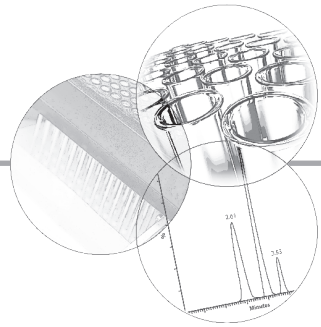

Tetracycline sensing using novel doxycycline derivatives immobilized on different surface plasmon resonance biosensor surfaces

Background: This article aims to explore novel doxycycline derivatives for analyzing low concentrations of tetracyclines in biological matrices and food in competitive assays. **Results:** Surface plasmon resonance (SPR) was employed in an indirect competitive format using a bacterial tetracycline-dependent regulatory protein as receptor. Three doxycycline derivatives were synthesized and covalently bound to the surface of four different sensor chips. Parameters that influence the immobilization of the doxycycline derivatives and subsequent binding of the receptor protein were studied. **Conclusion:** The novel doxycycline derivatives were successfully used as competitors in an indirect SPR assay.

Surface plasmon resonance (SPR) is a powerful tool for the analysis of biological fluids or tissues [1] and has also recently gained importance in detecting residues and contaminants in food due to its easy sample preparation, automation and speed of analysis [2]. For analytes of low molecular weight (e.g., tetracycline antibiotics), a competitive format is typically chosen with an analogous compound immobilized onto the sensor surface and a high-molecular weight recognition element to elicit a sensitive response even at low analyte concentrations. This recognition element is most often an antibody. Receptor proteins offer the advantage of reproducible preparation through genetic engineering and group-specific recognition. For this study, a bacterial tetracycline-dependent regulatory protein (TetR) that binds tetracyclines specifically with high affinity (Ka ~ 10^9 M^{-1}) and better crossreactivity compared with commercially available antibodies was used [3]. The challenge was to synthesize a suitable tetracycline derivative that can be recognized by TetR after being covalently immobilized to a sensor chip. The doxycycline scaffold provides excellent recognition by TetR and substantially higher chemical stability than tetracycline, because dehydrogenation resulting in the formation of anhydrotetracycline can be excluded. Three doxycycline derivatives with different spacer length and four SPR sensor chip surfaces were evaluated. The analytical procedure was as follows: the receptor protein and tetracycline-containing standard or sample solution were mixed and then injected into the flow cell that holds the immobilized doxycycline derivatives. Tetracyclines present in solution are bound to the tetracycline binding sites at TetR, so the binding sites are 'loaded' and TetR thus cannot bind to the immobilized doxycycline derivatives. If no tetracyclines are present in solution, TetR-binding sites for tetracyclines remain free and TetR will bind to the immobilized doxycycline derivatives. This mechanism is displayed in Figure 1.

Experimental

■ Instrumentation & reagents

The Biacore Q sensor system, HBS-EP and HBS-P buffer (10-mmol l^{-1} HEPES, 150-mmol l^{-1} sodium chloride, 3-mmol l^{-1} EDTA (in the case of HBS-EP buffer) and 0.005 % (v/v) surfactant 20, pH 7.4), as well as the amine-coupling kit (containing ethanolamine HCl, pH 8.5 1 mol l^{-1}; 0.4-mol l^{-1} 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide [EDC], aqueous solution; 0.1-mol l^{-1} *N*-hydroxysuccinimide [NHS] in water) were from GE Healthcare (former Biacore International AB, Uppsala, Sweden)

For the running buffer, 5-mmol l^{-1} magnesium sulfate was added to the HBS-P-buffer, because magnesium ions are necessary for the binding of tetracycline to the receptor [4].

TetR was obtained as a recombinant protein and purified with cation-exchange chromatography and gel filtration [5].

Sensor chips CMDP, HC 1000 m and CMTEG were purchased from XanTec Bioanalytics GmbH, Münster, Germany, and carboxymethyl dextran sensor chips (CM5) were from GE Healthcare.

Ulrike Andree[1], Erika Mueller-Seitz[1], Igor Usai[2], Stefan Loeber[2], Peter Gmeiner[2], Cornelius Wimmer[3], Wolfgang Hillen[3] & Michael Petz[1†]

[†]Author for correspondence

[1]Department of Food Chemistry, Faculty of Mathematics and Natural Sciences, University Wuppertal, Gauss-Str. 20, 42119 Wuppertal, Germany

Tel.: +49 202 439 2783

Fax: +49 202 439 3785

E-mail: petz@uni-wuppertal.de

[2]Department Chemistry and Pharmacy, University Erlangen–Nuernberg, Schuhstrasse 19, 91052 Erlangen, Germany

[3]Institute of Microbiology, University Erlangen–Nuernberg, Staudtstr. 5, 91058 Erlangen, Germany

FUTURE SCIENCE part of fsg

SURFACE PLASMON RESONANCE

Biosensor technique for studying the mechanisms and kinetics of ligands binding to receptors and for label-free real-time analysis

TETRACYCLINES

Group of broad-spectrum antibiotics widely used in human and veterinary medicine

Tetracycline hydrochloride, doxycycline hyclate and sodium dodecyl sulfate (SDS), were purchased from Sigma, Steinheim, Germany. Magnesium sulfate heptahydrate, sodium acetate and sodium borate were from Merck, Darmstadt, Germany. Anhydrotetracycline, trifluoroacetic acid, dicyclohexyl carbodiimide, diisopropylethylamine (DIPEA), dichloromethane and *N,N*-dimethylformamide were purchased from Acros Organics, Geel, Belgium. Doxycycline was donated by Heumann Pharma, Nuernberg, Germany. All chemicals were of analytical grade.

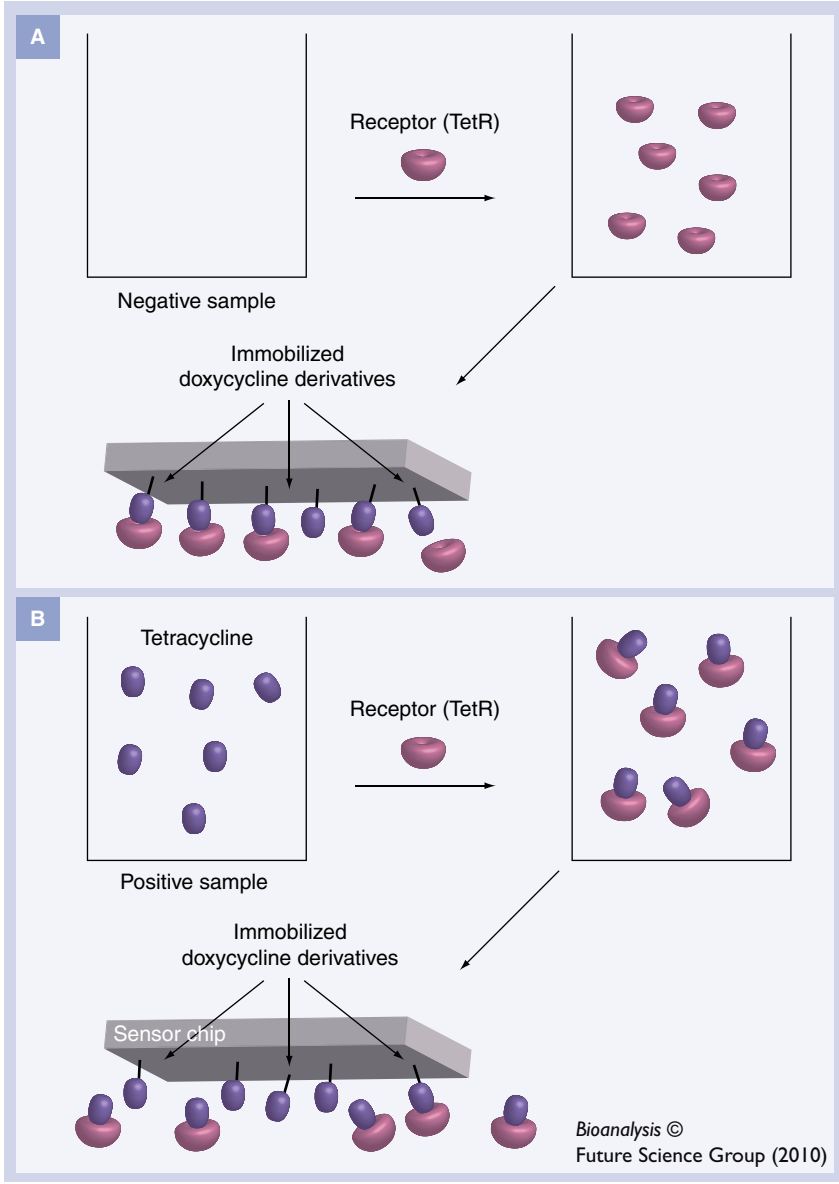

Figure 1. Indirect competitive format: (A) with no tetracyclines present in the sample, the binding sites at TetR are not occupied. Thus, TetR binds to the immobilized doxycycline derivatives at the sensor chip surface. **(B)** With positive samples the binding sites at TetR are loaded with tetracycline present in the sample. Thus, no or less TetR is bound to the immobilized derivatives.
TetR: Tetracycline-dependent regulatory protein.

■ Fluorescence measurement

All fluorescence measurements were performed using a SPEX Fluorolog® 3, Horiba Europe, Darmstadt. Measurements were carried out in a buffer containing 100-mM NaCl, 100-mM Tris-HCl (pH 8.0) and 25-mM $MgCl_2$. The temperature was set to 28°C. For characterization of the TetR(BD) doxycycline derivative interaction, the excitation wavelength was set to 295 nm and the emission was recorded at 337 nm. TetR(BD) was titrated with increasing amounts of each doxycycline derivative. The K_D value was calculated by a nonlinear regression fit.

■ Synthesis of doxycycline derivatives

The synthesis of the functionalized doxycycline derivatives started from 9-aminodoxycycline **1**, which is readily available by nitration and subsequent catalytic hydrogenation [6]. As depicted in **FIGURE 2**, the desired derivatives featuring an aliphatic primary amino group were synthesized by *N*-acylation with Boc-protected ω-amino acid anhydrides, which were obtained from the respective carboxylic acids upon treatment with 0.5 equivalents of dicyclohexylcarbodiimide **(SUPPLEMENTARY INFORMATION)**. The resulting amides **2A–C** were treated with trifluoroacetic acid to obtain the corresponding primary amines DOX-5, DOX-8 and DOX-11. The final products were purified by preparative reversed-phase HPLC and subsequent lyophilization.

■ Sensor chip preparation & biosensor conditions

In the Biacore Q system, four flow channels (flow cells) are located on each sensor chip. Each cell was operated independently with separate chip-surface preparation and immobilization of the derivative. Sensor chips were prepared according to a protocol from Fägerstam *et al.* [7]. The chip surface was activated by injecting 35 µl of a 1:1 mixture of EDC/NHS. Activation levels for all CM5 chips used were between 130 and 160 response unit (RU). For the immobilization of the doxycycline derivatives, 150-µl derivative solution (0.5-mg ml^{-1} derivative in HBS-EP buffer, except when stated otherwise) were injected. Injections of 15-µl ethanolamine were performed to deactivate remaining NHS esters. All steps were performed at a temperature of 25°C and a flow rate of 5 µl min^{-1}.

Tetracycline standard solutions were prepared freshly each day from a 1-mg ml^{-1} stock solution of tetracycline in running buffer (HBS-P-buffer + 5-mM $MgSO_4$). Tetracycline stock solutions were stored at 4–8 °C and discarded after 1 week.

Tetracycline-dependent regulatory protein was obtained from isolation and purification in a concentration of 0.78 mmol l^{-1} in Tris-buffer with glycerol (stock solution). The stock solution was stored at -18°C. A working solution of 1 µM was prepared freshly each day in running buffer. Unless explicitly stated, 25-µl working solution was injected for biosensor analysis at a flow rate of 5 µl/min.

For the competition experiments, TetR working solution was mixed with the tetracycline standard solution by the instrument prior to injection.

To test the surface regeneration, a protocol according to Andersson was used [8]. The following regeneration solutions were employed:

- Ionic: potassium thiocyanate (0.46 mol l^{-1}); magnesium chloride (1.83 mol l^{-1}), urea (0.92 mol l^{-1}), guanidinium hydrochloride (1.83 mol l^{-1}), solution prepared in purified water;
- Nonpolar: DMSO, formamide, ethanol, acetonitrile and n-butanol were mixed in equal amounts (v/v/v/v/v);
- Chelating: EDTA solution (20 mmol l^{-1});
- Detergent: CHAPS (3-[(3-chloramidopropyl)dimethylammonio]-1-propane-sulfonate) (0.3%, w/v), Zwittergent 3–12 (3-dodecyldimethylamino-propane-1-sulfonate) (0.3%, w/v), Tween 80 (0.3%, v/v), Tween 20 (0.3%, v/v), Triton X-00 (0.3%, v/v), solution prepared in purified water.

As a result of the regeneration scouting, a solution of 0.2% SDS (w/v) in running buffer was used; 5 µl were injected at a flow rate of 20 µl/min for surface regeneration.

To monitor the stability of the chip surface (baseline), the response was taken 10 s before the first injection of a measurement cycle. The response taken 60 s after injection and the baseline response were used to calculate the ΔRU value for the injected solution. Criteria to describe the immobilization process were activation level and immobilization level. The activation level is the difference in RU after and before injection of EDC/NHS; the immobilization level is given by the difference between the RU value after the injection of the doxycycline derivative and baseline response.

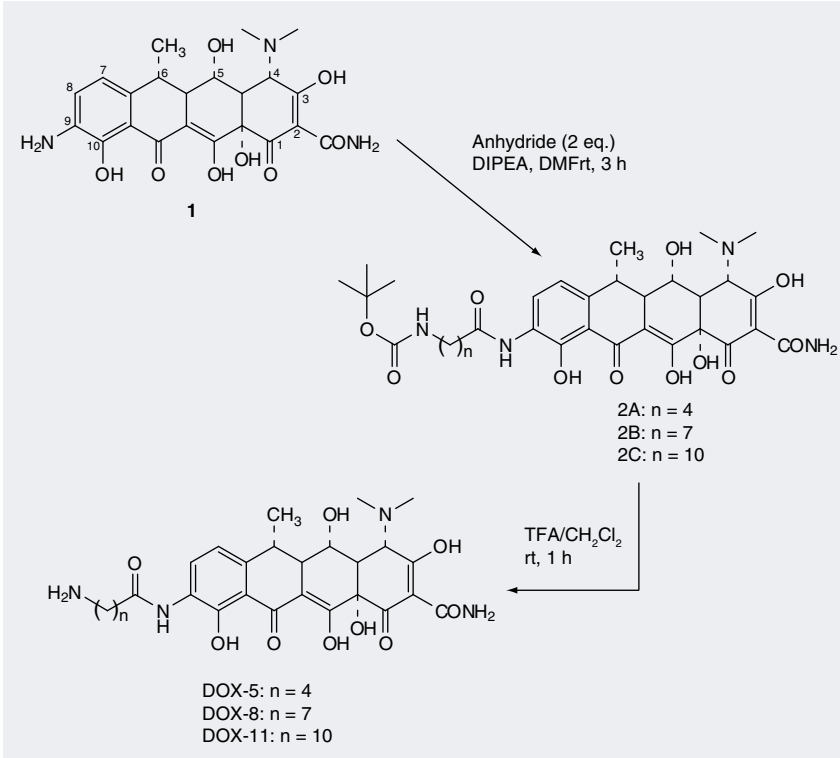

Figure 2. Synthesis of DOX-5, DOX-8 and DOX-11.

Results & discussion

■ Immobilization of doxycycline derivatives

For the indirect format, doxycycline derivatives had to be immobilized at the sensor chip surface. All sensor surfaces used had carboxyl groups allowing amine coupling of derivatives. The carboxyl groups on the surface were activated by derivatization with NHS, mediated by EDC. The resulting NHS esters react to form a covalent bond with the primary amines of the side chain (spacer) of the doxycycline derivative. After immobilization of the doxycycline derivative, remaining NHS esters on the chip surface were deactivated with ethanolamine.

Although tetracycline derivatives feature many functional groups, which could theoretically be utilized for the attachment of a linker unit, most of them are essential for the induction process. In particular, the A-ring functionalities and the 1,3-diketone unit strongly interact with

Spacer
Part of a molecule providing a connection of variable distance between two other parts of the molecule

SPR sensor chip
Consists of a gold-coated glass surface with a carboxymethylated dextran layer to attach biomolecules

Regeneration
Analytical step to remove bound analyte and matrix components from the sensor chip surface

ΔResponse unit
Difference between two absolute surface plasmon resonance responses

Table 1. Sensor chips used and their characteristics.

Chip type	Material	Thickness
CMDP (planar)	Carboxymethyl dextran, planar	<5 nm
CM5	Carboxymethyl dextran	100 nm
HC1000m	Polycarboxylate, linear hydrogel	1000 nm
CMTEG (planar)	Carboxymethylated tetraethylene glycol	<5 nm

Table 2. Activation and immobilization levels shown for different sensor chip surfaces exemplarily for DOX-11[*]. Binding of TetR[‡] was measured directly after immobilization.

	CMDP	CM5	HC1000m	CMTEG
Layer thickness (nm)	<5	100	1000	<5
Activation level EDC/NHS (ΔRU)	40	161	34	193
Immobilization level (ΔRU)	351	2362	7134	636
TetR-binding (ΔRU)	175	447[§]	672	576

[*]Injection of 150 µl of 0.5 mg ml^{-1} derivative in HBS-EP buffer pH 7.4.
[‡]1 µM, injection volume 40 µl except for CM5: 25 µl.
[§]Less injection volume: 25 µl TetR instead of 40 µl.
RU: Response unit; TetR: Tetracycline-dependent regulatory protein.

the TetR protein [4]. Coupling using these recognition elements would result in a decreased accessibility for the binding site of the receptor. Recently, we demonstrated that derivatizations at position 9 of doxycycline are possible under preservation of the inducing potency [9]. Thus, we intend to exploit this structural tolerance for the aspired linker attachment. Thereby, an alkyl spacer with a remote amine function for coupling should provide a sufficient distance to the chip surface to allow for the binding with the receptor protein TetR. Thus, three doxycycline derivatives with different spacer lengths can be synthesized **(Figure 2)**.

The amount of derivative immobilized depends on parameters such as chip surface, activation of the chip surface, length of the spacer, pH, concentration and contact time of the derivative solution with the surface. Control experiments did not show any binding of underivatized doxycycline to the surface.

Table 3. Immobilization level and subsequent TetR binding depending on pH of the immobilization solution, CM5 surface, derivative DOX-5[*].

pH	4.5	7.4	8.5
Buffer	Acetate buffer 10 mmol l^{-1}	HBS-EP buffer 10 mmol l^{-1}	Borate buffer 50 mmol l^{-1}
Immobilization level (ΔRU)	3064	674	515
TetR binding (ΔRU)	174	997	825

[*]1.79 mM, injection volume: 150 µl; TetR injection volume 40 µl for acetate and HBS-EP-Buffer, 80 µl for borate buffer.
RU: Response unit; TetR: Tetracycline-dependent regulatory protein.

Table 4. TetR binding depending on derivative concentration used for the immobilization[*].

	Flow channel	DOX-5 (mM)	Immobilization level (RU)	TetR binding (RU)
Chip 1	1	0.18	228	33
	2	0.18	221	17
	3	0.35	251	90
	4	0.35	264	187
Chip 2	1	0.89	365	959
	2	1.34	495	999
	3	1.79	674	997

[*]CM5 surface, DOX-5, TetR 1-µM injection volume 25 µl for chip 2, 40 µl for chip 1.
RU: Response unit; TetR: Tetracycline-dependent regulatory protein.

Sensor chip surfaces

The chips listed in **Table 1** have different properties as they were designed for different applications. Carboxymethyl dextran chips provide a hydrophilic environment that is favorable for biomolecular interactions. Straight polymer chains (carboxymethyl dextran and linear polycarboxylate) are highly flexible and allow a certain mobility of the bound molecules within the surface. A thick chip surface provides a high capacity for immobilization. Very thin surfaces allow only monolayer immobilization, while thicker layers allow 'multilayer' immobilization and, thus, enhancement of the signal.

Surface material as well as thickness of the layer will influence the system regarding activation of the surface and immobilization of the derivatives and, hence, subsequently affect the binding characteristics of the receptor protein. The immobilization level is expected to be high for thick surfaces due to their increased surface capacity.

Table 2 shows activation and immobilization levels for the four chip surfaces as well as TetR binding. Activation and immobilization were performed as described before and were not optimized for the different sensor chips or derivatives. It can be seen from **Table 2** that high activation levels will not necessarily result in high immobilization levels and that these do not correlate with subsequent TetR binding. The activation levels differed by a factor of nearly five and immobilization levels differed by a factor of up to 20.

With the applied format, the accessibility of the immobilized derivatives for the receptor is crucial and possibly not all of the immobilized derivatives are accessible for the receptor, especially at those chips with a high immobilization level. Furthermore, it is known that several water molecules are needed for the embedding of tetracyclines into the binding pocket of TetR [10]. Thus, increased hydrophobicity due to a longer alkane chain of the spacer may lead to decreased TetR binding. As the CM5 sensor surface gave good results for immobilization and TetR binding, this chip type was selected to study the influence of pH and derivative concentration as well as spacer length.

pH

pH affects the charge of the derivatives as well as the charge of the surface layer. For tetracyclines, the pH range is limited due to epimerization and isomerization of the molecule at acidic and alkaline pH. Epimers of tetracyclines at position 4 (dimethylamino function) show a 300-fold-reduced binding to TetR [3], so conditions must be avoided that favor epimerization. To test the effect of pH on the immobilization level, three buffers were employed to dissolve the doxycycline derivatives; the pH of the running buffer in the subsequent step was always 7.4.

Table 3 shows the influence of pH on the immobilization level. The highest amount of DOX-5 was immobilized at pH 4.5. However, this high immobilization level did not yield high TetR binding after injection of the receptor. The surfaces immobilized at pH 7.4 and 8.5 showed higher TetR binding despite the lower immobilization level. Possibly the five- to six-fold higher immobilization level obtained at pH 4.5 leads to a high density of derivative, so that there is sterical hindrance for the TetR to bind to the doxycycline derivatives. Hence, all further immobilizations were carried out at pH 7.4.

Derivative concentration

The concentration of the doxycycline derivative used for immobilization affected the immobilization level. Higher derivative concentrations resulted in higher immobilization levels **(Table 4)**. However, from 0.89 mM onwards, no gain in TetR binding was achieved through higher immobilization levels. Possibly, surplus amounts of derivative immobilized from concentrations above 0.89 mM were not accessible for the receptor.

■ Spacer length

Three doxycycline derivatives were used that differed only in the length of the alkane chain of the spacer with five, eight or 11 C-atoms. Accordingly, these derivatives differed in molecular weight, structure, hydrophobicity, dielectric constants and polarizability. The molecules' properties are crucial for two reasons: on the one hand, the spacer must ensure a sufficient distance between surface and doxycycline structure in order to provide accessibility for the receptor without sterical hindrance; on the other hand, the derivative must still match the binding site of TetR. Concerning the first, derivatives with longer spacers should provide better properties; the latter is discussed later.

Table 5. Influence of spacer length on immobilization level*.

Derivative	Molecular mass (g/mol)	Amount injected (nmol)	Immobilization level (ΔRU)
DOX-5	558.6	0.134	477
DOX-8	600.7	0.125	1050
DOX-11	642.7	0.117	2362

CM5 surface, injection of 150 µl of 0.5 mg l^{-1} derivative in HBS-EP buffer pH 7.4. For corresponding TetR binding on these flow cells see* **Table 6. *RU: Response unit.*

On all chips, the highest immobilization levels were obtained for DOX-11, followed by DOX-8 and DOX-5. The longer the spacer, the higher were the immobilization levels **(Table 5)**. The slightly different molar amounts used in this experiment are negligible compared with the effect of spacer length: immobilization level increases by a factor of approximately two from DOX-5 to DOX-8 and again from DOX-8 to DOX-11. The length of the spacers' alkane chain seems to have an influence on the reactivity of the amino function and, thus, lead to better immobilization of derivatives with higher spacer length.

Epimers

Tetracycline diastereomers differing in configuration of the stereogenic center at the C-4 position

■ TetR binding

Tetracycline-dependent regulatory protein has a high affinity to tetracyclines. The specific binding of TetR to immobilized doxycycline derivatives is an essential requirement for the principle of the analytical format **(Figure 1)**. Derivatization of doxycycline on the one hand and the fixation of the derivatives on the other hand can both lead to a decrease in affinity with TetR. Parameters influencing TetR binding are spacer length of the derivatives and concentration of TetR. Ideally, TetR binding should be specific, reproducible and stable for the time needed to analyze the samples. It should also be possible to completely regenerate the chip surface (removal of bound TetR) as a final step in each measurement cycle.

■ Specificity of TetR binding/nonspecific binding to chip surface

Tetracyclines bind to TetR only in the presence of divalent cations, (e.g., magnesium ions). In the absence of divalent cations the binding constant drops from 10^9 to approximately $10^5\ M^{-1}$ [11]. The amount of nonspecific binding of TetR to the chip surface can therefore be measured by injecting TetR in absence of magnesium ions. Specific and nonspecific binding were determined on all chip surfaces.

Figure 3 shows that almost no binding of TetR to the surface takes place in the absence of Mg^{2+} **(Figure 3A)** on a CM5 chip, as only a

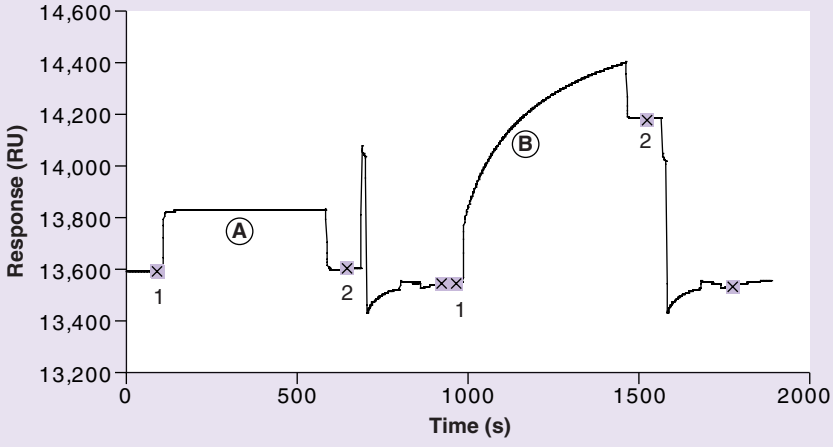

Figure 3. Injection of TetR in absence (A) and presence (B) of magnesium ions; TetR 1 µM in running buffer, injection volume 40 µl at 5 µl/min, exemplarily for DOX-8 on CM5 sensor chip: report points 1 and 2 for measuring ΔRU.
RU: Response unit; TetR: Tetracycline-dependent regulatory protein.

'bulk effect' (change of refractive index due to the TetR present in the injected solution) is detected at the beginning and the end of the injection. The resonance signal returns to the baseline reading when no more TetR is injected. In the presence of Mg^{2+} **(Figure 3B)**, a rising signal is observed in addition to the bulk effect. After the injection, the resonance signal drops by the amount of the bulk effect but does not return to the baseline reading due to the TetR bound to the surface. The difference between data points 2 and 1 **(Figure 3)** shows the amount of TetR binding (ΔRU). An SDS regeneration step after data point 2 was subsequent to every TetR injection.

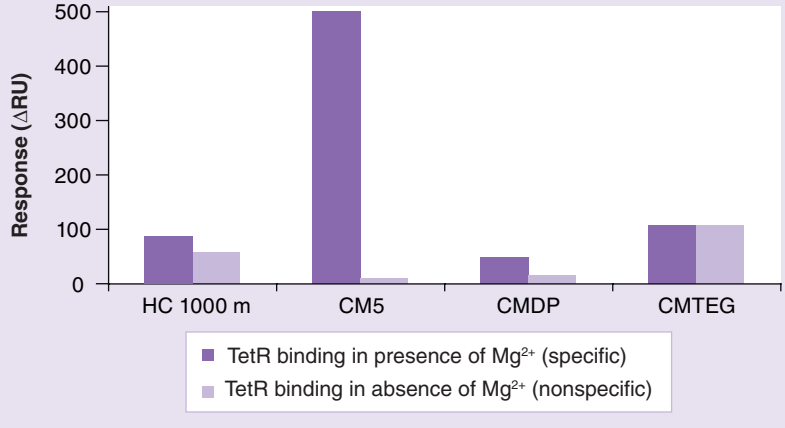

Figure 4. Binding of TetR in presence of Mg^{2+} (dark columns) and in the absence of Mg^{2+} (light columns) showing either specific or nonspecific binding to different chip surfaces, exemplarily for DOX-11. TetR was 25 µl except for CM5, where TetR 40 µl.
RU: Response unit; TetR: Tetracycline-dependent regulatory protein.

Nonspecific binding of TetR was determined for all chip surfaces. Results are shown in **Figure 4**. On the CMTEG chip, approximately equal amounts of TetR are bound in the absence or presence of Mg^{2+}. This does not comply with the rationale of the system and can be taken as an indication for massive nonspecific binding to the chip surface. This chip type cannot be employed in the development of a competitive assay, as specific and nonspecific binding cannot be distinguished. On the CMDP chip, the thinness of the surface layer might be responsible for nonspecific binding as the underlying intermediate layer or even the gold layer might influence the binding characteristics.

The high amount of nonspecific binding on the HC1000m surface may be caused by the thickness of the surface. A mass change at the surface occurs when TetR diffuses into the hydrogel; this mass change is detected regardless of whether TetR is bound to the derivatives or not. The thickness of the hydrogel might contribute to slow transport, with the effect that after the end of the injection, excess unbound TetR is not removed from the hydrogel and simulates TetR binding. Further elucidation of the nonspecific binding of TetR to the chip surface could have been obtained by injecting TetR to different chip surfaces before a derivative is immobilized. Almost no nonspecific binding of TetR to the CM5 surface was observed; this demonstrates the suitability of this chip to be used in a competitive assay format. If this format is applied to samples other than analyte in buffer, the nonspecific binding of matrix compounds must be taken into account in addition to the nonspecific binding of the receptor.

Stability of TetR binding at different chip surfaces

The complex of immobilized doxycycline derivative and TetR [Dox_{im}-TetR] is a result of **Reaction 1**. The complex dissociates according to **Reaction 2** whereby TetR is removed by running buffer:

$$Dox_{im} + TetR \rightarrow [Dox_{im} - TetR] \quad \text{(Fast)}$$

Reaction 1

$$[Dox - TetR] \rightarrow Dox_{im} + TetR \quad \text{(Slow)}$$

Reaction 2

Poor reproducibility of responses is expected when **Reaction 2** is too fast and irreproducible. Hence, the degree and rate of dissociation were determined. TetR was loaded to the sensor

surface and then the signal was monitored for 60 min. During this time, buffer was running through the flow cell with a flow rate of 5 µl/min, by which dissociated TetR was transported out of the flow cell. TetR binding is sufficiently stable on all surfaces. Looking at the degree of relative dissociation, the CM5 chip gave the best result **(FIGURE 5)**.

TetR concentration

Tetracycline concentrations in buffer or sample solutions will be measured indirectly via the amount of remaining free TetR, which is captured by the immobilized doxycycline derivative. Therefore, the dependence of TetR binding on TetR concentration was determined. On all surfaces, higher TetR concentrations led to higher signals and all showed a linear correlation for TetR concentrations ranging from 0.1 to 1.5 µM (results not shown).

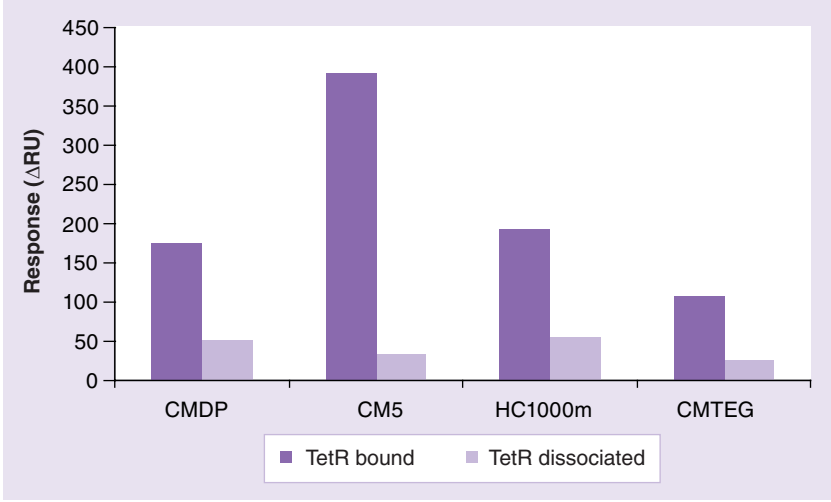

Figure 5. Dissociation of TetR within 60 min at buffer flow rate of 5 µl/min compared with initial TetR binding on different chip surfaces, exemplarily for DOX-11.
RU: Response unit; TetR: Tetracycline-dependent regulatory protein.

Influence of spacer length on TetR binding: derivatives in solution

Spacer length is a critical parameter not only for immobilization but also for TetR binding. The affinity of the doxycycline derivative relative to doxycycline was measured using an SPR assay format described by Moeller *et al.* [12]. In this assay, TetR is bound in a first step to a specific DNA sequence. Tetracyclines present in the test solution bind to TetR, evoke a conformational change and, as a result, TetR is released from the DNA and this mass change is detected.

The doxycycline derivatives, as free molecules dissolved in buffer, react as ligands with the receptor protein TetR. They induce the conformational change needed to detach the TetR from the DNA operator sequence *tetO*. At the concentration of 100 pmol, DOX-5 and DOX-8 react identically and display 53% of the activity observed for unsubstituted doxycycline. DOX-11, with the longest alkyl spacer chain, reacts with a lower activity, with only 33% of the activity of doxycycline **(FIGURE 6)**.

The derivatization of doxycycline decreases its ability to bind to TetR. This is probably due to different molecular structure and hydrophobicity, as well as different hydratization of the molecule. Spacer length has an adverse effect; DOX-11 does not fit to the TetR binding site as well as DOX-8 or DOX-5.

Fluorescence titration measurements were used to determine the binding constants of these doxycycline derivatives [13]. The Langmuir fits and Scatchard analyses showed a decreased ability of TetR to bind doxycycline derivatives with increased spacer length **(FIGURE 7)**. The best binding was achieved with DOX-5, which had a binding constant of $K_A = 7.9 \pm 0.5 \times 10^8\ M^{-1}$. Binding constants of DOX-8 and DOX-11 were $K_A = 3.45 \pm 1.08 \times 10^8\ M^{-1}$ and $K_A = 1.1 \pm 0.05 \times 10^8\ M^{-1}$, respectively.

SCATCHARD ANALYSIS
Method for calculating the affinity constant of a ligand with a protein

Influence of spacer length on TetR binding: immobilized derivatives.

In solution, spacer length of the doxycycline derivatives is negatively correlated with TetR binding. On the chip surface, however, a higher

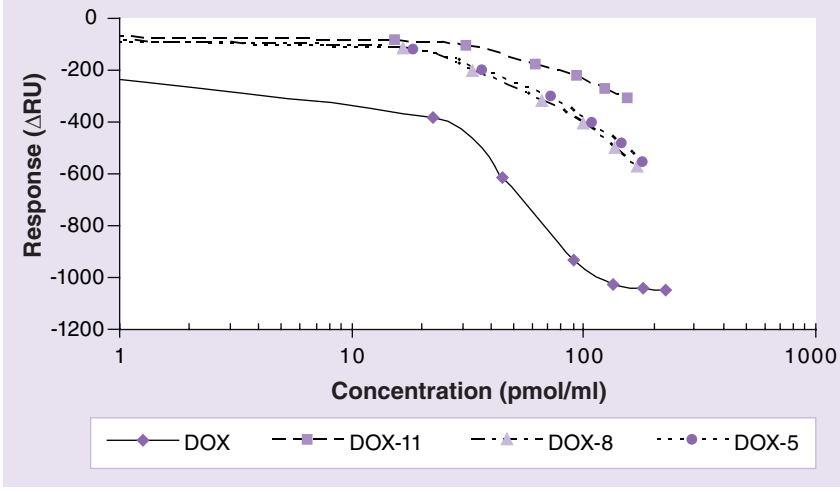

Figure 6. Release of TetR from the *tetO* DNA fragment caused by doxycycline derivatives compared with the release caused by doxycycline.
RU: Response unit; TetR: Tetracycline-dependent regulatory protein.

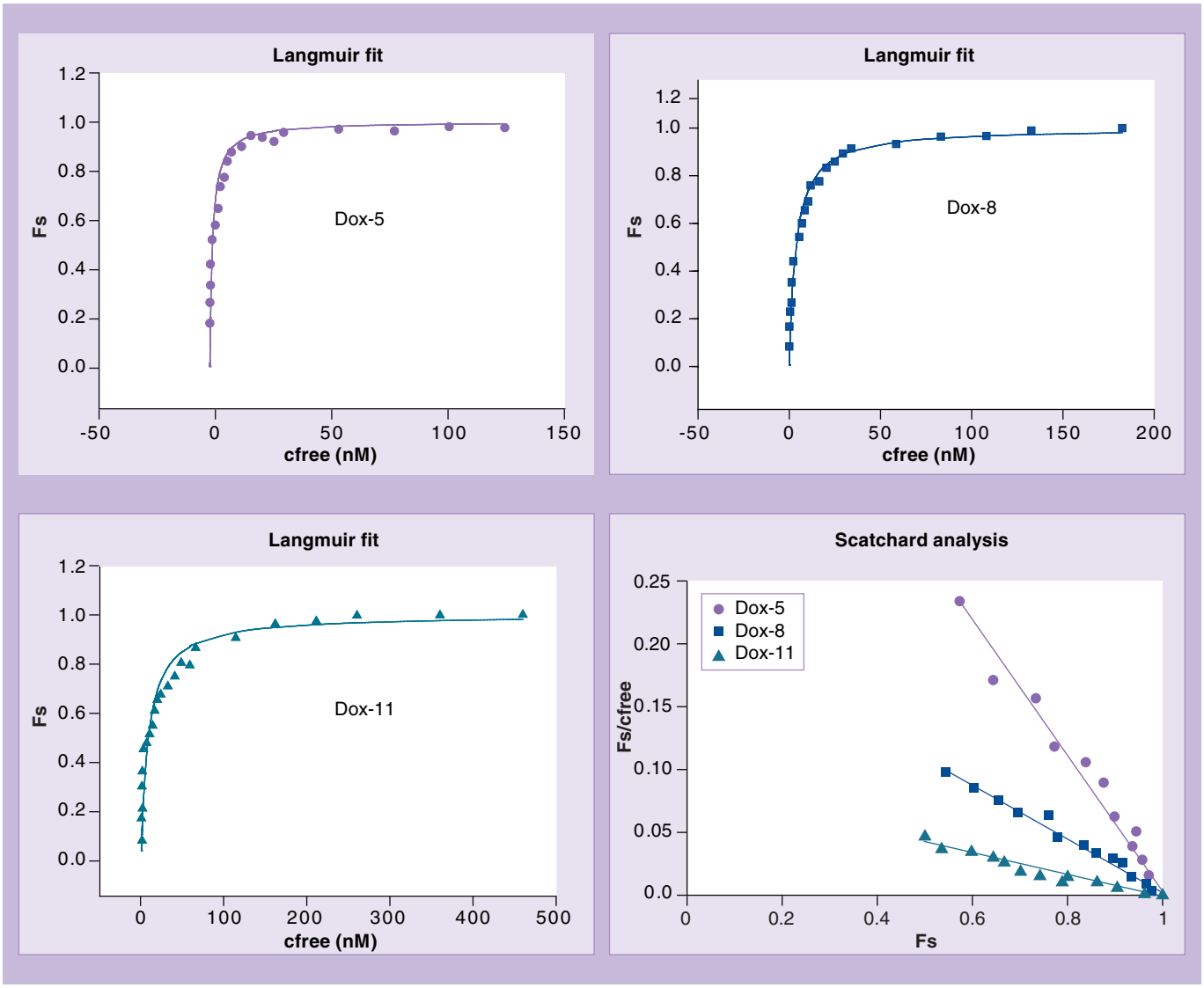

Figure 7. Langmuir fits and Scatchard analysis by fluorescence titration for the three doxycycline derivatives.
Fs: fractional saturation (percentage of TetR with bound doxycycline derivative); TetR: Tetracycline-dependent regulatory protein.

spacer length might provide a better flexibility and accessibility for TetR and, so, the influence of the derivatives fixed on the CM5 surface was studied.

Signal height after TetR injection decreased in the sequence DOX-8 → DOX-11 → DOX-5 at the chip surface **(Table 6)**. However, this is a result of both parameters, different immobilization levels for these derivatives and altered binding characteristics of the derivatives. DOX-8, immobilized at twice the level of DOX-5, also led to twice the amount of TetR binding. DOX-11, immobilized at a five-fold higher level, showed only 1.26-fold TetR binding. The longer spacer with 11 C-atoms obviously reduced the recognition in the ligand–receptor system, irrespectively, whether the doxycycline derivatives were freely dissolved or immobilized. The enhanced distance from the surface and the increased flexibility by a longer spacer did not improve the accessibility of the doxycycline structure on the CM5 sensor chip for the receptor molecule.

Table 6. TetR binding as influenced by spacer length*.

Derivative	Immobilization level (ΔRU)	TetR binding (ΔRU)	Ratio TetR/ immobilization level
DOX-5	477	356	0.75
DOX-8	1050	668	0.64
DOX-11	2362	447	0.19

**CM5-surface, TetR 1 µM, injection volume 25 µl.*

However, the higher spacer length of DOX-11 seemed to be an advantage when doxycycline derivatives were immobilized on the thin-layered CMDP chip. Despite the poorer recognition by the receptor, the ratio of TetR binding to immobilization did not differ significantly from that for DOX-8 and DOX-5. The interference of nonspecific TetR adsorption to the chip surface **(FIGURE 4)** renders this chip unsuitable for the development of an assay system. This holds true for the other two sensor chips, HC1000m and CMTEG.

■ Competition with tetracyclines on CM5 surface

All the parameters that influence TetR binding also influence the ability of the assay format to detect tetracyclines in a sample after incubation with added TetR, as they are indirectly determined by the amount of TetR, which remains for binding with the immobilized doxycycline derivatives. Competition on the different sensor chips was tested.

Tetracycline-dependent regulatory protein–tetracycline mixes were incubated for 3–12 min to find out if TetR binding was influenced by different incubation times. No differences were observed. It can therefore be concluded that the formation of the complex, according to **REACTION 1**, is very fast. This observation seems logical against the background that TetR is a regulatory protein in bacteria and that, in a resistant bacterial cell, tetracycline must be bound quickly to prevent it from reaching its original target, the ribosome.

FIGURE 8 shows that TetR binding is in negative correlation with the tetracycline concentration; when more tetracyclines in the solution, less TetR is available for binding to the surface.

The lines displayed for the different derivatives do not differ much in slope, indicating that neither of the derivatives used provide a higher sensitivity over the other; low TetR binding for the negative injection (0 ng/ml) in this experiment might be due to extensive use of the sensor chip and the stability issues discussed in 'surface regeneration and system stability'.

In addition to tetracycline, the structurally different tetracyclines doxycycline and anhydrotetracycline were tested as competitors on a CM5 surface. The magnesium complexes of various tetracyclines have different association equilibrium constants with TetR [3]. Anhydrotetracycline was thus expected to bind better to TetR than doxycycline and to doxycycline better than tetracycline. However, no significant difference was noticed for these three tetracyclines (data not shown).

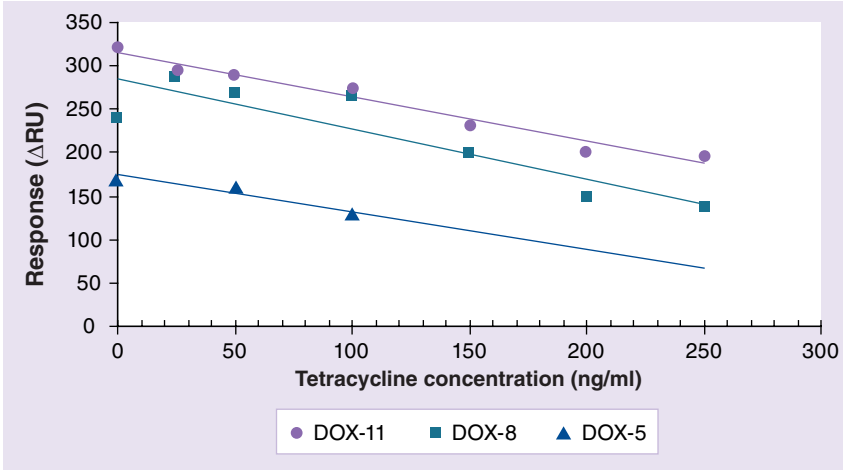

Figure 8. Response of TetR binding related to tetracycline concentration in buffer (CM5 surface, TetR 1 µM, injection volume 25 µl).
RU: Response unit; TetR: Tetracycline-dependent regulatory protein.

■ Surface regeneration & system stability

The sensor surface must be regenerated before the next injection cycle. The receptor protein and matrix components from samples of biological origin must be removed. If TetR is accumulated on the chip surface, less binding sites for TetR are available and fewer TetR can be bound in subsequent cycles, thus affecting the reproducibility and sensitivity of the system. Different regeneration reagents and mixtures were tested systematically using a protocol described by Andersson *et al.* on all surfaces [8]. As pH changes are critical for tetracycline stability, acidic and basic regeneration solutions were not tested, in order not to provoke epimerization. The main properties of the different solutions were:

- Ionic
- Nonpolar
- Chelating
- Detergent
- Water

Cocktails of these solutions were mixed and tested for their regeneration characteristics. SDS was tested in addition as it is not contained in the detergent solution mentioned above, but was successfully employed for regeneration in the tetO/TetR format [12]. On all surfaces, only

SDS (0.3% dissolved in running buffer) and ICw (solution containing KSCN, $MgCl_2$, urea, guanidine hydrochloride and EDTA) showed a certain capacity in removing TetR. All other solutions showed either no effect at all or even an additional binding of compounds to the surface. Regeneration with the ICw solution seemed to 'activate' the CM5 surface; runs regenerated with high ionic strength showed more TetR binding in later cycles but led to decreased reproducibility.

Throughout all measurements it was observed that the reproducibility of the signal in repeated injections was poor. TetR solutions and/or mixes of TetR and tetracycline solution always showed a lower TetR binding compared with the preceding injections. For all sensor surfaces except the CM5 surface, part of an explanation can be incomplete regeneration. On the CM5 sensor surface the phenomenon was observed, although regeneration was complete. However, the ability of the derivatives to bind TetR decreases quickly with the number of injections, thus displaying a certain instability of the system. Possibly, alterations of the hydrodynamic structure of the derivatives took place that led to a loss of binding capacity for TetR.

Conclusion

A major goal in this investigation was to select a suitable surface providing appropriate immobilization rates for the doxycycline derivatives as competitors in tetracycline sensing. A minimum of nonspecific binding and good regeneration characteristics were aimed for. In that regard, the carboxymethyl dextran chip CM5 with a 100-nm layer outperformed the other three chips with different surface materials and/or layer thickness.

The degree of immobilization of the derivative and the subsequent binding of the receptor protein TetR was highly dependent on the alkyl spacer length between the doxycycline structure and the amino function needed for coupling to the CM5 surface. A longer spacer led to a higher degree of immobilization and eventually more flexibility for the binding process. However, this was counteracted by a reduction of binding activity in the ligand–receptor system. Compared with doxycycline, the binding activity dropped to 53% for the derivatives with a five or eight C-atom spacer and to 33% with an 11 C-atom spacer, when analyzed in an assay system of the freely soluble molecules. These relations were likewise seen for the derivatives immobilized to the CM5 surface with comparable binding characteristics for the five and eight C-atom spacer derivative and a further reduction using the 11 C-atom derivative. When immobilized on the monolayer CMDP chip, the derivative with the 11 C-atom spacer did not display lower receptor binding. In this case, a presumed increased flexibility and distance from the surface might have counteracted the loss of binding activity for the doxycycline structure. The described indirect competitive format using immobilized doxycycline derivates as competitors was applicable for tetracycline detection, although was not as sensitive as that reported elsewhere [12].

Future perspective

The novel doxycycline derivatives employed in this study provide an option to establish bioassays for the detection and determination of tetracyclines in various assays applying indirect formats. In the next step, the poor reproducibility has to be addressed.

Acknowledgements

The authors wish to thank the Biacore section of GE Healthcare for providing the Biacore Q instrument and reagents and Terry McGrath (Xenosense, Belfast) for helpful discussions.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

We gratefully acknowledge the financial support by the German Research Foundation (DFG), project No. PE 306/7-2. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Supplementary information

Supplementary information accompanies this paper at www.future-science.com/loi/bio.

Executive summary

- Novel doxycycline derivatives were synthesized via functionalizing doxycycline at the C9 position.
- The terminal amino function of the spacer allowed covalent immobilization to carboxylated sensor chip surfaces for surface plasmon resonance analysis.
- These derivatives were recognized by the tetracycline-specific receptor protein TetR as solutes, as well as covalently attached on the sensor chip.
- Different alkane spacer length and different chip surfaces had large effects on derivative immobilization levels and TetR recognition.
- Proof of principle was provided for the applicability of these derivatives to analyze tetracyclines by bioassay in an indirect competitive format.
- The sensor chip regeneration step and reproducibility of the assay need further optimization.

Bibliography

1 Yu D, Blankert B, Viré J-C, Kauffmann J-M. Biosensors in drug discovery and drug analysis. *Anal. Lett.* 38(11), 1687–1701 (2005).

2 Petz M. Recent applications of surface plasmon resonance biosensors for analyzing residues and contaminants in food. *Monatsh. Chem.* 140(8), 953–964 (2009).

3 Lederer T, Kintrup M, Takahashi M, Sum PE, Ellestad GA, Hillen W. Tetracycline analogs affecting binding to Tn10-encoded Tet repressor trigger the same mechanism of induction. *Biochemistry* 35(23), 7439–7446 (1996).

4 Saenger W, Orth P, Kisker C, Hillen W, Hinrichs W. The tetracycline repressor – a paradigm for a biological switch. *Angew. Chem. Int. Ed. Engl.* 39(12), 2042–2052 (2000).

5 Ettner N, Müller G, Berens C *et al.* Fast large-scale purification of tetracycline repressor variants from overproducing Escherichia coli strains. *J. Chromatogr. A* 742(1–2), 95–105 (1996).

6 Beereboom JJ, Ursprung JJ, Rennhard HH, Stephens CR. Further 6-deoxytetracycline studies: effect of aromatic substituents on biological activity. *J. Am. Chem. Soc.* 82(4), 1003–1004 (1960).

7 Fägerstam LG, Frostell-Karlsson A, Karlsson R, Persson B, Rönnberg I. Biospecific interaction analysis using surface plasmon resonance detection applied to kinetic, binding site and concentration analysis. *J. Chromatogr.* 597(1–2), 397–410 (1992).

8 Andersson K, Hamalainen M, Malmqvist M. Identification and optimization of regeneration conditions for affinity-based biosensor assays. A multivariate cocktail approach. *Anal. Chem.* 71(13), 2475–2481 (1999).

9 Berens C, Lochner S, Löber S *et al.* Subtype selective tetracycline agonists and their application for a two-stage regulatory system. *ChemBioChem.* 7(9), 1320–1324 (2006).

10 Orth P, Cordes F, Schnappinger D, Hillen W, Saenger W, Hinrichs W. Conformational changes of the Tet repressor induced by tetracycline trapping. *J. Mol. Biol.* 279(2), 439–447 (1998).

11 Takahashi M, Altschmied L, Hillen W. Kinetic and equilibrium characterization of the Tet repressor-tetracycline complex by fluorescence measurements. Evidence for divalent metal ion requirement and energy transfer. *J. Mol. Biol.* 187(3), 341–348 (1986).

12 Moeller N, Müller-Seitz E, Scholz O, Hillen W, Bergwerff AA, Petz M. A new strategy for the analysis of tetracycline residues in foodstuffs by a surface plasmon resonance biosensor. *Eur. Food Res. Technol.* 224(3), 285–292 (2007).

13 Scholz O, Schubert P, Kintrup M, Hillen W. Tet repressor induction without Mg^{2+}. *Biochemistry* 39(35), 10914–10920 (2000).