

DSM Science & Technology Awards 2004

Name	Carolin Peter
University	Westphalian Wilhelms University, Münster (D)
Department	Institute for Inorganic and Analytical Chemistry
PhD Supervisor	Prof. Dr. K. Cammann

Evanescent field DNA biosensor for the rapid, time-resolved detection of multiple hybridization events – application for animal species differentiation in food and for identification of microorganisms

(candidate: Carolin Peter)

Introduction

Specific hybridization between a nucleic acid target sequence and complementary nucleic acid primer or probe sequences [1] is the basic principle of nearly all DNA based analysis methods which have come into widespread use in the last decade. Generally, an amplification step, e.g. by the polymerase chain reaction (PCR), is indispensable to compensate for the low concentration of a given DNA sequence under study. Application of those methods promising highly sensitive and specific results ranges from human and veterinary medicine to forensic science, food quality control and environmental monitoring. In recent years, DNA biosensors have been shown to be well suited for the rapid analysis of nucleic acid samples, combining the sequence-specific hybridization with a quantitative detection of DNA targets. By definition, a biosensor couples the specificity of a biological detection reaction with a physical transducer that generates an electronic signal proportional to the concentration of the analyte. DNA biosensors exploit the hybridization of target DNA in a sample solution with complementary DNA probes immobilized on the solid surface of the transducer. A variety of transducers such as optical [2,3,4], electrochemical [5,6,7] and piezoacoustic [8,9] ones has been applied for the detection of nucleic acids. However, most of the biosensor research published so far dealt with the detection of single, short target oligonucleotides, and only recently some reports address the analysis of real samples [10,11,12]. Additionally, only very few manuscripts describe the parallel detection of more than one target sequence [2,13,14].

Summary of the PhD thesis

In this work, a DNA biosensor system was used which was based on a general patent [15] of the Institute for Chemical and Biochemical Sensor Research (ICB GmbH, Münster) for ligand receptor analysis using evanescent field detection. This was further developed (partly in frame of this thesis) into a platform for the rapid, time-resolved detection of multiple hybridization events. For the first time, the application of the sensor system for different questions in DNA analysis of real samples was established and a sensitive and reliable PCR-based method for animal species differentiation in food was developed to demonstrate the usefulness of the sensor system in routine controls [16].

In first experiments the sensor system was optimized and characterized in detail concerning fundamental aspects by analysis of interactions between model oligonucleotides [17]. For

this purpose hybridization of fluorophor-labelled model oligonucleotide targets with complementary probe sequences covalently attached to the sensitive glass surface of the sensor chip was investigated. Since different probe sequences were immobilized in arrays of distinct spots using a piezoelectric nano-dispenser, the spatially resolved detection of the fluorescent light with a CCD camera enabled simultaneous detection of multiple target sequences. With a flow rate of at least 1 $\mu\text{l/s}$ for the target solution and a probe concentration of 5 μM to ensure sufficient excess of binding sites, a linear increase of the fluorescent signal in time was always observed for the initial phase of hybridization. Based on alkaline denaturation of the formed hybrids the binding of all target molecules was perfectly reversible resulting in an excellent stability of the sensor signal in successive measurements with one sensor chip. E.g., in 20 measurements a drift of $\leq -0.5\%$ per measurement and a coefficient of variation of only 5% were observed. From theoretical considerations it can be inferred that the slope of the fluorescence signal during the initial phase of hybridization is directly proportional to the target concentration. Thus, by using this slope as sensor signal hybridization in the DNA biosensor chip was in principle quantitative. E.g., for a single-stranded, Cy5-labelled 46mer oligonucleotide DNA target the calibration curve revealed a linear range of nearly two decades with a sensitivity of 12.8 counts/(s \cdot nM) and a lower limit of detection of 13 pM.

By variation of diverse parameters of hybridization it was demonstrated that intensity and selectivity of the interaction between probe and target were influenced by temperature and hybridization buffer as well as by probe length and the number of mismatches in the basepairing sequence. Thus, the hybridization to an 18mer probe in the DNA biosensor chip was almost completely suppressed by a single mismatch in the centre of the probe sequence.

Since due to an optimized geometry of the flow channel mass transport in the sensor chip was nearly not diffusion-limited, the interaction between target and probe was mainly kinetically controlled. Therefore, the DNA biosensor system was suited not only for the analysis of the initial binding phase of hybridization, even kinetic studies observing the succession of association, equilibrium and dissociation were possible. Determination of rate constants k_{on} for the association and k_{off} for the dissociation and also values obtained for the equilibrium dissociation constant K_D showed that both, mismatches and the length of the basepairing sequence affected all three phases of the binding reaction. Kinetic data obtained for the interaction between different target and probe sequences in this work were in the same order of magnitude as values from literature generated with other DNA biosensor systems which, however, could identify mismatches only in the dissociation phase. Thus, one main advantage of the DNA biosensor chip was the possibility to detect mismatches already during the association phase, i.e. the earliest possible time point.

After the studies on fundamental aspects of the hybridization were completed, it was demonstrated that the sensor system is also suited for the hybridization of fluorophor-labelled PCR products. The fluorophor was usually incorporated by means of a Cy5-labelled primer. As expected, double-stranded PCR products had to be denatured before the measurement to achieve the optimum in hybridization efficiency. Nevertheless, the basic characteristics of the hybridization in the DNA biosensor chip, as e.g. the linear signal increase in time, the sensor stability and the linearity of calibration, were also valid for the PCR products. Hence the DNA biosensor system is a powerful tool for the rapid screening of PCR products obtained in most kinds of genetic analysis.

As a first step towards a new method for animal species differentiation in food a consensus PCR system for the amplification of a DNA fragment from the mitochondrial cytochrome *b* (*cytb*) gene was established focusing on the six most important farm animals (cattle, pig, chicken, turkey, sheep and goat) processed in meat and cheese. Universal primers *cytb*403 and Cy5*cytb*779 (numbers according to their position within the *cytb* gene) were designed to bind to highly conserved regions of the *cytb* gene, yielding a 377 bp PCR product with extensive internal regions of inter-species variations. In the food diagnostic routine, consensus PCR products from different species are usually visualized by gelelectrophoresis and differentiated by subsequent restriction fragment length polymorphism (RFLP) analysis, which is, however, laborious, time-consuming and especially in case of mixed samples not always unambiguous. In contrast, PCR products were rapidly and reliably detected in this work by hybridization to species-specific probe sequences in the DNA biosensor chip. Sufficient specificity of hybridization was ensured by choosing 20mer probe sequences from those regions within the PCR products with high inter-species variations. The analysis of PCR products obtained from reference DNA for the six different species revealed only for probe goat684 unexpected cross-hybridization with the 377 bp pig PCR product although *cytb* genes of both species differed by 9 mismatches in the probe binding region. Nevertheless, this cross-hybridization is less relevant since raw material from pig is only processed into meat products whereas from goat only the milk is used for the production of dairy products so that both species are normally not expected to be present in the same real sample. A first attempt to design an alternative goat-specific probe was complicated by the closely related *cytb* sequences from sheep, goat and cattle and the low GC contents in the few regions with sufficient sequence diversity.

By real-time hybridization analysis of the 377 bp *cytb* PCR products in the DNA biosensor chip it was discovered that the standard consensus PCR leads to an excess of the Cy5-labelled antisense strand (although equal amounts of both primers were used). Obviously, the course of the PCR became asymmetric in the last few cycles, probably caused by different binding efficiencies of both primers at the chosen annealing temperature of 62°C.

This peculiarity was studied in some detail in order to exploit the asymmetric ratio of PCR product strands for analysis without the preceding denaturation step thereby further reducing the analysis time. Moreover, higher signal intensities were obtained since hybridization between antisense strand and the probe sequence was not impaired by any re-annealing of the complementary sense strand as in case of double-stranded PCR products.

Although incorporation of the fluorophor via a Cy5-labelled primer proved to be excellently suited for a cost-effective and simple application in routine PCR and allowed even the hybridization of non-purified PCR products, labelling of PCR products with Cy5-dUTP nucleotides was investigated as an alternative method frequently reported in the literature. With this, label degrees above 1 and concomitantly higher signal intensities were achieved. However, this method was much more expensive and therefore not suitable for the envisaged routine applications. It would, however, be useful for optimization of primer sequences by means of unlabelled, i.e. low-priced primers.

After the hybridization of pure PCR products from single species mixed samples were analyzed to allow a systematic evaluation of this new method for species differentiation in food, including an estimation of the lower limits of detection in real samples. Amplicons of different species obtained from reference DNA samples could be detected simultaneously without any interference. Moreover, due to the high sensitivity of the PCR admixtures of 1 % were easily detected both, in mixtures of template DNA (i.e. DNA mixed prior to the PCR) and on the level of raw meat (i.e. meat mixed prior to DNA extraction). As expected, the results also demonstrated that the PCR-based sensor chip analysis, owing to the exponential amplification step involved, allowed only for a semi-quantitative detection of the different species. For the same reason admixtures in low concentrations (e.g. 1 %) were preferentially amplified. Even admixtures of 0.1 % of chicken in beef and of beef in pork, respectively, were still detected above the threshold, taking advantage of the broad dynamic range of at least two decades and of the possibility to adjust the sensitivity of the detection by the flexible choice of the analyzed PCR product volume. The limit of detection in both meat mixtures for the minor components of clearly below 1% matches the required sensitivity for the application in routine controls of food samples. Positive results with minor contents of a particular species, which are often obtained with species specific PCR systems, are not desirable.

Finally, numerous commercial meat and cheese samples from the routine control of the relevant public authority (Federal Chemical and National Veterinary Office, Münster, Germany) were analyzed. Regardless whether the samples were processed or contained single or multiple species, the results from reference methods such as enzyme-linked immunosorbent assay (ELISA), species-specific PCR and PCR-RFLP were always definitely confirmed by using the DNA biosensor system. In contrast to those the sensor chip analysis

of PCR products is at least semi-quantitative and, moreover, immediately provides full information about the species present in a sample. Hitherto different methods or conditions had to be applied for each species of interest. Additionally, it is much faster than PCR-RFLP or species-specific PCR since results can be obtained within approximately 15 min after PCR and results of the hybridization method are easier to interpret as those from PCR-RFLP especially for samples with three or more different species.

Apart from real-time hybridization in the DNA biosensor chip the 377 bp *cytb* PCR products for species identification have also been analyzed by conventional state of the art microarray hybridization [18]. Indeed, this method showed various disadvantages compared to the real-time hybridization, e.g. concerning the time required for analysis, the statistical precision of results or the consumption of glass slides with immobilized probe arrays (for the conventional chip analysis each array can only be used once). In addition, real-time hybridization suffered less from unspecific binding due to the reduced period of interaction between targets and probes. However, both methods showed similar sensitivities because 0.1 % admixtures in meat samples were still detectable in each case and after considering the characteristic cross-hybridizations for each species also the conventional microarray hybridization method gave the same results as the reference methods for nearly all samples. The rationale for studying also conventional microarray hybridization of the 377 bp *cytb* PCR products was that it could be implemented in laboratories equipped with a commercial microarray scanner, whereas the DNA biosensor system is yet a prototype.

Finally, the analytical potential of the real-time hybridization in the DNA biosensor chip was also demonstrated for the detection of microorganisms. As a model system a set of 132 oligonucleotide probe sequences for the detection of all known sulphate reducing prokaryotes (SRPs) had previously been developed in the group of Prof. Dr. K. Schleifer (Chair for Microbiology, TU München) for the conventional hybridization analysis on the so-called SRP-PhyloChip [19]. As part of a co-operative study fundamental experiments for the transfer of the SRP detection to the DNA biosensor chip were carried out in this work by detecting the three species *Desulfovibrio halophilus*, *Desulfomicrobium aspheronum* and *Desulfohalobium retbaense* with dedicated 18mer probe sequences.

Due to the low inter-species diversity of the 16S rRNA gene, which was again amplified by consensus PCR, even very closely related sequences with just a few mismatches had to be differentiated for an unequivocal detection of the three species. Actually, a definite discrimination between perfect-match and one- or two-mismatch pairings was possible during the association phase of hybridization in most cases. Only a G-T-mismatch did not affect the binding efficiency between probe and target sequence enough to enable clear mismatch discrimination. This phenomenon has been attributed to the presence of two stable hydrogen bonds which still can be formed by a G-T-mismatch. In conclusion, the results

demonstrated that for the design of specific probe sequences not only the length of the basepairing sequence and the number of mismatches, but also position and nature of the mismatches must be taken into consideration.

During the application of the DNA biosensor system for the detection of SRPs a special advantage of the integrated fluidics could be utilized for a fine tuning of the stringency yielding an optimal specificity for each probe sequence. Whereas in the conventional hybridization on the SRP-PhyloChip only mono-stringent conditions were possible, i.e. each variation of a stringency parameter required a separate DNA chip [19], the DNA biosensor chip enabled dynamic studies. This could be achieved by a systematic increase of stringency (via the formamide concentration of the washing buffer) during the dissociation phase. Thus, on the one hand now even the discrimination of the G-T-mismatch was possible and, even more important for the development of medium density chips, it was taken into account that the conditions of optimal stringency for the numerous SRP probe sequences differed widely in some cases. When designing a probe set this problem inevitably occurs since hybridization efficiencies are usually not yet predictable from the probe sequence. Therefore the real-time hybridization analysis of PCR products with the DNA biosensor chip offers a promising opportunity for the rapid and unambiguous identification of closely related target sequences even on a complex probe array.

References

- 1 Kricka LJ (1999) *Clin Chem* 45: 453-458
- 2 Peter C, Meusel M, Grawe F, Katerkamp A, Cammann K, Börchers T (2001) *Fresenius J Anal Chem* 371: 120-127
- 3 Watts H, Yeung D, Parkes H (1995) *Anal Chem* 67: 4283-4289
- 4 Nelson BP, Grimsrud TE, Liles MR, Goodman RM, Corn RM (2001) *Anal Chem* 73: 1-7
- 5 Mascini M, Palchetti I, Marrazza G (2001) *Fresenius J Anal Chem* 369: 15-22
- 6 Ozkan D, Erdem A, Kara P, Kerman K, Meric B, Hassmann J, Ozsoz M (2002) *Anal Chem* 74: 5931-5936
- 7 Ju HX, Ye YK, Zhao JH, Zhu YL (2003) *Anal Biochem* 313: 255-261
- 8 Wang J, Nielsen PE, Jiang M, Cai X, Fernandes JR, Grant DH, Ozsoz M, Beglieter A, Mowat M (1997) *Anal Chem* 69: 5200-5202
- 9 Mannelli I, Minunni M, Tombelli S, Mascini M (2003) *Biosens Bioelectron* 18: 129-140
- 10 Bianchi N, Rutigliano C, Tomassetti M, Feriotto G, Zorzato F, Gambari R (1997) *Clin Diagn Virol* 8: 199-208
- 11 Kai E, Sawata S, Ikebukuro K, Iida T, Honda T, Karube I (1999) *Anal Chem* 71: 796-800
- 12 Feriotto G, Borgatti M, Mischiati C, Bianchi N, Gambari R (2002) *J Agric Food Chem* 50: 955-962
- 13 Schuderer J, Akkoyun A, Brandenburg A, Bilitewski U, Wagner E (2000) *Anal Chem* 72: 3942-3948
- 14 Healey BG, Matson RS, Walt DR (1997) *Anal Biochem* 251: 270-279

- 15 Meusel M, Trau D, Katerkamp A, German patent DE 19711281
- 16 Peter C, Brünen-Nieweler C, Cammann K, Borchers T, Real-time DNA biosensor chip for the rapid animal species identification and differentiation in food. Manuscript submitted for publication in J Agric Food Chem.
- 17 Peter C, Wienhausen F, Meusel M, Cammann K, Borchers T, Evanescent Field DNA biosensor for real-time detection of multiple hybridization events. Manuscript submitted for publication in Anal Bioanal Chem.
- 18 Peter C, Brünen-Nieweler C, Cammann K, Borchers T, Differentiation of animal species in food by oligonucleotide microarray hybridization. Manuscript submitted for publication in Eur Food Res Technol.
- 19 Loy A, Lehner A, Lee N, Adamcyk J, Meier H, Ernst J, Schleifer KH, Wagner M (2002) Appl Environ Microbiol 68: 5064-5081