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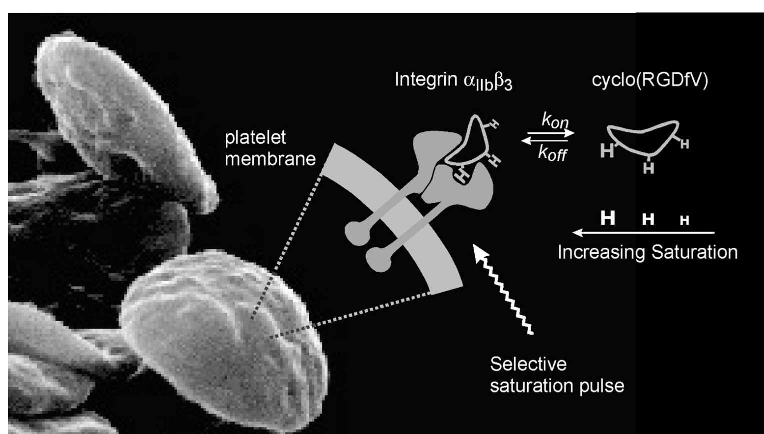

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Direct Observation of Ligand Binding to Membrane Proteins in Living Cells by a Saturation Transfer Double Difference (STDD) NMR Spectroscopy Method Shows a Significantly Higher Affinity of Integrin $\alpha_{IIb}\beta_3$ in Native Platelets than in Liposomes

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Abstract: About 30% of the proteins in mammalian systems are membrane bound or integrated (e.g., GPCRs). It is inherently difficult to investigate receptor−ligand interactions on a molecular level in their natural membrane environment. Here, we present a new method based on saturation transfer difference (STD) NMR to characterize at an atomic level binding interactions of cell surface proteins in living cells. Implemented as a double difference technique, STD NMR allows the direct observation of binding events and the definition of the binding epitopes of ligands. The binding of the pentapeptide cyclo(RGDfV) to the surface glycoprotein integrin $\alpha_{IIb}\beta_3$ of intact human blood platelets can be detected by saturation transfer double difference (STDD) NMR in less than an hour. A 5-fold higher STD response reflects a significantly higher affinity of integrin $\alpha_{IIb}\beta_3$ in native platelets than in liposomes, which demonstrates the importance of studying membrane proteins in their natural environment. Also, the binding mode of cyclo(RGDfV) in the arginine glycine region is slightly different when interacting with native integrin in platelets compared to integrin reintegrated into liposomes.

Introduction

Many interesting targets in drug design are membrane-bound proteins, an important subclass of which are the G-protein coupled receptors (GPCRs).[1] These proteins are often difficult to deal with. They lose their structure and hence their functionality when removed from their natural membrane environment. Methods, such as total internal reflection fluorescence microscopy (TIRFM)[2] and surface plasmon resonance (SPR),[3] have been used for binding studies. However, none of them gives insight into the binding epitope of the ligand at an atomic level. X-ray structures could theoretically provide this information. However, membrane-spanning proteins have been difficult to crystallize in an active form.

Many NMR methods have been developed to screen and to characterize binding processes at a molecular level, for examples, transferred nuclear Overhauser enhancement (trNOE) spectroscopy,[4,5] structure activity relationship (SAR) by NMR,[6,7] NOE pumping,[8,9] and competitive binding spectroscopy.[10] However, these methods do not allow the observation of the binding of ligands to membrane-bound proteins in their natural environment. Saturation transfer difference (STD) NMR is an efficient method to study protein−ligand recognition events (cf. Figure 1).[11] STD NMR does not require any labeling or immobilization of either the ligand or the receptor and runs under close to physiological conditions. We could demonstrate that STD NMR is suitable for the analysis of membrane-bound proteins when reintegrated into liposome membranes.[12]

Integrin $\alpha_{IIb}\beta_3$ is the most abundant surface glycoprotein on platelets and consists of two noncovalently linked α and β subunits. It recognizes proteins and peptides presenting the peptide motif RGD.[13,14] Platelets are cells without a nucleus but with an active energy metabolism. In the activated state, the integrin binds to fibrinogen and then mediates thrombus formation by platelet aggregation. Here, we characterize the interaction between intact human platelets and the inhibitor cyclo(RGDfV) by a new STD NMR method. Cyclo(RGDfV) was designed as a highly potent ligand for integrin $\alpha_V\beta_3$ with

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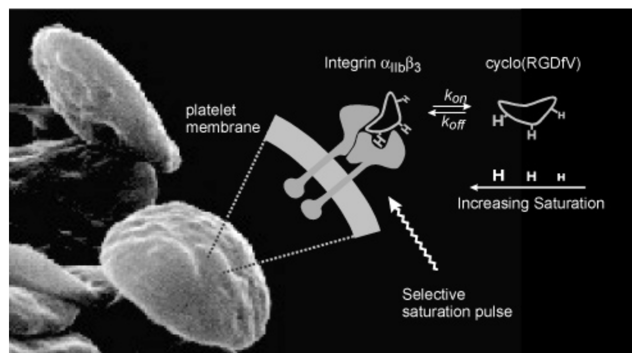

Figure 1. Schematic view of the NMR analysis performed here to identify ligand binding to membrane proteins. On the left side, platelets are shown in an electron micrograph image. The schematic expansion to the right shows the membrane-anchored integrin with bound and free ligands. In STD NMR experiments, information of the bound ligand is transferred from the protein into solution by dissociation of the ligand from the bound state. The basis for STD NMR is a selective saturation of the protein that is spread all over the protein by intramolecular spin diffusion. That saturation is also transferred to the bound ligand by spin diffusion. Saturation of the ligand is carried into solution, where it is detected. When subtracting the saturated spectrum from one without protein saturation, a difference spectrum is obtained showing signals only of ligands. Additionally, the ligand's binding epitope can be determined from the STD spectra because the intensity of the STD signal is correlated to the proximity of the individual proton of the ligand to the protein.[18]

nanomolar binding affinity but also shows micromolar binding with integrin $\alpha_{IIb}\beta_3$.[2,15]

STD NMR requires only small amounts (in the picomol range) of receptor proteins. A large excess of ligand amplifies the information from the binding process to create a sufficiently strong NMR signal of the ligand.[16] The expression level of integrin $\alpha_{IIb}\beta_3$ on platelets results normally in $\sim$ 40 000 integrin molecules per platelet.[17] This and the low cytoplasmic volume of the platelets result in $\sim$350 pmol receptor in the NMR sample. NMR analysis of complex cellular systems is complicated by a huge number of signals originating from large and small compounds present. STD NMR analysis of such a system shows signals originating from many different binding events. Also, residual signals of large molecules will be picked up. Only resonances of nonbinding small molecules are eliminated by subtraction. An epitope mapping of the binding ligand is very difficult because of numerous overlapping NMR signals. To characterize the binding event of interest, it is necessary to remove all or most unwanted signals.

Materials and Methods

Preparation of Cell Suspension. Human platelet concentrates were received from the blood donation service, Hamburg Eilbek (Blutspendedienst Hamburg Eilbek). The concentrates contained pooled platelets from five blood donations ($>2.5 \times 10^{11}$ platelets) suspended in citrate−phosphate−dextrose (CPD) plasma (to 0.8 mL of human plasma was added 0.2 mL of CPD stabilizer solution). The remaining numbers of erythrocytes and leukocytes were specified as $<5 \times 10^8$ and $<1 \times 10^6$, respectively.

For all preparation steps, a deuterated TRIS-buffered saline (TBS buffer) was used that is prepared in deuterium oxide (D_2O, 99.9%) and contains 10 mM perdeuterotris(hydroxymethyl)aminomethane (TRIS-d_{11}), 150 mM NaCl, 4 mM NaN_3, 3 mM KCl, and 1 mM 4-(2-aminoethyl)benzolsulfonylfluoride hydrochloride (AEBSF). All preparations were done at room temperature.

First, 16 mL of CPD plasma ($\sim$14 $\times$ 10^9 platelets) was pipetted into a 50 mL Falcon tube and centrifuged for 30 min at 1600 *g*. The supernatant was discarded and the remaining pellet cautiously resuspended in 2 mL of TBS buffer containing an additional 1 mM disodium ethylendiaminetetraacetate (TBS-EDTA buffer). The suspension was centrifuged at 1600 *g* for 15 min. The supernatant was discarded, and the pellet was resuspended in 2 mL of TBS-EDTA buffer. These steps were repeated six times. The following four steps were carried out analogously. However, for the first time, TBS buffer without additives was used for resuspension, and for the remaining three times, TBS buffer containing also 1 mM $CaCl_2$ (TBS−$CaCl_2$ buffer) was utilized. After the last centrifugation, pellets of about 500 μL volume were obtained and resuspended in 500−750 μL of TBS−$CaCl_2$ buffer. The suspension was split into two NMR tubes, and 150 nmol cyclo(RGDfV) (3 mM stock solution in TBS buffer) was added to one sample.

Acquisition of NMR Spectra. All NMR spectra were recorded at a temperature of 283 K with a spectral width of 10 ppm on Bruker Avance DRX 500 MHz and Avance DRX 700 MHz spectrometers, each equipped with a 5 mm inverse triple-resonance probe head. Selective saturation of the protein was achieved by a train of Gauss-shaped pulses of 50 ms length each, truncated at 1%, and separated by a 1 ms delay. A number of 40 selective pulses was applied, leading to a total length of the saturation train of 2.04 s. The on-resonance irradiation of the protein was performed at a chemical shift of −1.1 ppm. Off-resonance irradiation was set at 114 ppm, where no protein signals are present. The spectra were subtracted internally via phase cycling after every scan using different memory buffers for on- and off-resonance. Total scan number in the STD experiments was 2K. NMR spectra were multiplied by an exponential line-broadening function of 0.5 Hz prior to Fourier transformation. Spectra processing was performed on Silicon Graphics Octane workstations using Xwinnmr 3.1 software (Bruker). For the spectra, which were recorded with a $T_{1\rho}$-filter, spin lock pulses of 30 and 80 ms were applied.

Results and Discussion

The pentapeptide cyclo(RGDfV) was developed by Kessler et al. and binds to integrin $\alpha_{IIb}\beta_3$ with a binding constant of K_D = 5 μM[2]. Preparation of the platelet suspension yielded two NMR samples containing the same composition of platelets in D_2O−TBS buffer, that is, approximately 7 $\times$ 10^9 platelets equivalent to 100−600 pmol integrin $\alpha_{IIb}\beta_3$, assuming that 1−5 $\times$ 10^4 molecules integrin[17] are present on each cell. To one sample was added cyclo(RGDfV) (sample A), while the other one served as a reference (sample B) (Figure 2). Saturation is transferred from integrin $\alpha_{IIb}\beta_3$ to cyclo(RGDfV) by irradiating the protein at −1.1 ppm for 2.04 s. This had a negligible effect ($<$1%) on ligand resonances (data not shown).

The results of the STD NMR experiments are presented in Figure 3. Figure 3A shows the STD spectrum of the platelet suspension containing 150 nmol cyclo(RGDfV) equivalent to a 250−1500-fold excess over integrin. Except for the resonances of Val $H\gamma/\gamma'$ and Phe $H\delta,\epsilon,\zeta$, the other signals of cyclo(RGDfV) are overlaid with the broad signals originating from the platelets and therefore cannot be assigned unambiguously.

Although the STD spectrum allows an observation of the specific binding of cyclo(RGDfV) to integrin $\alpha_{IIb}\beta_3$, epitope mapping and identification of all signals is limited. The application of a $T_{1\rho}$-filter is an established NMR method to suppress NMR signals of large molecules. But even such a spin lock field did not eliminate signals originating from the biological components of platelets sufficiently (cf. Figure 3B).

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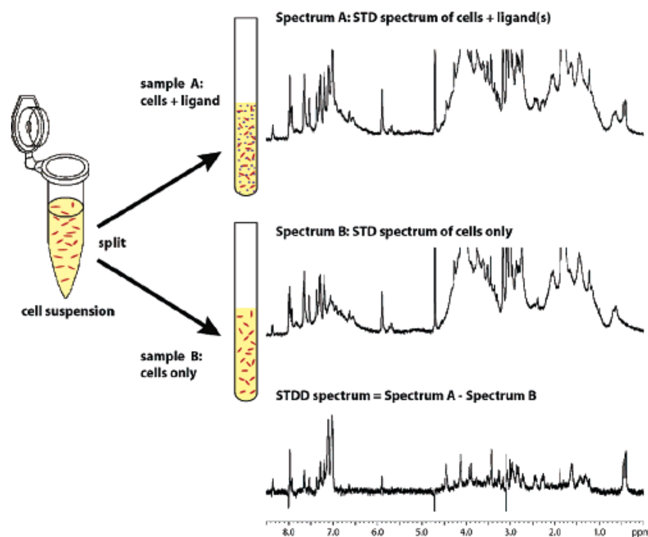

Figure 2. Schematic overview of the new Saturation Transfer Double Difference (STDD) NMR method to detect binding of ligands (denoted by purple dots) to membrane-bound proteins in living cells (denoted by red dots). A cell suspension is split up into two NMR tubes. To one tube (sample A) are added ligands. Sample B contains only the cell suspension. Top: STD NMR spectrum of ligand and cells containing the transmembrane receptor. As a result, one obtains a spectrum with the STD signals from the added ligand and the other binding processes in the cell. Middle: STD NMR spectrum of cells containing the transmembrane receptor. The STD NMR spectrum contains all signals from binding processes within the cell. No ligand receptor interactions can occur. By subtracting the cell STD spectrum (middle) from the cell/ligand STD spectrum (top), it is possible to obtain the STDD spectrum (bottom) in less than 1 h, revealing only signals from the ligand receptor interactions.

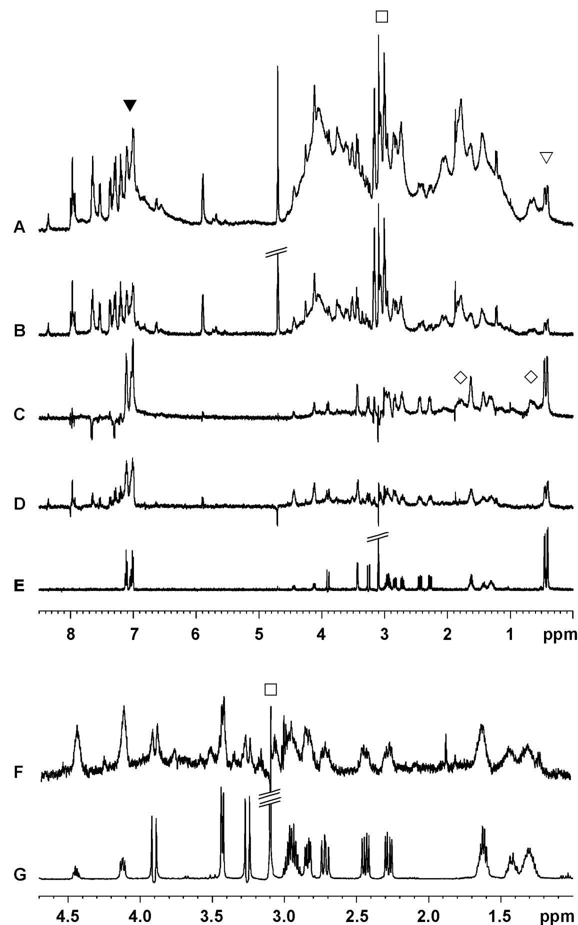

Figure 3. Filter effect of the STDD technique. The crowded ^{1}H STD NMR spectrum of a platelet suspension with cyclo(RGDfV) at 500 MHz is shown in A. Only the signals marked by triangles can be quantified without problems (▼ Phe Hδ,ϵ,ζ; ▽ Val Hγ,γ'). Residual signals of other proteins and binding events overlay the spectrum of the ligand of interest. The unsatisfying suppression of the platelet's signals is shown in spectrum B recorded at 500 MHz using a $T_{1\rho}$-filter of 30 ms. (C) Saturation Transfer Double Difference (STDD) spectrum at 700 MHz showing clearly the filter effect of the double difference method when compared to the reference spectrum of the ligand shown in E (◇ platelet signals). (D) Saturation Transfer Double Difference (STDD) spectrum at 500 MHz showing the same effect as for C. (E) ^{1}H NMR reference spectrum of cyclo(RGDfV) at 500 MHz. F and G show expansions of spectra D and E, respectively, to prove the outstanding filtering effect of STDD. Water suppression was achieved by a WATERGATE pulse sequence (w5) except for spectrum C where a WATERGATE 3-9-19 sequence was applied. All spectra were acquired in deuterated TBS buffer at 283 K (□ TRIS).

Therefore, a new type of filter is required to eliminate the background signals of the platelets. Hence, we developed the STDD-filter, for which the STD reference spectrum of sample B has to be subtracted from the STD spectrum of sample A. This subtraction results in a double difference spectrum (cf. spectra C and D of Figure 3) that shows only resonances of the inhibitor peptide. Comparison of spectra C and D in Figure 3 with the spectrum of cyclo(RGDfV) (Figure 3E) clearly reveals that disturbing signals originating from the platelets are almost perfectly canceled out. This becomes more evident when looking at the spectral region between 1.1 and 4.6 ppm (cf. spectra F and G of Figure 3). In this region, the peptide resonances were totally covered by the platelet envelope in the STD spectrum 3A. It is unclear why the spectrum at 700 MHz (Figure 3C) is somewhat less clean than that at 500 MHz (Figure 3D).

On the basis of the STDD spectra, an assignment of the ligand's signals as well as an epitope mapping becomes easily possible. Spectra can be obtained in less than an hour. Moreover, use of a spin lock to suppress signals of large molecules in STD spectra results in loss of saturation from the ligand by T1 and T2 relaxation processes, which leads to a decreased signal-to-noise ratio. For the double difference spectroscopy introduced here, no spin lock pulses were used. Therefore, the double difference spectrum shown in Figure 3D shows a 1.5-fold higher signal-to-noise ratio than that shown in Figure 3B.

The examination of the binding epitope of cyclo(RGDfV) in Figure 4B shows that the individual protons receive a saturation corresponding to their proximity to the protein. The results are compared to earlier STD NMR studies carried out in our research group where the interaction between cyclo(RGDfV) and integrin $\alpha_{IIb}\beta_3$ embedded into liposomes was characterized.[12] Native integrin $\alpha_{IIb}\beta_3$ in platelets interacting with cyclo(RGDfV) has STD percent values about 5-fold higher than those of the solubilized integrin reintegrated into liposome membranes (cf. Figure 4A). The total concentration of the receptor protein in the platelet preparation was ~0.6 μM and in the previous liposome preparation[12] 5 μM (counting only the receptors facing outward). Even though the receptor density was significantly less in the platelet preparation, the intensity of the STD effects was 5 times larger using the same ligand excess.

This indicates a significantly higher affinity of cyclo(RGDfV) to integrin $\alpha_{IIb}\beta_3$ on native platelets. This high activity of the integrin is probably due to the fact that the receptor protein on the platelets is left in its natural environment, whereas the reintegration protocol requires that the membrane proteins have to be isolated, solubilized, and purified before reintegration into the liposome membranes. This process is usually accompanied

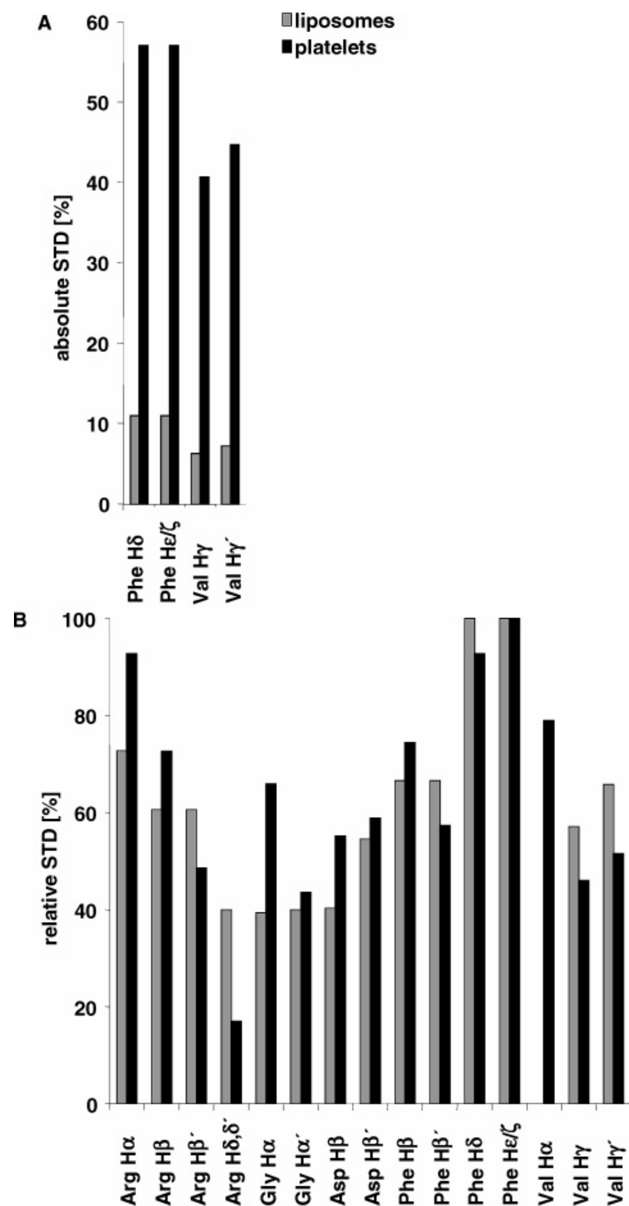

Figure 4. (A) The absolute STD percent for four groups is shown. It becomes clear that the STD effects in platelets (black) are significantly higher than those in liposomes (grey). (B) STD NMR-based epitope mapping of cyclo(RGDfV) when bound to integrin $\alpha_{IIb}\beta_3$ in human platelets (black) and solubilized integrin $\alpha_{IIb}\beta_3$ reintegrated into liposomes (grey). The resonance of Val Hα could not be analyzed in the liposome spectra. Even though the absolute intensities of the STD signals (shown in A) are 5-fold higher for the native integrins in platelets than for those in liposomes, their relative intensities (shown in B) are, except for a few protons, very similar, indicating a comparable binding mode to native integrin. However, the differences suggest a slightly different binding mode of the cyclopeptide to the native integrin integrated in platelet membranes compared to the integrin reintegrated into liposome membranes.

by high losses and produces often a significant portion of non-native proteins. Three protons (Arg Hδ,δ', Gly Hα) show distinct deviations in the STD response, which reflects a slightly different binding mode to the native receptor (cf. Figure 4B). Otherwise, both systems show approximately the same relative STD effects. Thus, except for the three protons mentioned, the binding epitope from liposome reintegrated integrin has approximately the same binding epitope as the native integrin. This indicates a modified orientation of the arginine side chain and the glycine residue. This confirms that the STDD method is well suitable to observe and analyze ligand binding to membrane-bound proteins in living cells. The strongest STD effects are observed for Arg Hα,β and Phe Hδ,ϵ,ζ. The protons within the alkyl side chain of Arg receive decreasing saturation, indicating a hydrophobic interaction near the peptide backbone.

The integrin $\alpha_{IIb}\beta_3$ is the most dominant protein on the surface of blood platelets, comprising about 53% of all membrane integrated proteins.[19] Thus, contamination of the STDD signals arising from other proteins is very unlikely. Also, the binding epitope, as determined from the STDD spectra, is very similar to that of the liposome integrated integrin, which is also only found in cases if a similar binding mode is used by the ligand.

Conclusion

The benefit of the new STDD method becomes evident as detailed information of membrane-bound receptor−ligand interactions is easily accessible in living cells. Studying biochemical processes that involve membrane proteins on the cytoplasmic membrane as well as on subcellular organelles and the nucleus has become more feasible using the technique presented here. Use of cryo probes will enhance the sensitivity of the method by about a factor of 3.5. Only about 25 pmol of membrane protein will be sufficient such that many surface proteins with a high expression level can be analyzed. A quantity of 25 pmol of receptor protein is present in 10^8 cells, giving an expression rate of about 150 000 receptors per cell. Typical for GPCR expressing cell lines is a receptor density of about 10^6 per cell, well exceeding the requirements for the amount of receptor molecules. A quantity of 10^8 cells can easily be suspended in an NMR tube within the active volume.

The method is also of great importance for pharmaceutical research where many targets are seven helix transmembrane-spanning proteins (e.g., GPCRs), which could not be investigated at an atomic level so far. As a further outlook, the method provides a new basis for a detailed insight of a drug's mode of action in complex biological systems, such as tissue and organs.

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Supporting Information Available: Additional NMR spectra of cell suspension, ^{1}H NMR spectrum sample A, ^{1}H STD NMR reference spectrum sample B, ^{1}H STD NMR spectrum sample A ($T_{1\rho}$-filter 80 ms). Table of chemical shifts of resonances of cyclo(RGDfV) and their relative STD % values (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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