Molecular Targeting

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A Xenon-129 Biosensor for Monitoring MHC–Peptide Interactions**

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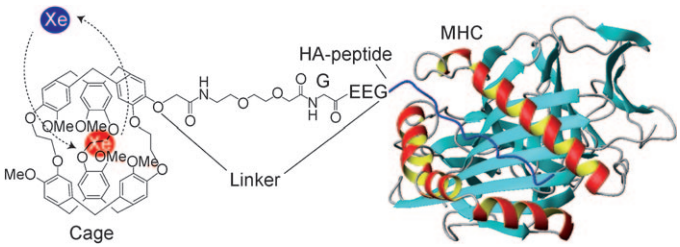
Nuclear magnetic resonance is a phenomenon that can yield spectroscopic and imaging information at the systemic and molecular levels. A highly attractive magnetic resonance (MR) probe in this respect is hyperpolarized ^{129}Xe, as it combines large signal enhancements (ca. 10000-fold compared to thermal polarization) with excellent chemical-shift dispersion (> 200 ppm in water). Its good solubility and compatibility with biological fluids has yielded important topological information at the level of proteins[1–3] or whole organs, for example, lung[4] or brain.[5–7] A critical step in molecular targeting was the development of cryptophane molecules that are able to trap xenon atoms for a time that is long enough for a separate signal to be observed in the NMR spectrum.[8–11] When coupled to a binding moiety, the interaction with the target molecule can be monitored through the ^{129}Xe signal in the NMR spectrum because of line-broadening, chemical shift changes, or resonance splitting.[8] By combining these indicators with chemical exchange saturation transfer (CEST), Wemmer, Pines, and co-workers were able to detect a biotinylated ^{129}Xe biosensor in a streptavidin-bound agarose phantom.[12] Successful attempts to improve the xenon affinity and solubility of the hydrophobic cage compound have been independently performed,[13,14] and the concept of multivalency was introduced by the development of cage dendrimers.[15] Recently, Dmochowski and co-workers extended the range of applicability of the biosensor to the enzymatic cleavage of a peptide substrate by matrix metalloprotein-7[16] and to targeting human carbonic anhydrase.[17] Herein, we introduce a further type of ^{129}Xe biosensor that enables the detection of complex formation between a peptide ligand and major histocompatibility complex (MHC) class II protein. MHC class II molecules are cellular surface molecules of immunological relevance that trigger T cell mediated immune responses when bound to an antigenic peptide molecule.[18] They also play a crucial role in the development of autoimmune disease.[19–22]

We chose the interaction of the hemagglutinin (HA) peptide with the sequence PKYVKQNTLKLAT with the human leukocyte antigen (HLA) DR1 as a model system for MHC class II peptide interactions. Our rationale for the linkage of the HA peptide to the cryptophane (Scheme 1) was based on previous work from other research groups[9] and had to fulfill two requirements: 1) the peptide linker moiety should enhance the water solubility of the hydrophobic cage and 2) the composition and length of the linker had to be designed such that the interaction of the cage with the MHC itself becomes unlikely but allows for sufficient proximity of the caged xenon to "sense" the binding event through a change of chemical shift in the NMR spectrum. Optimization of the N-terminal peptide extension and the polarity of the nonpeptide linker resulted in a cage–linker–HA construct (abbreviated cage–HA) with excellent solubility and no tendency to enhance oligomerization when bound to DR1 (Scheme 1). The construct was purified by HPLC methods and its composition confirmed by mass spectrometry.



Scheme 1. Visualization of the cage–linker–HA construct in complex with the human class II MHC molecule DR1 (PDB code: 1DLH[23]). HA is shown as a blue ribbon. G and E relate to the single letter code for the amino acids glycine and glutamate, respectively.

Subsequently, the cage–linker–peptide construct was implemented into the refolding protocol for MHC class II molecules in order to achieve complex formation. Specific binding was confirmed by an ELISA assay and observation of a peptide-bound SDS-stable dimer (Figure S1 in the Support-

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Supporting information for this article (synthetic details for the cage–linker–HA construct, bacterial expression of HLA-DR1, NMR experiments with ^{15}N-labeled DR1-HA complexes, and experiments with ^{129}Xe) is available on the WWW under http://dx.doi.org/10.1002/anie.200806149.

ing Information). An overlay of the ^{15}N–^{1}H HSQC spectra of DR1 loaded with wild-type HA (red spectrum) and cage–HA (blue spectrum) is shown in Figure 1. The two spectra are essentially identical, with only few minor chemical-shift differences. This indicates that cage–HA binds in a similar mode to DR1 as wildtype HA and that no additional epitopes are occupied by the linker–biosensor extension.

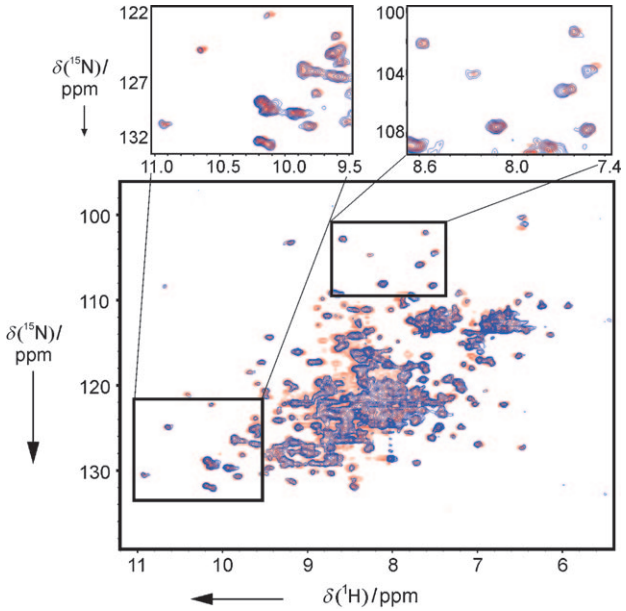

Figure 1. Superposition of spectra showing DR1 co-refolded in the presence of either wildtype HA (red) or the cage–HA (blue). Protein concentrations were 140 μM for DR1-bound wildtype HA and 40 μM for DR1-bound cage–HA. Insets show regions of tryptophan side-chain (upper left) and glycine backbone NH resonances (upper right) for better comparison.

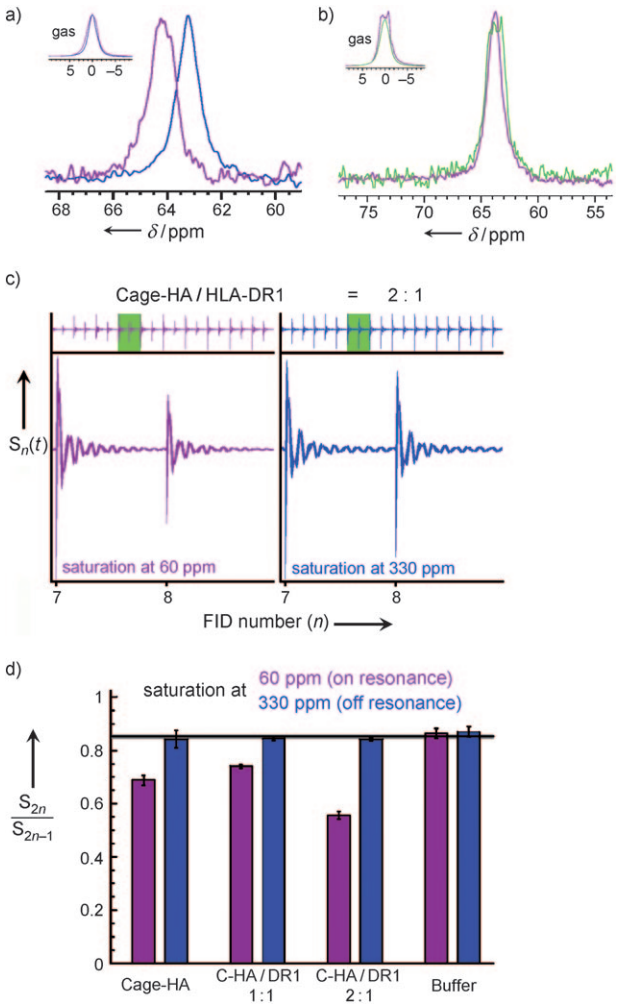

Figure 2. ^{129}Xe NMR spectra of complex formation. a) ^{129}Xe signal of cage–HA in the absence (blue) or presence (magenta) of equimolar amounts of DR1. b) Line observed upon addition of excess cage–HA (magenta) and calculated sum of the individual signals (green) observed in (a). The insets show ^{129}Xe gas signals of the corresponding spectra used as reference at $\delta = 0$ ppm. c) Loss of the bulk ^{129}Xe signal by hyper-CEST. Saturation was applied either on-resonance to the cage–bound xenon ($\delta \approx 60$ ppm, magenta) or off-resonance at the mirror frequency relative to the ^{129}Xe solution signal ($\delta \approx 330$ ppm, blue) for even numbered signals $S_{2n}(t)$, whereas no saturation was applied for odd numbers $S_{2n-1}(t)$. The free induction decay (FID) of two examples are highlighted and displayed in the lower panels to show the effect of saturation. d) Statistical analysis of amplitude loss S_{2n}/S_{2n-1}. The horizontal line at 0.85 indicates the damping that arises solely from pulse excitation and T_1 decay (see the Supporting Information).

The principle of DR1 binding to cage–HA with ^{129}Xe detection is shown in Scheme 1: Cage-bound xenon exchanges with bulk-solute xenon at an approximate rate of 30–50 ms,[8] thus cage-bound xenon can give rise to a separate ^{129}Xe signal whose chemical shift may depend on complex formation.[10] As expected, the ^{129}Xe NMR spectrum of cage–HA alone (10 μM) shows a resonance at 63.3 ppm (Figure 2a, blue line and Figure S2 in the Supporting Information) when the solution was loaded with hyperpolarized ^{129}Xe (88% enrichment, 9 mM concentration). Furthermore, the ^{129}Xe NMR spectrum of a 1:1 complex with DR1 (10 μM) shows a signal that is shifted by 1 ppm downfield relative to free cage–HA (Figure 2a, magenta line) and with a slightly enhanced width (37 Hz versus 31 Hz). When excess cage–HA (10 μM) was added to this 1:1 complex, we observed the signal of width 49 Hz (Figure 2b, magenta line), which can be effectively superimposed with the sum of the lines of width 66 Hz (Figure 2a, green line in Figure 2b).

We then used magnetization transfer from either free or DR1-bound caged ^{129}Xe to freely dissolved ^{129}Xe for signal enhancement, which is a prerequisite in more sensitive imaging applications. In the experiment, the magnetization of hyperpolarized caged ^{129}Xe is depleted by pulsed excitation and subsequent dephasing (saturation), which leads to the depolarization of bulk hyperpolarized ^{129}Xe through chemical exchange (hyper-CEST).[12] When the sample was saturated far off-resonance at 330 ppm downfield from the ^{129}Xe gas signal, a decrease in bulk signal amplitude of 15% is observed in all samples (Figure 2c, right panel and Figure 2d), which arises from the pulsed excitation scheme and T_1 decay (see Figure S3 and Figure S4 in the Supporting Information). In contrast, by applying the saturation on-resonance of caged ^{129}Xe at around 60 ppm, further reductions of the ^{129}Xe bulk

signal amplitude are exclusively observed in samples containing cage–HA (free and/or MHC-bound; Figure 2c, left panel and Figure 2d). The attenuation by hyper-CEST is 33% in the presence of free cage–HA and 27% for cage–HA in complex with DR1. The difference between the two values most likely represents a difference in concentrations. Furthermore, the mixture of these samples displays an accumulative signal loss of 48%, which clearly shows that magnetization from free and bound cage–HA is equally saturated and transferred by our pulse scheme (Figure S2 in the Supporting Information).

For in vivo imaging applications, it is essential that biomarkers are nontoxic and exert their specific binding function. To this end, fibroblast cells stably transfected with DR1 (L57.23) were loaded with cage–HA and used as antigen presenting cells (APC) in an in vitro T cell assay. When mixed with T cell hybridoma cells (EvHA/X5) that specifically recognize the DR1-HA complex, the loaded cells triggered the activation of the T cells, as shown by the release of the cytokine interleukin-2 (IL-2). The extent of activation was monitored in a secondary assay where the IL-2 dependent proliferation of CTL-L cells is determined by the incorporation of [^{3}H]thymidine.

As shown in Figure 3, both HA and cage–HA are able to activate HA-specific EvHA/X5 cells when added to DR1-expressing APC. The weaker response to the cage–HA was

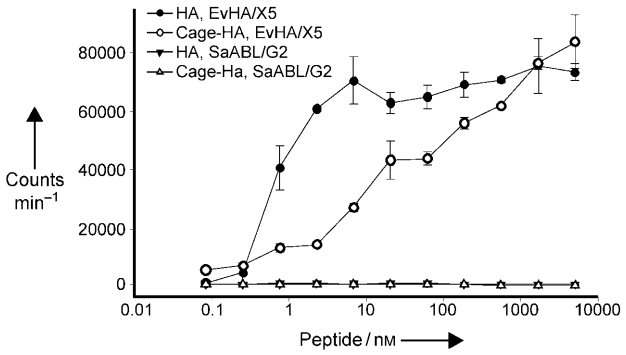

Figure 3. T cell response triggered by cage–HA. DR1-expressing L cells (L57.23) were incubated with increasing amounts of HA (solid symbols) or cage–HA (open symbols) in the presence of the HA-specific T cell hybridoma line EvHa/X5 (circles) or the control line SaABL/G2, specific for a peptide derived from the human ABL protein (triangles). Both T cell hybridoma lines recognize their antigens when associated with DR1, as measured by [^{3}H]thymidine incorporation in a secondary cell proliferation assay. The error bars indicate the range for two values from duplicate measurements.

consistent with the slightly reduced affinity to DR1 (Figure S1 in the Supporting Information). The cryptophane moiety alone did not evoke a T cell response (data not shown) and no stimulation was observed with a control cell line specific for another peptide antigen (SaABL/G2). Thus, cage–HA is nontoxic and the specific proliferation of T cells is antigen-specific and apparently not obstructed by the cage extension. Follow-up experiments also revealed that the cage–HA construct is nontoxic to living animals and that a cell-specific immune response can also be observed in DR1 transgenic mice (data not shown).

The observed chemical shift difference of approximately 1 ppm between free and MHC-bound biosensors is relatively small and calls for future improvements of linker length and composition that would potentially allow for the selective excitation of bound cage–HA. However, for in vivo applications, one might not need to distinguish between free and bound biosensor, given that one can prove that specific binding occurs predominantly at sites of high DR1 surface expression. One interesting application of the MHC class II peptide system should be the use of the ^{129}Xe biosensor to follow the release of DR1-bound cage–HA molecules. Modulation of peptide release by environmental risk factors or so-called MHC loading enhancers[24–26] could be experimentally validated in cell culture or transgenic mouse models. Optical imaging with fluorescence-labeled HA peptide would allow for cross validation of the novel NMR approach. The detection of DR1 peptide loading in deep tissues, however, requires the increased penetration depth provided by MR and, in combination with the ^{129}Xe biosensor described in this study, may offer a unique tool for the in vivo investigations of MHC-based dysfunctions of the immune system.

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