Electronic Supplementary Information for

Synthesis and Characterization of pH-sensitive, Biotinylated MRI Contrast Agents and Their Conjugates with Avidin

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Table S1. Elution conditions for analytical and semi-preparative HPLC. The flow rates of 1 and 10 mL/min were used for the analytical and semi-preparative HPLC respectively.

Time (minute)	% H_2O	% CH_3CN
0.0	80	20
5.0	80	20
17.0	20	80
23.0	20	80
26.0	80	20
30.0	80	20

Fluorescence assay

The interaction of biotin (Bio) or **GdL**^{3,4} with avidin (Av) was followed by the displacement of the fluorescence probe 2-anilinonaphthalene-6-sulfonic acid (ANS) using the fluorescence assay as previously described.[1]

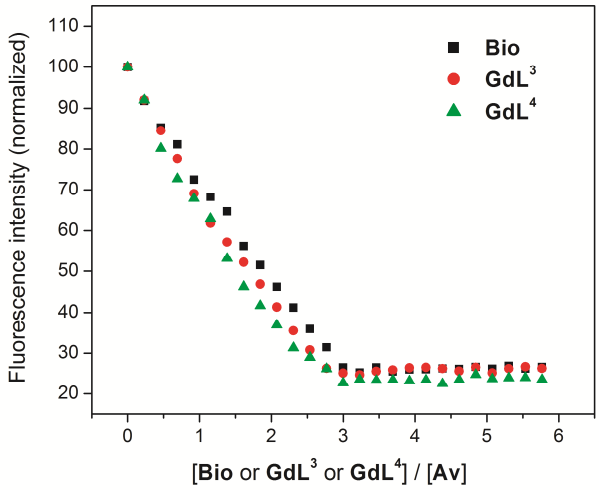

Figure S1. Fluorescence titration of the avidin-ANS complex with biotin and **GdL**$^{3-4}$. The corrected and normalized fluorescence signal is plotted as a function of the ratio of added biotin/**GdL**$^{3-4}$ to avidin.

Since three, rather than four binding sites are available on the avidin tetramer, this stoichiometry was used to develop a linear relationship used to determine the conditional stability constant value K_s for the reaction of avidin-2,6-ANS complex with biotin (Eq. 1), as well as with **GdL**^{3,4}.

$$\mathrm{Av(ANS)_3} + 3\mathrm{Bio} \rightleftharpoons \mathrm{Av(Bio)_3} + 3\mathrm{ANS}, \qquad K_s = \frac{[\mathrm{Av(Bio)_3}][\mathrm{ANS}]^3}{[\mathrm{Av(ANS)_3}][\mathrm{Bio}]^3} \tag{1}$$

If the basic avidin-ANS and avidin-biotin reactions are considered:

$$\mathrm{Av} + 3\mathrm{ANS} \rightleftharpoons \mathrm{Av(ANS)_3}, \qquad K_s^{\mathrm{I}} = \frac{[\mathrm{Av(ANS)_3}]}{[\mathrm{Av}][\mathrm{ANS}]^3} \tag{2}$$

$$\mathrm{Av} + 3\mathrm{Bio} \rightleftharpoons \mathrm{Av(Bio)_3}, \qquad K_s^{\mathrm{II}} = \frac{[\mathrm{Av(Bio)_3}]}{[\mathrm{Av}][\mathrm{Bio}]^3} \tag{3}$$

then K_s from Eq. 1 can be rearranged:

$$K_s = \frac{[\mathrm{Av(Bio)_3}][\mathrm{ANS}]^3}{[\mathrm{Av(ANS)_3}][\mathrm{Bio}]^3} \times \frac{[\mathrm{Av}]}{[\mathrm{Av}]} = \frac{K_s^{\mathrm{II}}}{K_s^{\mathrm{I}}} \quad (4)$$

to give the linear dependence:

$$\frac{[\mathrm{Av(Bio)_3}]}{[\mathrm{Av(ANS)_3}]} = K_s \times \frac{[\mathrm{Bio}]^3}{[\mathrm{ANS}]^3} \quad (5)$$

where [ANS] represents the fixed concentration of ANS (experimental details given in the Experimental section), [Bio] is the concentration of added biotin in each titration point, and $[\mathrm{Av(Bio)_3}]$ and $[\mathrm{Av(ANS)_3}]$ are the concentrations of avidin-biotin and avidin-ANS complexes, respectively. As these concentrations are proportional to measured fluorescence intensities,[2] the obtained experimental data (Figure S1) can be used for conditional stability constant determination:

$$\frac{Y_0 - Y}{Y - Y_{\mathrm{lim}}} = K_s \times \frac{[\mathrm{Bio}]^3}{[\mathrm{ANS}]^3} \quad (6)$$

where Y_0 stands for fluorescence intensity at the beginning of titration (no biotin added), Y_{lim} is an average value of all measured fluorescence intensities after the equivalence point was reached, and Y stands for measured fluorescence intensities during the titration (as reaction (1) proceeds). The measured fluorescence intensities were corrected for inner-filter effects[3] and normalized. The conditional stability constant values K_s for avidin-biotin, avidin-**GdL**^{3} and avidin-**GdL**^{4} are obtained as slopes from the linear fit based on Eq. 6, with R^2>0.98 (Figure S2).

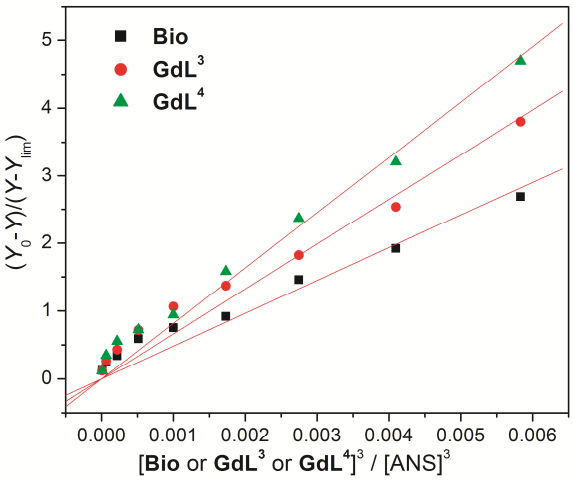

Figure S2. Conditional stability constant (K_s) determination according to Eq. 6.

K_s values are obtained as mean values of five times repeated titrations. Results are shown in Table S2.

Table S2. Conditional stability constant values (K_s) obtained as the slope of the linear fit based on Eq. 6

Sample fluorescence Titration	$K_s \pm$ SD		
	Av-Bio	Av-**GdL**^{3}	Av-**GdL**^{4}
1.	302 ± 11	413 ± 24	464 ± 19
2.	417 ± 16	735 ± 30	618 ± 22
3.	527 ± 22	541 ± 21	751 ± 29
4.	379 ± 12	625 ± 23	676 ± 16
5.	487 ± 18	662 ± 33	818 ± 29
$<K_s> \pm$ SD	(4.2 ± 0.9)x10^2	(6 ± 1)x10^2	(7 ± 1)x10^2

As the dissociation constant of the avidin(monomer)-ANS was already reported (K_d=203 μM)[3], K_s^I value (Eq. 2) is calculated ($K_s^I = \frac{1}{(K_d)^3} = 1.2\text{x}10^{11}$), and used for the overall stability

constants K_s^{II} (Eq. 4) calculation, resulting in $K_s^{II}(Av(Bio)_3)=5.1x10^{13}$, $K_s^{II}(Av(\mathbf{GdL^3})_3)=7.1x10^{13}$, $K_s^{II}(Av(\mathbf{GdL^4})_3)=8.0x10^{13}$.

MRI phantom experiments

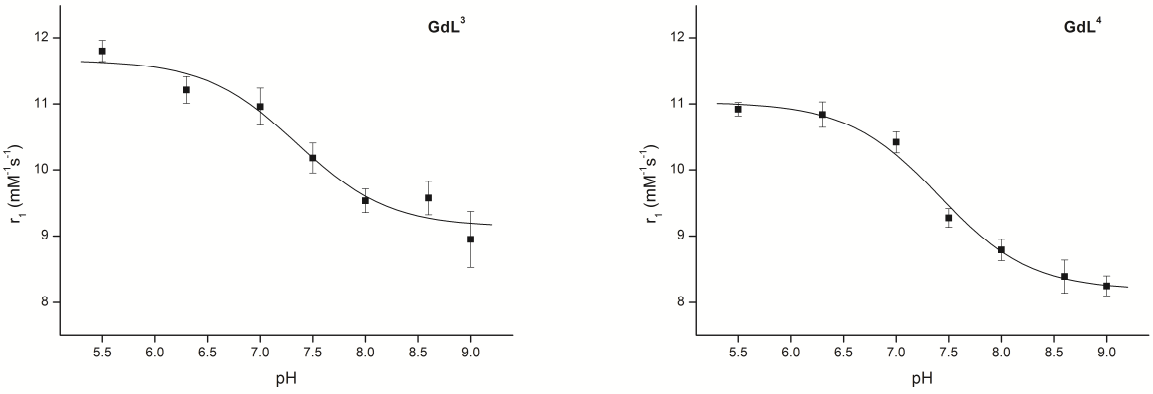

Figure S3. pH dependant r_1 response of $\mathbf{GdL^3}$ (left) and $\mathbf{GdL^4}$ (right) in the absence of avidin (3T MRI scanner, 21 °C). Values are presented as mean ± SEM of five independent experiments. The lines represent result of the sigmoidal fit and are displayed to aid a better visualization of the pH dependent r_1 decrease.

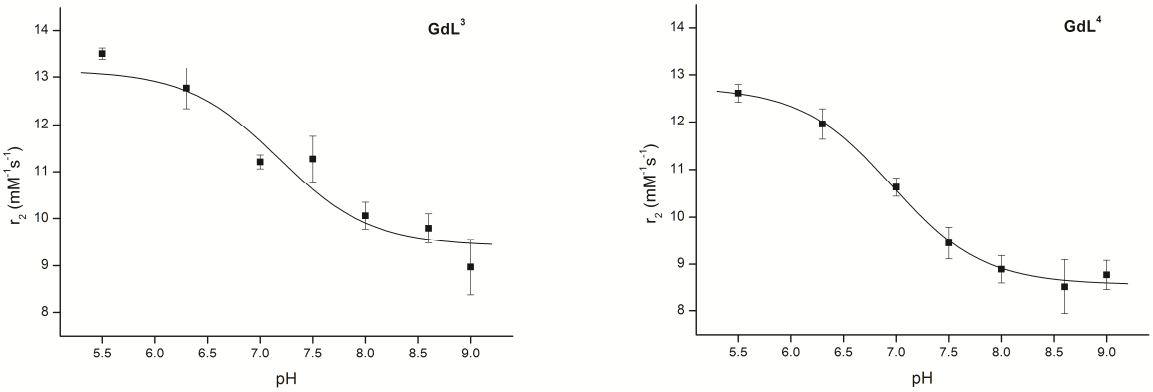

Figure S4. pH dependant r_2 response of $\mathbf{GdL^3}$ (left) and $\mathbf{GdL^4}$ (right) in the absence of avidin (3T MRI scanner, 21 °C). Values are presented as mean ± SEM of five independent experiments. The lines represent result of the sigmoidal fit and are displayed to aid a better visualization of the pH dependent r_2 decrease.

MRI phantom experiments/E-titrations

The curves obtained from the MRI E-titrations were fitted based on the previously published formula,[4] however they were slightly modified to use relaxivity values instead of relaxation rates (Eq. 7).

$$r_{1,2}^{obs} = 1000 \times \left\{ \left(L_0 \times r_{1,2}^{f}\right) + 0.5 \times \left(r_{1,2}^{b} - r_{1,2}^{f}\right) \times \left((n \times c_{Av}) + L_0 + {K_a}^{-1} - \sqrt{\left((n \times c_{Av}) + L_0 + {K_a}^{-1}\right)^2 - 4 \times n \times L_0 \times c_{Av}} \right) \right\} \quad (7)$$

where:

$r_{1,2}^{obs}$: observed longitudinal or transversal relaxivity

L_0: concentration of SCA in M (set to 0.001 M since relaxivities are used)

$r_{1,2}^{f}$: longitudinal or transversal relaxivity of the free SCA

$r_{1,2}^{b}$: longitudinal or transversal relaxivity of the avidin bound SCA

n: number of binding sites on the avidin tetramer (set to n=3 as obtained from fluorescence displacement assay)

c_{Av}: normalized concentration of avidin in M

K_a: binding constant of respective SCA (set to 7.1×10^{13} and 8.0×10^{13} for **GdL^3** and **GdL^4**, respectively, as obtained from the fluorescence displacement assay).

References:

1. D. M. Mock, G. Langford, D. Dubois, N. Criscimagna and P. Horowitz, *Anal. Biochem.*1985, **151**, 178-181.

2. B. Valeur, *Molecular Fluorescence: principles and applications*, Wiley-VCH, Weinheim; Chichester, 2002.

3. D. M. Mock, G. Lankford and P. Horowitz, *Biochim. Biophys. Acta* 1988, **956**, 23-29.

4. T. N. Parac-Vogt, K. Kimpe, S. Laurent, L. Vander Elst, C. Burtea, F. Chen, R. N. Muller, Y. C. Ni, A. Verbruggen and K. Binnemans, *Chem. Eur. J.* 2005, **11**, 3077-3086.