Multivalent targeting of AT1 receptors with angiotensin II-functionalized nanoparticles

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Abstract

The angiotensin II receptor type 1 (AT1R) is a G protein-coupled receptor of paramount significance since it is overexpressed in a number of diseased tissues that are highly attractive for nanoparticle targeting. However, it is also expressed at physiological levels in healthy tissue. Multivalent interactions mediated by multiple AT1R-binding moieties per nanoparticle could promote a high binding avidity to AT1R overexpressing cells and concomitantly spare off-target tissue. To investigate the feasibility of this approach, angiotensin II was thiolated and conjugated to PEGylated quantum dots. Nanoparticle binding, uptake and affinity to several cell lines was investigated in detail. The colloids were rapidly taken up by clathrin-mediated endocytosis into AT1R-expressing cells and showed no interaction with receptor negative cells. The EC50 of the thiolated angiotensin II was determined to be 261 nM, whereas the ligand-conjugated Qdots activated the receptor with an EC50 of 8.9 nM. This 30-fold higher affinity of the nanoparticles compared to the unconjugated peptide clearly demonstrated the presence of multivalent effects when using agonist-targeted nanoparticles. Our study provides compelling evidence that, despite being immediately endocytosed, Ang II-coupled nanoparticles exert potent multivalent ligand–receptor interactions that can be used to establish high affinities to an AT1R overexpressing cell and tissue.

Introduction

The angiotensin II receptor type 1 (AT1R) is an attractive target for nanoparticle-based drug delivery due to its marked overexpression in severe pathologies such as myocardial infarctions [1,2], cancer [3,4] or choroidal neovascularization [5]. With the present work, we demonstrate that nanoparticles to which the physiological ligand angiotensin II (Ang II) is conjugated are able to bind cells expressing the AT1 receptor in a multivalent nature. Although particles with similar targeting moieties have already been prepared for targeting the infarcted heart [2] and for studying receptor trafficking routes [6,7], the ability of AT1R-targeted nanoparticles to bind several receptors simultaneously has never been investigated. In situations such as this, where targeting of a receptor present in both healthy and diseased tissue is desirable, multivalency allows for preferential binding of ligand-targeted nanoparticles to cells that overexpress the targeted receptor [8]. Concomitantly, interactions with off-target cells that express the receptor at a lower level can be minimized. In the case of the AT1R this is of fundamental importance since AT1 receptors, which are usually known for their role in blood pressure regulation and fluid retention, are not only overexpressed in diseased tissue but also expressed at physiological levels within off-target tissues like the endothelium [9] or the kidney glomerulus [10,11]. Both are exposed to intravenously administered nanoparticles during their circulation in the blood [12] and are prone to off-target nanoparticle binding [13,14].

We recently demonstrated that angiotensin receptor blockers (ARBs), when immobilized on a nanoparticle surface, bind AT1 receptors by a multi-ligand binding mechanism [15]. However, ARBs do not provoke receptor internalization after binding [16]. This gives the ARB-conjugated particles sufficient time to reach out to further receptors on the cell surface and consequently build up a strong multivalent binding. In contrast to the antagonists, the agonist Ang II immediately induces receptor internalization after binding [17], which we hypothesized could impair the formation of stable multivalent interactions. Furthermore, we were interested if nanoparticles would still be taken up by cells, despite crosslinking several receptors, and thus, exhibiting multivalent attachment to the cell surface. Multivalent interactions between cells and nanoparticles have already been reported for other important receptors such as transferrin receptor [18,19], folate receptor [20,21] or the human epidermal growth factor receptor-2 (HER2) [22,23].
To gain insight into the multivalent nature of Ang II-coupled nanoparticles, the ligand was coupled to highly fluorescent core-shell quantum dots (Qdots). Flow cytometry and confocal microscopy were utilized to investigate nanoparticle endocytosis, subcellular localization and mechanism of uptake. Changes in intracellular calcium concentrations induced by both the soluble ligands and the ligands conjugated to the nanoparticle surface were measured to quantify the degree of nanoparticle multivalency towards AT1 receptors.

**Material and methods**

**Materials**

Qdots® 655 ITK™ amino PEG (#Q21521MP; Life Technologies, Carlsbad, CA) were used as nanoparticle starting material. Losartan carboxylic acid, also known as EXP3174, was purchased from Santa Cruz (Heidelberg, Germany). All chemicals were obtained from Sigma Aldrich (Taufkirchen, Germany) in analytical grade unless stated otherwise and used without further purification. Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA). Dulbecco’s phosphate-buffered saline (DPBS) pH 7.40, which was used for nanoparticle purification and cell experiments, consisting of 1.5 mM KH2PO4, 8 mM Na2HPO4, 2.7 mM KCl and 138 mM NaCl, was purchased from Life Technologies (Carlsbad, CA).

**Cell culture**

Rat mesangial cells were a kind gift from Dr. Armin Kurtz (Department of Physiology, University of Regensburg, Germany) and were cultured in RPMI1640 medium containing 10% fetal bovine serum (Sigma Aldrich, Taufkirchen, Germany) supplemented with insulin–transferrin–selenium (Life Technologies, Carlsbad, CA) and 100 nM hydrocortisone. Human adrenal gland carcinoma cells NCI-H295R (ATCC No. CRL-2128) were maintained in the same medium. HeLa cells (ATCC No. CCL-2) were cultured in Eagle’s Minimum Essential Medium (EMEM) containing 10% fetal calf serum (FCS) and 1 mM sodium pyruvate. All cells were cultured in T-75 cell culture flasks (Corning, Corning, NY).

**Thiolation of angiotensin II**

1.5 µmol angiotensin II (Bachem, Bubendorf, Switzerland) was reacted with 1.875 µmol 2-iminothiolane HCl in borate buffer pH 8.00 (50 mM) for 24 h at room temperature. The resulting N-substituted 2-iminothiolate intermediate was hydrolyzed for 48 h in acetate buffer pH 5.00 (100 mM), to which ethylenedinitrilotetraacetic acid disodium salt (Merck, Darmstadt, Germany) in a concentration of 1 mM was added.

To quantify the amount of reactive thiol groups, an Ellman’s assay was performed. Briefly, a sample of thiolated peptide was incubated with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in phosphate buffer pH 8.00 (100 mM) for 15 min and analyzed on a UVIKON 900 double-beam spectrophotometer (Kontron instruments, Milan, Italy) at a wavelength of 412 nm. Using a molar extinction coefficient of 14 150 M⁻¹cm⁻¹ the concentration of the thiolated peptide was quantified. Purity and conversion were further analyzed by high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). The HPLC system consisted of a Shimadzu LC-10ATVP pump (Shimadzu, Duisburg, Germany), a Shimadzu SIL-10A VP autosampler connected to a Shimadzu SPD-10A UV-Vis detector. A Luna® 5 µm C18(2) 100 Å 250 × 4.60 mm LC column (Phenomenex, Aschaffenburg, Germany) was used as stationary phase. The peptides were analyzed based on a linear acetonitrile–trifluoroacetic acid (TFA)–water gradient with a flow rate of 1 mL/min. Elution was obtained by using the following gradient of solvents A [0.1 % (v/v) TFA in water] and B [0.1 % (v/v) TFA in acetonitrile]: 90:10 (A:B) to 30:70 in 15 min and re-equilibration to 90:10 in the following 7 min. The column was operated at 35°C and the peptides were detected at 275 nm, at which angiotensin II has an absorbance maximum. ESI-MS spectra were recorded on a Q-TOF 6540 UHD system (Agilent Technologies, Böblingen, Germany).

**Conjugation of thiolated Ang II to PEGylated Qdots**

160 pmol Qdots® 655 ITK™ amino PEG were activated in borate buffer pH 8.50 (50 mM) with 160 nmol sulfo-succinimidyl-14-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (Thermo Fisher, Waltham, MA) at room temperature. After 1 h of gentle shaking, the activated Qdots were purified from unreacted sulfo-SMCC by size exclusion chromatography using a Sephadex G-25 resin in a PD-10 column (GE Healthcare, Munich, Germany) with DPBS as eluent. Fractions containing activated Qdots were collected and pooled. Subsequently, 16 nmol of thiolated angiotensin II (100-fold excess) were added to the pooled, purified and maleimide-activated Qdots and reacted for 1 h at room temperature to yield a stable thioether bond. Unreacted maleimide groups on the nanoparticle surface were inactivated with a 100-fold excess of 2-mercaptoethanol for 30 min. The reaction mixture was purified from excess peptide and 2-mercaptoethanol by ultrafiltration using a 100 kDa molecular weight cut-off Amicon Ultra-4 filter unit (Millipore, Billerica, MA) for 10 min at 1500g and size exclusion chromatography as described above. Finally the Qdot solution was up-concentrated by ultrafiltration. Qdot concentration was determined by fluorescence measurements using a FluosStar Omega fluorescence microplate reader (BMG Labtech, Ortenberg, Germany) with an excitation and emission wavelength of 450 and 650 nm, respectively.

**Flow cytometry**

The binding of nanoparticles to receptor positive and receptor negative cell lines was quantified by flow cytometry measurements. NCI-H295R cells, rat mesangial cells and HeLa cells were seeded into 24-well plates (Corning, Corning, NY) at a density of 150 000 cells/well (NCI-H295R) or 100 000 cells/well (HeLa cells, rat mesangial cells). After the cells had been seeded and allowed to grow inside the 24 well-plates for 48 h, they were washed with DPBS. Pre-warmed nanoparticle solutions were then added to the cell monolayer. Qdot solutions of non-targeted and Ang II-targeted Qdots were prepared in Leibovitz’s medium (Life Technologies, Carlsbad,
CA) at a concentration of 10 nM and supplemented with 0.1% BSA. After 1 h nanoparticle incubation at 37°C, the cells were vigorously washed with DPBS and trypsinized. Leibovitz’s medium containing 10% FCS was added to the cells to quench the trypsin activity. After resuspension of the cells, they were centrifuged (5 min, 200g), resuspended in DPBS, centrifuged again (5 min, 200g) and finally resuspended in 200 µL DPBS. The cells were analyzed for fluorescence with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Qdot fluorescence was elicited by excitation at 488 nm and the fluorescence was analyzed using a 661/16 nm FL4 bandpass filter. The resulting data was analyzed using WinMDI 2.9 (The Scripps Research Institute, San Diego, CA). Only the population of intact cells was gated and used for the analysis of cellular fluorescence. The geometrical mean, which was used for further analysis and statistics, was determined from the fluorescence histogram.

Confocal laser scanning microscopy

NCI-H295R cells and rat mesangial cells were seeded into 8-well µ-slides (Ibidi, Martinsried, Germany) at a density of 15 000 cells/well (NCI-H295R) and 10 000 cells/well (rat mesangial cells). After the cells had adhered to the cell culture plastic, nanoparticle binding was assessed. First, cells were washed with warm DPBS. Then, pre-warmed nanoparticle solutions in Leibovitz’s medium supplemented with 0.1% bovine serum albumin (BSA) were pipetted onto the cells and incubated for 1 h at 37°C. After the incubation period, cells were vigorously washed with DPBS. Live cells were analyzed in Leibovitz’s medium. Confocal analysis of nanoparticle binding to the cells was performed with a Zeiss Axiovert 200 microscope combined with a LSM 510 laser-scanning device using a 63 × Plan-Apochromat (NA 1.4) oil immersion objective (Zeiss, Jena, Germany). Qdot fluorescence was elicited by excitation at 488 nm with an argon laser and recorded after filtering with a 650 nm long pass filter. The confocal pinhole was set to 150 µm, which corresponds to a focal plane of 1.1 µm. For image acquisition AIM 4.2 software (Zeiss, Jena, Germany) was used. Images were processed using ImageJ (NIH, Bethesda, MD; http://imagej.nih.gov/ij/).

For colocalization studies, NCI-H295R cells and rat mesangial cells were simultaneously incubated with 10 nM Ang II-Qdots and 5 µg/mL Alexa Fluor® 488-labeled transferrin (Life Technologies, Carlsbad, CA) in Leibovitz’s medium supplemented with 0.1% BSA for 30 min. After washing with DPBS the adhering cells were analyzed. The green Alexa Fluor® 488 dye was excited with a 488 nm argon laser and its fluorescence recorded using a 505–530 nm bandpass filter.

Intracellular calcium measurements

The intracellular calcium concentration was measured via a microscope and a plate reader-based system using the ratiometric fluorescent calcium chelator Fura-2. For microscopic analysis, NCI-H295R cells were grown on cell culture dishes (Corning, Corning, NY) and loaded with 5 µM Fura-2AM (Life Technologies, Carlsbad, CA) in a Ringer type buffer (5 mM HEPES, 138.9 mM NaCl, 3.6 mM KCl, 1 mM MgCl₂, 1.3 mM CaCl₂, 1.6 mM Na₂HPO₄ and 5.4 mM NaH₂PO₄ at pH 7.40) for 1 h at room temperature. After extensive washing, the cellular fluorescence was measured at 510 nm following excitation at 340 and 380 nm using a Zeiss examiner A1 microscope (Zeiss, Jena, Germany), equipped with a WN-Achromat 40 × objective (Zeiss, Jena, Germany). Images were continuously acquired at 1 image/s using Axiovision 4.8.2.

Results

Angiotensin II modification and nanoparticle labeling

To enable conjugation to amine-decorated PEGylated nanoparticles, angiotensin II was covalently modified at the primary amine group of its N-terminal aspartic acid by reaction with 2-iminothiolane (also known as Traut’s reagent) in a 2-step reaction (Figure 1A). Reversed-phase high-performance liquid chromatography (RP-HPLC) and Ellman’s assay with DTNB confirmed the complete conversion of the native peptide into the thiolated form (Figure 1B). Electrospray ionization mass spectrometry (ESI-MS) further verified the successful reaction (Figure 1C). Absence of the (M+3H)³⁺ peak in the mass spectrograph indicated that the conjugation chemically altered a basic group, which clearly suggests reaction at the N-terminal aspartic acid.

The modified Ang II peptide was then coupled to the amine groups of the PEGylated Qdots using the heterobifunctional linker sulfo-SMCC (Figure 1D). Qdots were activated by reacting their peripheral amine groups with the sulfo-NHS ester of sulfo-SMCC. After purification and removal of unreacted sulfo-SMCC, the maleimide-activated nanoparticles were covalently conjugated with the thiolated Ang II in a thiol-ene Michael addition click reaction to form a stable thioether bond. Transmission electron microscopy revealed that the conjugation of the ligand to the Qdot
PEG corona did not lead to aggregation. After coupling the Qdots remained monodisperse and evenly distributed (Supplementary Figure S1).

Nanoparticle binding and uptake

Nanoparticle binding to selected cell lines was investigated by flow cytometry and confocal laser scanning microscopy. AT1R-expressing NCI-H295R cells and rat mesangial cells as well as AT1R-negative HeLa cells were incubated with non-targeted and Ang II-targeted Qdots at a concentration of 10 nM. Although non-targeted Qdots showed some association to the AT1-expressing cell lines, binding of Ang II-targeted Qdots to NCI-H295R and rat mesangial cells was significantly higher (Figure 2). Conversely, ligand-coupled Qdots showed no association nor binding to receptor negative HeLa cells, indicating that ligand immobilization on the nanoparticle surface was indeed successful.

By confocal laser scanning microscopy, subcellular localization of the nanoparticles was analyzed in detail. From these data, it is evident that not only did the ligand-targeted Qdots bind to the cell surface, but they were also rapidly taken up into the cells. After 30-min incubation, the cells began exhibiting a massive amount of Qdot fluorescence. Nanoparticles were visible in vesicular regions throughout the cytoplasm and seemed to accumulate preferentially in the perinuclear region (Figure 3), whereas non-targeted Qdots appeared to adhere in a non-specific manner to the cell surface. When the cells were co-incubated with Ang II-targeted nanoparticles and losartan carboxylic acid, a

Figure 1. Strategy for angiotensin II conjugation to nanoparticles. (A) First, a thiol group was introduced at the N-terminal aspartic acid of angiotensin II using 2-iminothiolane. (B) Reversed-phase HPLC analysis verified the successful two-step reaction. (C) ESI-MS of the native and the modified peptide. (D) To conjugate the thiolated angiotensin II to nanoparticles, PEGylated amine-terminated Qdots were activated with sulfo-SMCC and subsequently coupled to the ligand. A thorough purification was performed after each step.
specific and potent AT1R antagonist, most of the intracellular fluorescence was eliminated, verifying that the nanoparticle uptake was predominantly mediated by the AT1 receptor.

With the help of fluorescently labeled transferrin, the predominant uptake route of the nanoparticles was studied in more detail. The distinct endocytic vesicles of Ang II-targeted Qdots clearly colocalized with the Alexa Fluor® 488-transferrin fluorescence (Figure 4); this was the case for both the NCI-H295R cells and the rat mesangial cells.

Receptor binding affinity and activation

The cellular binding affinity and thus the multivalency of both the ligand alone and the ligand-conjugated nanoparticles was determined by intracellular calcium measurements with the help of the fluorescent calcium chelator fura-2. First, a microscope-based single cell tracking approach was used to examine the influence of the highly absorbing semiconductor Qdot core on fura-2 emission. When NCI-H295R cells were perfused with 10 nM of non-targeted Qdots the fura-2 emission ratio was unchanged (Figure 5A, top). However, when the cells were perfused with 10 nM angiotensin II, a sharp increase in the emission signal was observed, indicating a calcium influx into the cytosol. When the cells were perfused with 10 nM Ang II-targeted Qdots, an increase in the fura-2 emission ratio was observed, to an even greater extent than the change elicited by the Ang II peptide alone (Figure 5A, bottom). In addition, a plate reader-based calcium mobilization approach was used to pinpoint the receptor binding affinities. Again, non-targeted Qdots showed no alteration of the basal calcium signal over a wide range of concentrations (Figure 5B, top). In contrast, the native angiotensin II peptide bound and activated the receptor with an EC50 of 70.1 ± 1.8 nM. Modification of the N-terminal aspartic acid of Ang II by introducing a thiol moiety decreased the receptor affinity to 261 ± 25 nM. Likewise, introduction of a 5 kDa PEG chain to the N-terminal amino acid lowered the peptide’s affinity to 320 ± 42 nM (Supplementary Figure S2). Ang II-targeted nanoparticles activated the AT1 receptor with

Figure 2. Nanoparticle binding to receptor positive and receptor negative cell lines by flow cytometry after 1h incubation. Ang II-targeted Qdots strongly associated with AT1R-expressing rat mesangial cells and NCI-H295R cells, whereas they showed no association with AT1 negative HeLa cells. Levels of statistical significance are indicated as: †p < 0.01 compared to fluorescence of untreated control cells; **p < 0.01 compared to cellular fluorescence of non-targeted Qdots. Data is expressed as mean ± standard deviation (n = 3).

Figure 3. Nanoparticle uptake as investigated with confocal microscopy. Ang II-targeted Qdots are readily endocytosed by rat mesangial and NCI-H295R cells and located in the perinuclear region of cells. Arrowheads mark cell nuclei. The AT1R antagonist losartan carboxylic acid (10 µM) markedly inhibited the uptake of ligand-modified Qdots. Non-targeted Qdots showed only weak association to cells.
Figure 4. Coincubation of cells with fluorescently labeled transferrin reveals the endocytosis route. Receptor positive cells were incubated with Ang II-targeted Qdots and Alexa Fluor® 488 labeled transferrin and incubated for 30 min. Colocalization of Qdots and Alexa Fluor® 488 indicate that Qdots are taken up by clathrin-mediated endocytosis.

Figure 5. Influence of Ang II-targeted Qdots on cytosolic calcium concentrations. (A) Perfusion of NCI-H295R cells with non-targeted Qdots does not alter their intracellular calcium concentration, whereas incubation with 10 nM Ang II-targeted Qdots led to an influx of calcium ions into the cytosol, demonstrating receptor activation. (B) When quantified in a plate reader-based approach, non-targeted Qdots again show no influence on the cytosolic calcium (top). Angiotensin II and N-modified angiotensin II bind and activate the receptor with an EC₅₀ of 70.1 ± 1.8 nM and 261 ± 25 nM, respectively, whereas Ang II-targeted Qdots activate the AT₁ receptor with an EC₅₀ of 8.9 ± 0.3 nM, demonstrating a distinct multivalent effect.
an EC$_{50}$ of 8.9 ± 0.3 nM indicating the multivalent nature of the nanoparticle binding. Qdot concentrations higher than 30 nM could not be tested since the broad and potent Qdot absorbance quenched the Fura-2 emission signal.

**Discussion**

An N-terminal thiolated angiotensin II derivative was synthesized and covalently coupled to PEGylated Qdots. The thiol moiety required for coupling to Qdots was introduced at the amino-terminus of the peptide since the negatively charged carboxy terminus is essential for receptor binding and activation [25,26]. Thus, the alternative modifications using an amide or ester formation at the C-terminus would lead to loss of the negative charge and a subsequent loss of the peptide’s and conjugate’s ability to bind the AT$_1$ receptor.

However, the reaction of the primary amino group of Asp$^1$ with 2-iminothiolane (Traut’s reagent) was anomalous and did not immediately form the mercapto group. Instead, an N-peptidyl-2-iminothiolane intermediate was generated that had to be hydrolyzed at a reduced pH of 5.00 (Figure 1A). After acid-catalyzed hydrolysis an N-peptidyl-4-mercaptobutyramide was formed with the intended reactive thiol moiety. This quite uncommon reaction pathway has also been observed for bombesin antagonists [27] and has been systematically investigated using short dipeptides [28]. The success of the subsequent angiotensin II conjugation to the nanoparticle surface was verified by flow cytometry and confocal microscopy. HeLa cells, which do not express functional AT$_1$ receptors [15], were not observed to associate with the obtained nanoparticles. In contrast, Ang II-targeted Qdots bound extensively to NCI-H295R and rat mesangial cells, both of which are AT$_1$R positive [29,30]. Qdots were mainly taken up into the cells by clathrin-mediated endocytosis, since most of the Qdots were colocalized with fluorescently labeled transferrin, a typical pathway marker for both clathrin-mediated uptake [31,32] and early and recycling endosomes [33]. Similar to many other GPCRs, the AT$_1$ receptor is mainly internalized by clathrin-mediated endocytosis upon agonist binding, which explains why a potent inhibitor of clathrin-mediated endocytosis greatly hindered angiotensin II internalization [34].

For targeted drug or gene delivery with nanoparticles, uptake of colloids into cells is highly valuable. This is especially true for nucleic acid therapeutics like plasmids or siRNA, which have to reach intracellular targets. These molecules usually cannot cross the cell membrane by themselves and need potent carriers to promote their uptake. Since the AT$_1$ receptor is a G$_{q/11}$-coupled receptor [26], binding of agonist to the receptor leads to a calcium influx into the cytosol. Kobila and coworkers discovered that, although intracellular signal transduction via inositol 1,4,5-trisphosphate and Ca$^{2+}$ pathways had been completely desensitized, AT$_1$ receptors were still being rapidly internalized after Ang II binding due to the receptor’s immediate recycling [35]. This interesting feature of the AT$_1$R underscores the receptor’s feasibility as a nanoparticle target, since it allows for extensive nanoparticle uptake into the target cells.

Non-targeted Qdots, which were not endocytosed after 1 h of incubation, had no effect on the basal calcium concentration. In contrast, Ang II-targeted nanoparticles induced a calcium influx into the cytosol that was higher compared to the calcium influx that was induced by the same concentration of Ang II. When quantified, the EC$_{50}$ of the native peptide and the thiolated peptide were determined to be 70.1 and 261 nM, respectively. A salt bridge between the terminal amino group Asp$^1$ and the terminal carboxy group of Phe$^8$ gives Ang II its classical hairpin-like conformation [36], which also stabilizes the binding of the peptide to the receptor [26]. When the terminal Asp$^1$ amino group is converted to a thiol moiety, this ion pair formation is no longer possible, ultimately leading to nearly 4-fold loss of receptor binding affinity for the N-thiolated peptide (Figure 5B, bottom). Likewise, introduction of a 5 kDa mPEG chain to the amino group of Asp$^1$ lowered the receptor affinity by a factor of 4.5 (Supplementary Figure S2). This is in great contrast to the angiotensin receptor antagonist EXP3174, where PEGylation led to a 580-fold affinity loss [15]. Since the amino acids of the receptor that contribute to the angiotensin II binding are mainly located in the extracellular receptor regions [26], PEGylation only had a minor influence on its affinity. On the other hand, because the receptor residues that are implicated in antagonist binding are located deep in the transmembrane region [37] PEGylation of EXP3174 severely harmed its receptor affinity and led to a profound loss of affinity by a factor of 580 [15]. Qdots on which the thiolated Ang II was immobilized regained affinity with an EC$_{50}$ of 8.9 nM, which was ~30-fold more affine than the thiolated ligand alone.

Based on the number of available amine groups on the nanoparticle surface and the observed coupling efficiency, we estimate that 5 to 7 ligand molecules were conjugated to each Qdot. The same conjugation procedure using endothelial growth factor (EGF) instead of angiotensin II resulted in four EGF molecules per Qdot [38]. Since Ang II is a smaller peptide we believe that a higher ligand number could be conjugated to the nanoparticle surface. As defined by Montet [39], six Ang II ligands per Qdot would result in a multivalent enhancement (MVE, β) of five compared to the unbound ligand. Compared to the multivalent enhancement of AT$_1$R antagonist-modified nanoparticles, which was 22.5 [15], the MVE of the Ang II-targeted Qdots was substantially lower. This can be explained by the decreased contact time of the colloids with the cell surface. Due to their rapid internalization into the cell, the time available for each nanoparticle to bind to several receptors is limited. However, although this prompt endocytosis weakens the multivalent interactions between particles and cells, the increased uptake could be immensely useful as a method for increasing intracellular concentrations of molecules with otherwise poor membrane permeability. Since cells with a higher receptor expression can be expected to endocytose a significantly higher amount of bound nanocarriers, such multivalent interactions could lead to selective drug delivery into specific cells and tissues.

**Conclusions**

Ang II-targeted nanoparticles strongly interact with the AT$_1$ receptor in a highly multivalent fashion and show no association to cells that do not express the receptor. After binding, the colloids are rapidly taken up via clathrin-mediated endocytosis. This constitutes the first
experimental evidence that the binding of Ang II-targeted nanoparticles to their cognate type 1 receptors is multivalent in nature, which potentially allows for their use in the targeting of tissue that overexpresses this receptor.

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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online
Supplementary Figures S1 and S2