described before.

Product cleavage from the HMBA-linker

Peptide-decorated reversibly cross-linked resins **6** and **7** were treated with 10% triethylamine in methanol (1 ml) for 16 h at RT. After filtration the resin was washed with 1 ml DMF. The collected filtrate was evaporated. Protecting groups were removed by treating with 95 % trifluoroacetic acid, 2.5 % tri*iso*propylsilane and 2.5 % water for 3 h. After precipitation with cold diethyl ether (4 times) the peptides were lyophilized (*tert*-BuOH : H_2O 4:1). The peptides were analyzed with HPLC and ESI-MS.

Synthesis of fluorescein-labeled peptide-decorated scaffolds **8**

The synthesis of the peptides on resin **5**, respectively, was carried out as described in the experimental section. After removal the final Fmoc-group 5(6)-carboxy-fluorescein (75 mg, 0.2 mmol) was coupled to the peptide-decorated reversibly cross-linked resin, respectively (20 mg) using *N,N'*-di*iso*propylcarbodiimide (31 µl, 0.2 mmol) and HOBt (31 mg, 0.2 mmol) in DMF (1 ml) for 16 h. The resin was washed with DMF and 20 % piperidine in DMF until the solution was colorless in order to

remove ester-bound carboxyfluorescein (see R. Fischer, O. Mader, G. Jung, R. Brock, *Bioconjugate Chem.* **2003**, *14*, 653-660.) After washing again with DMF and DCM, the resin was dried in vacuo. The decomposition of the fluorescein-labeled resins was conducted as described in the experimental section.

Synthesis of the side-chain-labeled peptide sequence AVPIAQK (decorated scaffold **15**)

Conjugation of 5(6)-carboxyfluorescein to the ε-amino group of the carboxyterminal lysine side chains of the AVPIAQK or KQAIPVA peptides was carried out via incorporation of Fmoc-Lys(Dde)-OH (Calbiochem-Novabiochem, Bad Soden, Germany) as carboxyterminal building block in peptide synthesis. The last aminoterminal amino acid was incorporated as Boc-protected building block, in order to avoid acylation of the aminoterminus with 5(6)-carboxyfluorescein. Deprotection of the Dde-protecting group was then performed by treatment with 2% hydrazine monohydrate in DMF (v/v) twice for 3 min. The coupling of the fluorophore to the deprotected ε-amino group followed the same protocol as the one for amino-terminal labeling.

Cellular uptake of peptide-decorated multivalent scaffold **8**

To further characterize the biological applicability of the decorated scaffolds, we also investigated cellular uptake thereof by flow cytometry. Uptake into HeLa cells at different concentrations of the decorated scaffold **8** was determined. As shown in Figure 4 the fluorescein-labeled scaffold is incorporated into each individual cell of the HeLa cell population. Even at low concentrations such as 50 nM, all HeLa cells efficiently internalize the peptide-decorated scaffold **8**.

We also investigated cellular toxicity of the decorated scaffold **8**. Based on morphological parameters in the flow cytometric experiment no apparent toxicity was observed at concentrations of 1 μM or below.

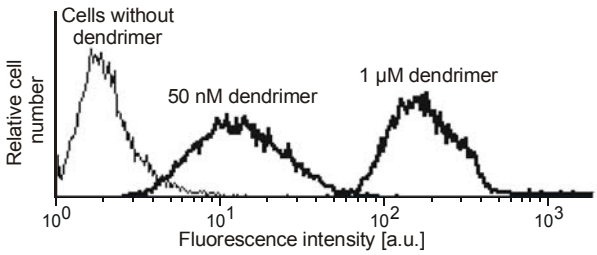

Figure 4: Cellular uptake of the fluorescein-labelled peptide-decorated multivalent scaffold **8**. HeLa cells were incubated for 2 h with serum-free medium containing the indicated amount of fluorescein-labelled scaffold. Then the cells were washed, trypsinized and analyzed by flow cytometry.

Flow cytometry. HeLa cells were seeded at a density of 50,000 per well in 24 well plates (Sarstedt, Nümbrecht, Germany) in serum-containing RPMI 1640. One day later, the cells were washed with serum-free RPMI 1640 and incubated in 200 µL serum-free RPMI 1640 containing the indicated amount of fluorescein-labeled peptide-decorated scaffold 8. Each condition was tested in duplicate. After a 2 h incubation, cells were washed with PBS, detached by trypsinization for 10 min, suspended in ice cold PBS containing 0.1 % (w/v) BSA, and measured immediately by flow cytometry. The fluorescence of 10,000 vital cells was acquired. Vital cells were gated based on sideward scatter and forward scatter.

Cell culture. HeLa cells were grown in a 5 % CO_2 humidified atmosphere at 37 °C in RPMI 1640 medium with stabilized glutamine and 2.0 g/L $NaHCO_3$ (PAN Biotech, Aidenbach, Germany) supplemented with 10 % fetal calf serum (PAN Biotech), 100 U/mL penicillin, and 100 µg/mL streptomycin (Biochrom, Berlin, Germany). HeLa cells were passaged by trypsinization with trypsin/EDTA (0.05/0.02 % (w/v)) (Biochrom) in PBS every third to fourth day.

Confocal laser scanning microscopy. Confocal laser scanning microscopy was performed on an inverted LSM510 laser scanning microscope (Carl Zeiss, Göttingen, Germany) fitted with a Plan-Apochromat 63x 1.4 N.A. lens. Measurements were performed with living, non-fixed cells.
HeLa cells were seeded at a density of 10,000/well in eight-well chambered cover glasses (Nunc, Wiesbaden, Germany). Two days later cells were washed once with

serum-free RPMI 1640. The fluorescein-labelled peptide-decorated scaffold 8 and the AlexaFluor 647-dextran (anionic; MW 10,000 Da, Mobitech, Göttingen) were then added to the culture medium. After 2 h the cells were washed three times with serum-free RPMI 1640 followed immediately by confocal microscopy at RT.

For double detection of the compound **8** and AlexaFluor 647-dextran the 488 nm line of an Argon Ion laser and the light of a 633 nm Helium/Neon laser were directed over an HFT UV/488/633 beam splitter and fluorescence was detected using an NFT 545 beam splitter in combination with a BP 505-530 band pass filter for fluorescein detection and an LP 650 long pass filter for AlexaFluor 647-detection.

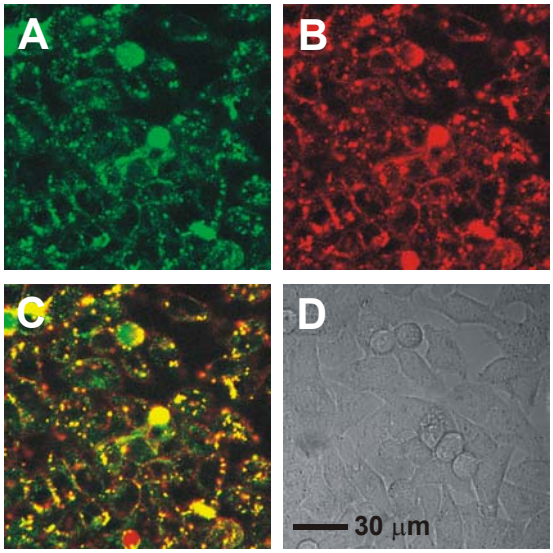

Figure 5. HeLa cells were incubated with serum-free medium containing 300 nM bafilomycin A1, 10 µM of AlexaFluor 647-dextran and 400 nM fluorescein-labelled peptide-decorated scaffold **8** for 2 h, washed and analyzed by multi-channel confocal laser scanning microscopy. Bafilomaycin A1 was included in the medium 15 min prior to the addition of the peptide-decorated scaffold and the dextran. Panel A shows the fluorescein fluorescence, panel B the AlexaFluor 647-dextran fluorescence, panel C the superposition of both fluorescence channels and panel D shows the transmission picture.