Biochemical Detection of cGMP From Past to Present: An Overview

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Contents

Abstract Cyclic guanosine monophosphate (cGMP), generated via the guanylate cyclase (GC)-catalyzed conversion from GTP, is unequivocally recognized as crucial second messenger, intimately involved in the regulation of a broad range of physiological processes such as long term potentiation, blood pressure regulation, or platelet aggregation (for review: Hobbs 2000). Since its first identification in rat urine by Ashman and co-workers (1963), various approaches have been conceived and established to quantify cGMP in biological samples, or to detect cGMP as the reaction product of enzymatic assays, allowing the determination of kinetic parameters. These approaches have evolved from laborious handling of small numbers of samples with average sensitivity to highly developed biochemical detection assays allowing the processing of very large numbers of samples. The present article focuses upon the history of biochemical cGMP detection from the pioneering work of the early years to the actual state-of-the-art approaches for the detection of this important biological messenger.

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1 Introduction

Cyclic guanosine monophosphate (cGMP) is generated via the catalytic activity of the enzyme family of guanylate cyclases (GC). This class includes the transmembrane particulate GCs (pGC) like GCA or GCB, which represent receptors for peptide ligands such as ANP, BNP, or CNP (for review: Lucas et al. 2000), as well as the cytosolic receptor for the gaseous messenger nitric oxide (NO) soluble guanylate cyclase (sGC) (for review: Hobbs 2000). Upon activation, both classes of GC catalyze the conversion of guanosine triphosphate (GTP) into the second messenger cGMP, which in turn regulates various effector systems such as ion channels, protein kinases, and phosphodiesterases. The breakdown of cGMP to guanosine monophosphate (GMP) is catalyzed by the enzyme family of phosphodiesterases (PDEs) including PDE2, PDE9, or PDE5 (for review: Bender and Beavo 2006). The latter represents the target for the cGMP elevating drugs Sildenafil (Viagra), Vardenafil (Levitra), and Tadalafil (Cialis) (Neumeyer and Kirkpatrick 2004; Ghofrani et al. 2006). The clinical impact of these drugs and the perspective that further cGMP elevating drugs such as NO-independent sGC activators (Evgenov et al. 2006; Stasch et al. 2006) might offer new therapeutic approaches have attracted broad attention to this scientific field. The biochemical detection of cGMP has therefore become an important prerequisite for both the quantification of tissue cGMP levels upon treatment with drugs such as PDE inhibitors, as well as the characterization of novel drugs using purified GC preparations.

The numerous assays which have been conceived and established over the last four decades to biochemically detect cGMP can be classified into two broad classes whose differences based on their application are, however, fading. The detection of GC catalytic activity in biological samples (e.g., homogenized cells or tissues) has been and is still performed by the addition of labelled substrate such as radioactive GTP. The subsequent separation of substrate and product to estimate GC catalytic activity has been developed from the initial attempts using thin layer chromatography (TLC) to chromatography on alumina column, which is still the basis of most radioactive cGMP assays.

In contrast to this, the approach, which has become most widely accepted for the detection of unlabelled cGMP in biological samples, is the competition of physiological cGMP with an exogenous labelled cGMP derivative for a common binding protein. At first, this approach was performed with undefined cGMP-binding protein homogenates, limiting the sensitivity of this competition-binding assay (Murad et al. 1971). The ground-breaking discovery for the biochemical detection of cGMP that still impacts on the actual assays formats was the development of specific anti-cGMP antibodies by Steiner and co-workers (Steiner et al. 1969, 1972a). Although most assays established within the last ten years are based on the competition of labelled and unlabelled cGMP for the specific binding sites of anti-cGMP antibodies, the approaches to detect the formation of these immuno-complexes have been strongly improved over the last years. cGMP detection assays have been developed from laborious hands-on RIAs to automatable homogenous assay formats capable

of processing a large number of samples with high sensitivity. This development also resulted in the stepwise replacement of classical radioactive enzyme activity assays.

The present article reviews the historical development of both major lines and some sideways of the biochemical detection of cGMP from the pioneering work of the first publications to the developments of high throughput screening applications, which have recently become commercially available.

2 Biochemical Detection of cGMP

2.1 Detection of cGMP from Enzymatic Guanylate Cyclase Assays

Guanylate cyclase assays with purified enzyme, cell lysates, or tissue homogenates are indispensable for the investigation of the mechanisms involved in the activation/deactivation of the enzyme, the characterization of its kinetic parameters, and for identifying compounds that modulate the catalytic activity. The general approach is based on the detection of labelled cGMP formed from labelled GTP via the enzymatic activity of GC. Historically, GTP is labelled radioactively although PDE assays have been published using fluorescent derivatives of cGMP (Ohba et al. 2001). The lynchpin of radioactive GC assays is the separation of radioactive cGMP from its precursor GTP. To achieve this aim, various methods have been established since the late 1960s and most of them are not longer applied, with the exception of radioactive sGC assays, which are based on column chromatography.

2.1.1 Radioactive Guanylate Cyclase Assays

cGMP Detection by Thin Layer Chromatography (TLC)

One of the first published methods to quantify cGMP generated in enzymatic assays using cell lysates was based on the conversion of radioactive GTP to radioactive cGMP and its subsequent purification by thin layer chromatography (TLC) (Schultz et al. 1969; White and Aurbach 1969; Bohme 1970; Fleischman 1982). ^{14}C-labelled GTP was converted to ^{14}C-cGMP by sGC-containing supernatants of crude cell lysates, the reaction mixture was spotted on PEI cellulose and separated by TLC. To validate that the observed TLC-signal was due to radioactive cGMP, the corresponding spot was eluted and half of the sample was subjected to a combination of phosphodiesterase (PDE) and alkaline phosphatase (AP) treatment to specifically degrade generated cGMP to radioactive guanosine. Finally, anion-exchange resins adsorbed remaining ^{14}C-cGMP of both samples and the difference of radioactivity measured in the supernatant (due to the radioactive guanosine) of both samples reflected the amount of generated cGMP. Although TLC is able to separate different

nucleotides in parallel, this approach is nowadays only of historical interest due to its limitations with respect to sample throughput and sensitivity. Nevertheless, it allowed the quantification of sGC activity in cellular lysates and anticipated various ideas, which have been incorporated in later approaches for the biochemical detection of cGMP.

cGMP Detection by Column Chromatography

The technical limitations, complex procedure, and the low throughput of the TLC-based purification of radioactive cGMP resulted in the development of various column-based approaches to separate radioactively labelled cGMP from GTP (Murad et al. 1971; Schultz et al. 1973; Krishnan and Krishna 1976; Ward and Brenner 1977; Karczewski and Krause 1978; Gerzer et al. 1981). These techniques are usually applied as part of activity assays with purified GC or enzyme containing cell lysates in which cyclase catalytic activity is monitored via the formation of radioactive cGMP. Although these assays have been established more than two decades ago, they are still routinely applied in many laboratories (Friebe and Koesling 1998; Koglin et al. 2002; Martin et al. 2003; Schmidt et al. 2004; Schindler et al. 2006) due to their high sensitivity, the direct measuring of cGMP, and the possibility to use crude GC-containing cell or tissue lysates as enzyme source. Various articles have been published over the years optimizing the protocol of cGMP purification from cell free assays and cell or tissue lysates. These methods are in general based on the combination of at least two of the following four methods in alternating order: Chromatography of samples on positively charged ion-exchange (IEX) (Murad et al. 1971; Nakazawa and Sano 1974; Schultz et al. 1974; Krishnan and Krishna 1976; Ward and Brenner 1977) or aluminium oxide columns (White and Zenser 1971; Nakazawa and Sano 1974; Krishnan and Krishna 1976; Ward and Brenner 1977; Karczewski and Krause 1978; Gerzer et al. 1981) to retain GTP/GDP and ATP/ADP; separation on negatively charged IEX columns to retain cGMP (Krishna et al. 1968; Schultz et al. 1973; Schultz et al. 1974; Durham 1976); and co-precipitation of adenosine and guanosine mono-, di-, and triphosphates with $ZnCO_3$ which has only slight impact on cyclic nucleotides (Krishna et al. 1968; Schultz et al. 1973; Gerzer et al. 1981). The recovery of cGMP (usually α^{32}P-cGMP) is generally monitored by the addition of trace amounts of ^{3}H-cGMP to the purification procedure.

Still many of the present publications are based on a combination of $ZnCO_3$ precipitation followed by aluminium oxide column chromatography resulting in a cGMP recovery of 65–75% (Gerzer et al. 1981; Schultz and Bohme 1984). As column chromatography is able to directly quantify the increase of cGMP via its radioactivity, there is no need for any enzymatic or fluorescent detection system that could be vulnerable to interfering compounds or colour quenching. These properties in combination with the capability to process up to 96 samples in parallel are making this approach a robust, sensitive, and cheap alternative for many applications especially in the case where crude tissue lysates are used as a source for the enzyme.

2.2 Detection of cGMP in Biological Samples

In addition to the direct determination of the catalytic activity of GCs, there has been and still is a need to determine lowest cGMP levels in biological fluids and tissues e.g., to track physiological pathways affecting GC activity. During the last 40 years, various assays have been developed to achieve this aim thereby pushing the boundaries of sensitivity and sample throughput to the limit of what was technically possible at the respective time. As the commercial available assay kits have become increasingly powerful and sensitive, they are meanwhile also routinely used as read-out for GC catalytic activity, replacing the established radioactive assays described above.

2.2.1 Direct Detection of cGMP

cGMP Detection by High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a well-established approach for the detection of substrates and products of enzymatic reactions in purified samples or complex lysates. As such, HPLC has been used to determine e.g., the enzymatic activity of calcineurin (Enz et al. 1994), dihydropyrimidine dehydrogenase (Lu et al. 1992), phosphodiesterase (Alajoutsijarvi and Nissinen 1987), as well as retinal guanylate cyclase (Koch and Stryer 1988). The major advantage of HPLC for the characterization of enzymatic reactions is its ability to detect unlabelled substrate and corresponding reaction products in parallel. This avoids the need of using labelled substrates such as radioactive of tagged derivatives, which might require special facilities or could influence the enzyme substrate interaction. HPLC-based methods for the detection of GC activity have been published and showed a good correlation with established assays such as RIA (Koch and Stryer 1988; Pietta et al. 1997). Pietta and co-workers reported an optimized HPLC-based protocol for the determination of the enzymatic activity of sGC using a standard C_{18} column and UV-detection at 254 nm. By avoiding a gradient elution profile, the duration of a single HPLC run was reduced to less than 15 min (Pietta et al. 1997). The detection limit of this approach was reported to be 10 pmole cGMP per HPLC run, underlining that the sensitivity of standard HPLC is much lower than commercially available RIAs. One approach to increase the sensitivity of HPLC-based detection of cGMP takes advantage of the fluorogenic reagent (3,4-dimethoxyphenyl) glyoxal (DMPG), to generate fluorescent derivatives of guanosine-containing compounds such as GTP, GDP, or cGMP (Ohba et al. 2001; Soda et al. 2001). The generated fluorescent derivative of cGMP (excitation: 400 nm, emission: 510 nm) was detectable to an amount close to 10 fmol, increasing the detection limit to a comparable level than the antibody-based approaches described below. However, a gradient elution profile was applied resulting in the need for column re-equilibration between two sample injections thus increasing the duration of each run (Soda et al. 2001). A further approach that has been published combined HPLC with mass spectrometry (MS). The

combination of both methods allowed the detection of femtomolar amounts of different nucleotides (e.g. cAMP, cGMP, AMP, and GMP) in parallel (Lorenzetti et al. 2007).

Taken together, HPLC is technically able to detect cGMP in biological samples with high sensitivity (see Table 1). It is the only method that allows the *direct* quantification of cGMP without any need of pre-purification of samples, enzymatic detection systems, labelled cGMP derivatives, radioactivity, or sophisticated data acquisition systems. HPLC offers sensitive quantification of cGMP at affordable costs, if the required hardware (HPLC/MS) is already at hand and established. Although HPLC-based enzyme assays have been shown to be automatable (Pazhanisamy et al. 1995), the major drawback of this approach is the slow serial processing of samples, which limits the use of this versatile method.

2.2.2 Enzymatic Detection of cGMP

cGMP Detection by Enzymatic Cycling

One of the earliest methods for the quantification of cGMP in tissues was the approach of enzymatic cycling described by Hardman and co-workers (Hardman et al. 1966). Enzymatic cycling allowed the direct and sensitive detection of tissue and urine cGMP levels down to femtomolar amounts (Hardman et al. 1966; Goldberg et al. 1969; Ishikawa et al. 1969; Goldberg and Haddox 1974). As enzymatic cycling detects cGMP via its conversion to GDP/GTP, samples had to be purified from such pre-existing contaminants by thin layer or column chromatography as described above. Tracer amounts of ^{3}H-cGMP were added to the samples to calculate the recovery rate during purification (Goldberg and Haddox 1974).

The detection system that allowed the quantification of nanomolar amounts of cGMP was the approach of enzymatic cycling, which consists of two enzymatic reactions: The reaction of Succinate-CoA and GTP to Succinyl-CoA and GDP catalyzed by succinate-thiokinase. Subsequently, the formed GDP is re-phosphorylated to GTP by the enzyme pyruvate kinase resulting in the consumption of Phosphoenolpyruvate and, in parallel, the generation of pyruvate. Thus, the GDP/GTP cycling results in the accumulation of pyruvate equivalents up to three orders of magnitude the amount of the incipiently present GDP (Goldberg et al. 1969). In their initial publication, Goldberg and co-workers quantified the accumulated pyruvate via its conversion to lactate and the consumption of the fluorescent co-substrate NADH. The loss of fluorescence was directly correlated with the amount GDP/GTP present in the assay (Goldberg et al. 1969). To further increase the sensitivity of the assay to femtomolar amounts of cGMP, the detection of NADH was replaced by the direct detection of NAD^{+} upon its conversion into the fluorescent derivative hydroxynicotinaldehyde via the addition of sodium hydroxide (Goldberg and Haddox 1974); an approach that has been rediscovered 25 years later by Seya and co-workers (Seya et al. 1999). To use enzymatic cycling for the detection of cGMP, the sample had to be purified from any contaminating GMP, GDP, and GTP

Table 1

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/ Kit/Price	References
Direct	HPLC	≈10 pmol (standard) ≈10 fmol (fluorescent) ≈30 fmol (LC/MS)	Tubes <30 samples	+ direct detection of cGMP + high sensitivity (fluorescent derivatisation) + detection of various nucleotides in parallel (LC/MS) + cheap (if HPLC or LC/MS equipment is available) + non-radioactive assay + no pre-purification of cGMP necessary + detection is not affected by interfering compounds 0 HPLC is automatable to some extend − slow sample processing and analysis − determination of kinetic parameters not possible − low sample throughput	–	Pietta et al. 1997; Soda et al. 2001; Lorenzetti et al. 2007
Enzymatic	Enzymatic cycling	≈100 pmol (standard) ≈5–100 fmol (fluorescent)	Tubes <30 samples	+ very high sensitivity (fluorescent detection) + high specificity for cGMP + cheap assay + non-radioactive assay − labour-intensive pre-purification of cGMP − possibility of nucleotide-contaminations of enzymes − not automatable − cycling reaction needs to be carefully optimized − compounds might interfere with cycling reaction − low sample throughput − determination of kinetic parameters not possible	–	Hardman et al. 1966; Goldberg et al. 1969, 1974; Seya et al. 1999

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/Kit/Price	References
	GDP-formation and quantification	≈100 fmol	Tubes <30 samples	+ high specificity for cGMP + cheap assay 0 good sensitivity − radioactive assay: demanding special training/facilities − limited half-life of ^{32}P-ATP − labour-intensive pre-purification of cGMP − internal ^{3}H-cGMP standard needed to estimate cGMP recovery − radioactive waste − possibility of nucleotide-contaminations of enzymes − not automatable − compounds might interfere with enzymatic reactions − low sample throughput − determination of kinetic parameters not possible	–	Schultz et al. 1973
	Luciferase bioluminescence	≈10 pmol (384 plate)	96/384/1536 plates >10,000 samples	+ automatable assay format + very high sample throughput − expensive − very low sensitivity for cGMP − designed to detect PDE-induced cGMP degradation − not useful to detect cGMP in biological samples	*Promega PDE-Glo* (V1361, 1,000 test points) (V1362, 10,000 test points) *Costs: 0.35 €/test point*	–

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/Kit/Price	References
Competition-binding	Receptor binding displacement	≈0.5–1 pmol	Tubes <30 samples	+ cheap assay 0 average specificity for cGMP − low sensitivity − radioactive assay: demanding special training/facilities − undefined protein mixture containing cGMP-binding protein − variations in quality of cGMP-binding protein preparations − internal ^{3}H-cGMP standard needed to estimate cGMP recovery − labour-intensive pre-purification of cGMP − not automatable − low sample throughput − determination of kinetic parameters not possible	–	Murad et al. 1971; Gilman and Murad 1974
	RIA	≈100 fmol (standard) ≈3–10 fmol (acetylated)	Tubes <30 samples	+ very high sensitivity (acetylated samples) + very high specificity for cGMP + no need for cGMP pre-purification from other nucleotides 0 relatively expensive kit − radioactive assay: demanding special training/facilities − limited half-life of ^{125}I-cGMP − gamma scintillation counter required − laborious processing of samples prior to assay − not automatable − low sample throughput − determination of kinetic parameters not possible	*GE Healthcare ^{3}H Biotrak cGMP RIA* (TRK500, 150 tubes) *Biomedical Technologies cGMP RIA* (BT-340, 200 tubes) *Izotop ^{125}I cGMP RIA* (RK-525, 100 tubes) *Immunotech cGMP RIA* (IM1118, 100 tubes) *IBL Hamburg ^{125}I cGMP RIA* (RE29071, 100 tubes) *Costs: 2.7–5.5 €/test point*	Steiner et al. 1972; Harper and Brooker 1975; Frandsen and Krishna 1976; Goldberg 1977

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format: Sample throughput	Pros and cons	Company/Kit/Price	References
	SPA	≈200 fmol (standard) ≈4 fmol (acetylated)	Tubes 96 well plates <1,000 samples	+ very high sensitivity (acetylated samples) + very high specificity for cGMP + no need to pre-purify cGMP from other nucleotides + homogenous assay format + automatable + determination of kinetic parameters possible 0 standard luminescence counter needed 0 medium sample throughput − expensive kit − laborious processing of samples prior to assay − radioactive assay: demanding special training/facilities − limited half-life of ^{125}I-cGMP	*GE Healthcare ^{125}I Biotrak SPA* (RPA 540, 100 tubes) *GE Healthcare ^{125}I Biotrak SPA* (RPA 557, 5 × 96plates) *Costs: discontinued*	Hart and Greenwald 1979; Udenfried et al. 1985
	ELISA	≈100–500 fmol (standard) ≈2–5 fmol (acetylated)	96 well plates <1,000 samples	+ very high sensitivity (acetylated samples) + very high specificity for cGMP + no need for cGMP pre-purification from other nucleotides + non-radioactive assay + standard 96 well filter-based plate reader is sufficient 0 medium sample throughput − expensive kit − limited half-life of kits − not automatable − determination of kinetic parameters not possible	*GE Healthcare cGMP direct Biotrak* (RPN 226, 96 plate) *Cayman chemicals cGMP EIA* (581021, 96 plate) *Cambridge Bioscience cGMP EIA* (STA-506, 96 plate) *Assay design cGMP EIA* (900–014, 96 plate) *Biomedical Technologies cGMP EIA* (BT-740, 2 × 96 plates) *IBL Hamburg cGMP ELISA* (CM581021, 8 × 12plate) *Costs: 2.3–5.8 €/test point*	Yamamotho et al. 1982; Pradelles and Grassi 1989; Tsugawa et al. 1991; Horton et al. 1992

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Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format Sample throughput	Pros and cons	Company/Kit/Price	References
	LOCI/ AlphaScreen	≈10 fmol (384 well plate)	96/384/1536 plates >10,000 samples	+ very high sensitivity + very high specificity for cGMP + no need for cGMP purification from other nucleotides + automatable + homogenous assay format + very high sample throughput + non-radioactive assay + direct measurement of cGMP in cell lysates + long excitation wavelength reduces background fluorescence 0 relative expensive kit 0 coloured compounds might quench signal 0 reagents are sensitive to ambient light − plate reader needs to be able to excite samples at 680 nm − compounds interacting with reactive oxygen species (anti- oxidants, metal ions) might be able to interfere with read-out	*Perkin Elmer cGMP AlphaScreen* (6760308M, 10,000 test points) (6760308R, 50,000 test points) + *additional Protein A kit* (6760617C, 500 test points) (6760617M, 10,000 test points) (6760617R, 50,000 test points) *Costs: 0.3–2.4 €/test point*	Ullman et al. 1994, 1996
	EFC	≈10 fmol (384 well plate)	96/384/1536/3456 plates >10,000 samples	+ very high sensitivity + very high specificity for cGMP + no need for cGMP purification from other nucleotides + works directly with cell lysates + automatable + homogenous assay format + very high sample throughput + non-radioactive assay + only assay that offers gain-of-signal read-out 0 standard luminescence readers are sufficient (96/384 plates) 0 relative expensive kit − compounds might interfere with enzymatic read-out	*GE Healthcare cGMP HitHunter* (90-0039-01, 100 test points) *discontinued* (90-0039-02, 800 test points) (90-0039-03, 10,000 test points) (90-0039-04, 40,000 test points) *Costs: 0.3–0.8 €/test point*	Kumar et al. 2007 (cAMP); Weber et al. 2002, 2007 (cAMP)

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/Kit/Price	References
	HTRF	≈40 fmol (384 well plate)	96/384/1536 plates >10,000 samples	+ high sensitivity + very high specificity for cGMP + no need for cGMP purification from other nucleotides + works directly with cell lysates + automatable + homogenous assay format + very high sample throughput + non-radioactive assay − expensive kit − more sophisticated requirements for plate reader (detection of signal at 620 nm and 665 nm, delay between excitation and detection)	*Cisbio HTRF cGMP kit* (62GM2PEB, 1,000 test points) (62GM2PEC, 20,000 test points) *Costs: 0.2–0.6 €/test point*	Mathies 1999; Bazin et al. 2001 (review)
In situ	Immuno-fluorescence	–	Slides <30 samples	+ localisation of cGMP in tissue slides + high specificity for cGMP + acetylation of cGMP in slides has been reported + non-radioactive + cheap − low sensitivity − not suitable for cGMP quantification − labour-intensive preparation of tissues/cells − low sample throughput	–	Chan-Palay and Palay 1979; de Vente et al. 1996

(Goldberg et al. 1969). cGMP was then incubated with phosphodiesterase (PDE) to generate GMP, which was subsequently phosphorylated to GDP via an enzymatic reaction catalyzed by ATP–GMP phosphotransferase under consumption of ATP. The formed GDP was then used to initiate the above-described GDP/GTP cycling reaction allowing the determination of the initial cGMP concentration.

The detection of cGMP by enzymatic cycling is a powerful and non-radioactive approach that offers a detection limit close to 5–10 fmol cGMP per sample at a reasonable price. However, as the cycling reaction is triggered by GDP/GTP, all enzymes and compounds have to be free from any putative contaminations with these molecules to obtain the necessary signal to noise ratio. For the same reason, the cGMP of the samples has to be separated from other guanosine nucleotides. This laborious pre-purification is one of the major drawbacks of the enzymatic cycling approach and reduces dramatically the sample throughput of this method. Moreover, the loss of cGMP during its purification needs to be estimated, what has been usually achieved by the addition of tracer amounts of radioactive cGMP foiling, a least partially the advantage of enzymatic cycling as a non-radioactive approach. In addition, the cycling system needs to be fine tuned to detect cGMP at the highest possible sensitivity. This balance can also be susceptible for interference by chemicals making the enzymatic cycling approach less robust. Nevertheless, once these considerations are taken into account, enzymatic cycling offers a cheap alternative to commercially available antibody-based systems, especially as the direct quantification of NAD^+ as described by Seya and co-workers offers a gain-of-signal read-out in contrast to most other assay formats.

cGMP Detection by Enzymatic Formation of Radioactive GDP

A different enzymatic approach allowing the detection of femtomolar amounts of tissue cGMP was published by Schultz and co-workers (Schultz et al. 1973). Similar to the enzymatic cycling approach published by Hardman and co-workers, the first step for the detection of cGMP relied on its PDE-mediated conversion to GMP. In turn, the generated GMP was used as substrate for subsequent enzymatic reactions, allowing its quantification. In contrast to the non-radioactive approach of enzymatic cycling, the present method is based on radioactive substrates to quantify the generated GMP.

To allow the quantification of cGMP via its conversion to GMP, the sample had to be purified from any cellular GMP to ensure a high signal to noise ratio and to minimize any possible inference of cellular components with subsequent enzymatic reactions involved in the quantification of GMP. Purification of cellular cGMP was achieved by ethanol extraction from homogenized tissues followed by $ZnCO_3$ co-precipitation. Whilst most of the 5′ nucleotides co-precipitated with the formed $ZnCO_3$, cyclic nucleotides such as cAMP and cGMP were virtually not affected and remained in solution. Subsequently, column chromatography was used to purify the sample from residual zinc ions and to separate extracted cGMP from cAMP. The addition of tracer amounts of titrated cGMP to the homogenized sample allowed the

estimation of the overall cGMP recovery, which was calculated to be 40–50%. To quantify the purified cGMP, the sample was subjected to a radioactive enzymatic detection system.

The initial step of this enzymatic detection is comparable to the enzymatic cycling approach. The extracted cGMP was converted to GMP via the addition of PDE. Following heat inactivation of PDE, the generated GMP was phosphorylated by GMP kinase in the presence of the co-substrate γ^{32}P-ATP, resulting in the formation of β^{32}P-GDP and non-radioactive ADP. Remaining radioactive γ^{32}P-ATP was converted to ADP and radioactive inorganic phosphate by adding myosin to the assay mixture. Subsequently, the radioactive phosphate was precipitated by the addition of a mixture consisting of perchloric acid, ammonium molybdate, and triethylamine. The radioactivity found in the supernatant was basically due to the formed β^{32}P-GDP and reflected the amount of the initial cGMP.

This protocol allowed the sensitive quantification of femtomolar amounts of cGMP in biological samples such as cell and tissue homogenates. Although the described method to quantify cGMP via the enzymatic formation of β^{32}P-GDP showed a high sensitivity, it suffered from the same drawbacks than the enzymatic cycling approach described above. The purity of the sample's cGMP from GMP and other cellular contaminants, which might have an impact on the enzymatic detection system, is a prerequisite for the present method and demands a time consuming and labour-intensive purification of the samples. This sample processing obviated any automation of the protocol and resulted in a low sample throughput. In addition, the limited half-life of the radioactive substrate γ^{32}P-ATP and the problematic handling and disposal of radioactive waste would discriminate the present method against the enzymatic cycling approach in its improved version. Despite these considerations, the assay published by Schultz and co-workers offered a cheap option and has been used in the following years for the quantification of cGMP in various tissues (Rabinovitch et al. 1980; Asakawa et al. 1978; Hadden et al. 1976).

cGMP Detection by Luciferase Bioluminescence

Recently, Promega has commercialized a bioluminescent approach for the HTS detection of cellular cAMP, cAMP-Glo. The assay is based on the addition of inactive tetrameric protein kinase A (PKA), PKA substrate, and a luciferase-system to cellular lysates. As the cellular cAMP activates PKA, which in turn consumes ATP to phosphorylate the added substrate, the amount of cellular cAMP is translated into PKA-mediated ATP consumption. As a consequence, the reduced levels of available ATP result in the reduction of luciferase-mediated bioluminescence (Kumar et al. 2007). To protect cellular cAMP levels from degradation, the assay buffer contains also a pan-phosphodiesterase (PDE) inhibitor. The cAMP-Glo assay detects cAMP in cell lysates from low to high femtomolar amounts (Fan and Wood 2007; Kumar et al. 2007).

The same assay approach is commercialized as PDE-Glo to quantify the activity of purified cAMP or cGMP dependent PDEs. The PDE-Glo manual illustrates that not only changes in the cAMP but also in the cGMP concentration can be translated via PKA-activation and subsequent ATP consumption into alterations of the luciferase-induced bioluminescence. However, due to the much lower affinity of PKA for cGMP compared to cAMP, the EC_{50} for the detection of cGMP is increased to approximately 10 pmole. Although this low affinity does not foil the determination of the activity of purified cGMP-specific PDEs (e.g. PDE5), it prevents the use of this bioluminescence approach for the detection of low amounts of cGMP in biological samples. The much higher affinity of PKA for cAMP and the higher cellular concentrations of cAMP compared to cGMP would mask any cGMP-mediated bioluminescence signal foiling the use of this kit for the detection of cGMP in lysates. Although the purification of cGMP from other nucleotides as described for the enzymatic cycling approach would theoretically be able to eliminate cAMP-derived signals, the low affinity of PKA for cGMP would still foil the detection of this molecule with feasible sensitivity. Taken together, it can be concluded that despite PDE-Glo is able to detect PDE-induced cGMP changes in cell free assays, it is non-applicable for the detection of cGMP in biological samples.

2.2.3 Detection of cGMP by Competition-Binding Approaches

Most of the above-mentioned methods for the detection of cGMP in tissue or cell lysates require pre-purification of the samples such as the enzymatic cycling approach (Goldberg et al. 1969; Ishikawa et al. 1969; Goldberg and Haddox 1974) or have a limited sample throughput such as HPLC (Pietta et al. 1997; Soda et al. 2001). In order to overcome these obstacles, competition-binding assays for cGMP have been established. This approach, which forms the basis of most actual assay formats, is based on the competition of cGMP in biological samples and exogenous added labelled cGMP for binding to a common cGMP-binding site. Increasing amounts of unlabelled cGMP from the sample result in displacement of the labelled cGMP from the binding protein and usually (depending on the assay read-out) in a decreasing signal (see Fig. 1). First, competition-binding assays were developed using undefined protein extracts containing cGMP-binding proteins (Murad et al. 1971; Gilman and Murad 1974; Kleine and Kroh 1978); at a later date, the groundbreaking development of anti-cGMP antibodies by Steiner and co-workers (Steiner et al. 1970, 1972b; Richman et al. 1980) allowed the replacement of these protein mixtures by highly specific anti-cGMP antibodies.

Receptor Protein Binding Displacement for cGMP

Before cGMP immuno assays (e.g. RIA, ELISA) became available to a broad range of researchers, different methods based on cyclic nucleotides binding proteins were published (Murad et al. 1971; Gilman and Murad 1974; Kleine and Kroh 1978).

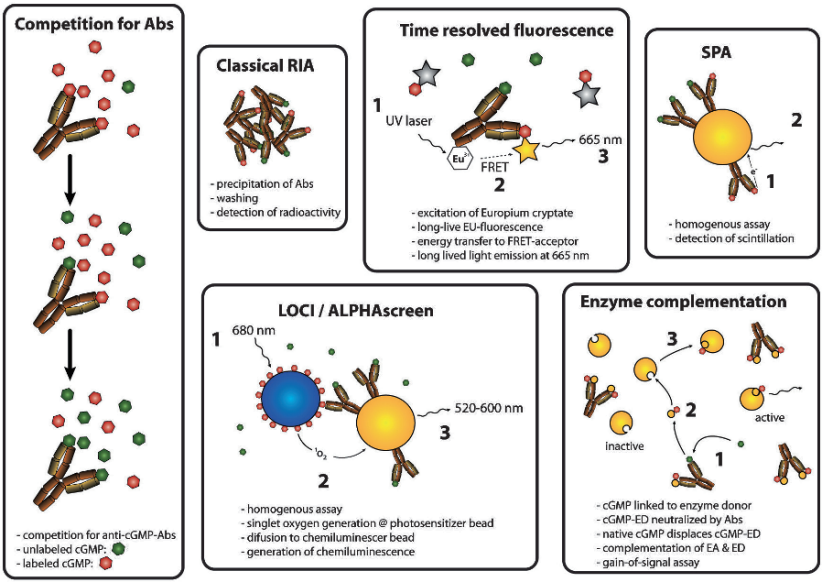

Fig. 1 *Comparison of different competition-based cGMP detection systems*. Virtually all commercially available cGMP detection assays are based on the competition of an unknown amount of unlabeled cGMP from the sample and a defined amount of tracer-labelled cGMP for binding to the anti-cGMP antibodies (Ab). This competition is shown on the left site of Fig. 1 where increasing amounts of unlabeled cGMP from the sample (green) displace tracer-coupled cGMP (red) from the Ab. The subsequent quantification of the displacement differs strongly depending on the assay design. *Classical RIA*: Classical radioimmuno assays (RIA) use radioactively labelled cGMP (usually ^{125}I-cGMP) as tracer and precipitate the Abs. The amount of bound radioactive cGMP can be directly measured in the precipitate. Maximal signal strength is achieved in the absence of sample and increasing amounts of unlabelled cGMP decrease the signal. *SPA*: Scintillation proximity assays (SPA) are comparable to RIAs as they use the same radioactively labelled cGMP. However, in contrast to RIAs the Abs are coupled to the surface of scintillator-containing beads. The radioactive decay of bound ^{125}I-cGMP in the proximity of the bead result in the scintillator-mediated emission of photons whereas electrons emitted from unbound radioactive cGMP are scavenged by the medium. SPA allowed the development of homogenous assay without the need for precipitation and washing steps. *LOCI/ALPHAscreen*: Luminescent oxygen channelling assay (LOCI) is based on the cross linking of two different kinds of microspheres via the interaction of bead-coupled anti-cGMP Abs with biotinylated cGMP (tracer) which in turn binds streptavidin-coupled beads. The proximity of both beads in the presence of Biotin-cGMP allows singlet oxygen molecules generated in one microsphere to react with the acceptor bead resulting in the emission of detectable photons. Singlet oxygen molecules, which are not generated in the proximity of an acceptor bead, are quenched due to the short half-life of this reactive molecule. *HTRF*: Homogenous time resolved fluorescence (HTRF) is based on Foerster resonance energy transfer (FRET) from a FRET-Donor, usually an Europium–Cryptate complex coupled to the Ab, and a FRET acceptor linked to cGMP (tracer). Binding of this fluorescent-cGMP to the antibody allows the detection of a long-lived FRET signal whereas unbound tracer cGMP shows no or only short-lived fluorescence. Maximal signal is achieved in the absence of unlabeled cGMP. *EFC*: Enzyme fragment complementation (EFC) based detection of cGMP is triggered by the spontaneous recombination of two inactive high affinity fragments of β-galactosidase to the active holoenzyme which catalytic activity serves as read-out. The smaller fragment (enzyme donor = ED) is coupled to cGMP (tracer) and neutralised by binding to the anti-cGMP Ab. Addition of unlabeled cGMP from a sample results in the displacement of the ED-cGMP from the Ab and as a result in the formation of active β-galactosidase. EC is the only gain-of-signal assay resulting in higher signal with increasing amounts of unlabeled cGMP

These approaches used undefined protein preparation from various sources containing cAMP- or cGMP-binding proteins such as PKA or PKG to bind the corresponding cyclic nucleotide. With respect to cGMP, Murad and co-workers published a receptor protein displacement assay, which used a protein homogenate from lobster tail muscle as binding protein for cGMP. By using increasing amounts of ^{3}H-cGMP, the authors showed specific binding to proteins from the lobster protein preparation with a K_D value of 2–10 nanomolar depending on the preparation. In the presence of 60 nM ^{3}H-cGMP saturated binding of the radioligand was achieved, thus enabling the detection of picomolar amounts of cGMP. Like for classical receptor binding assay with purified receptors, the addition of unlabelled ligand from purified or non-purified samples resulted in the displacement of radiolabeled cGMP from the lobster protein preparation allowing the quantification of the respective cGMP concentration.

The receptor protein displacement assay allowed the reproducible detection of cGMP in biological samples close to 500 fmol per sample. The major advantage of this method was that it was able to detect cGMP with sufficient sensitivity for many applications for a very reasonable price. In addition, as the assay directly detected the binding of cGMP to its target protein, it was less susceptible for interference by chemicals than enzymatic detection systems. The approach of protein binding displacement was further optimized to allow the parallel detection of cGMP and cAMP in urine samples (Kleine and Kroh 1978). However, the protein mixture used as cGMP receptor showed not only less sensitivity but also less specificity for cGMP compared to immunoassays. Especially, cAMP showed a much higher cross-reactivity to the cGMP-binding protein preparation compared to antibody-based methods. This lack of specificity demanded a labour-intensive pre-purification of cGMP from other nucleotides and resulted in a low sample throughput. Although the protein receptor displacement assay was a reliable and cheap approach, the above-mentioned drawbacks resulted in its replacement by immunoassays such as RIA or ELISA.

cGMP Radio Immuno Assay (RIA)

The development of cGMP-specific antibodies by Steiner and co-workers (Steiner et al. 1972a) enabled the design of a cGMP radio immuno assay (RIA) as described earlier for cAMP (Steiner et al. 1969). As cyclic nucleotides are not immunogenic, a 2′-O-succinyl derivative of cGMP was coupled to keyhole limpet hemocyanin, which was used to immunize rabbits (Steiner et al. 1972a). The generated antibodies showed a high selectivity for cGMP and virtually no cross-reactivity with other nucleotides rendering sample pre-purification unnecessary (Steiner et al. 1972a, b). The published RIA was a classical competition-binding assay comparable to the protein cGMP displacement assay described earlier (Murad et al. 1971) but with much higher sensitivity allowing the detection of tissue cGMP levels down to 100 fmol per sample. This increase in sensitivity was achieved by the higher affinity of the antibody for cGMP compared to cGMP-binding proteins and the use of

^{125}I-cGMP (Steiner et al. 1972a), which has a much higher specific activity than ^{3}H or ^{14}C labelled cGMP. Tissue cGMP displaced the radioactively labelled cGMP from the antibody until equilibrium between association and dissociation of both ligands was reached (Steiner et al. 1972b, Fig. 1). Subsequently, antibody-bound and free ^{125}I-cGMP were separated via precipitation of the anti-cGMP antibody by adding secondary anti-rabbit antibodies, ammonium sulphate (Steiner et al. 1972b), or ice-cold ethanol (Frandsen and Krishna 1976). Alternatively, unbound cGMP was adsorbed by charcoal (Goldberg 1977). The initial cGMP RIA as published by Steiner and co-workers (Steiner et al. 1972b) was further optimized to higher sensitivity by acetylation or succinylation of the sample's cGMP (Harper and Brooker 1975; Frandsen and Krishna 1976). By generating these derivatives, which reassembled more closely the cGMP derivative that was used for immunization (Steiner et al. 1972b; Harper and Brooker 1975; Frandsen and Krishna 1976; Brooker et al. 1979), the detection limit of this approach was reduced to 3–10 fmol per sample (Harper and Brooker 1975; Frandsen and Krishna 1976). This extremely high sensitivity combined with the lack of virtually any cross-reaction with other nucleotides made this assay extremely useful for the detection of tissue cGMP levels and formed the basis for actual commercial cGMP RIA kits.

Despite the unmatched sensitivity and specificity for cGMP, the classical RIA has several drawbacks that promoted the development of more sophisticated detection systems. The classical RIA relies on a time consuming extraction of cGMP from tissue or also cell homogenates. Usually this includes lyophilization and subsequent resuspension of the samples in assay buffer. As this process is not automatable, it hampers the analysis of large numbers of samples. Furthermore, the general RIA protocol requires incubation over night, and antibody-bound cGMP needs to be separated from free ligand by several precipitation and washing steps slowing the analysis of samples further down. Another drawback lies in the use of radioactive cGMP as tracer. Most commercially available RIAs use ^{125}I-labelled cGMP that offers, on the one hand, a high specific activity thereby increasing the sensitivity of the assay; on the other hand, it reduces the shelf life of the kit due to the short half-life of the isotope. In addition, the use of radioactive tracers demands special facilities and waste disposal procedures. Despite these obstacles and the high costs of commercially available cGMP RIAs, the high sensitivity and specificity are making this assay a well-established option to quantify cGMP in a manageable number of samples.

cGMP Scintillation Proximity Assays (SPA)

The development of RIAs enabled researchers to detect cGMP from biological samples or enzymatic assays with an unmatched sensitivity at that time. Nevertheless, as described above, classical RIAs suffer from several drawbacks mainly the labour-intensive sample processing. Moreover, RIAs rely on the equilibrium of binding of the ligand to the antibody; as such this approach does not allow the determination of association and dissociation kinetics. Although this doesn't affect the detection

of cGMP in biological samples, the ability to monitor binding kinetics of ligands to their corresponding target proteins has been desirable for various applications e.g. drug development. The scintillation proximity assay (SPA) resolved most of these obstacles as it allowed the development of homogenous assay formats without any need for precipitation or washing steps.

The term scintillation proximity assay for a special class of assay technique was coined by Hart and Greenwald (Hart and Greenwald 1979a, b). In these initial publications, the authors determined the interaction of antibody and antigen by using two different types of antigen-coated microspheres of which one was ^{3}H-labelled, whereas the other contained a scintillator. As the β-particles emitted from of the ^{3}H-labelled beads had an average energy of 6 keV, they were readily absorbed in aqueous buffers within very short distances of approximately 4 μm. However, once both types of microbeads were cross linked via the addition of appropriate antibodies, the β-particles were emitted in the direct proximity of the scintillator resulting in the emission of a photon, which could be detected by a standard scintillation counter (Hart and Greenwald 1979a, b). Although the described application was designed for the quantification of antibody titres, the authors already pointed out that coating of the microspheres with appropriate receptors such as antibodies could convert SPA into an extremely versatile technique (Hart and Greenwald 1979b).

In the following years, the SPA approach was optimized to increase its sensitivity and versatility. A major development was the use of antibody coated scintillator-containing microspheres in combination with radioactive ligands (Udenfriend et al. 1985, 1987; Bosworth and Towers 1989). By replacing the ^{3}H labelled microspheres of the original assay design (Hart and Greenwald 1979a) with directly radioactively labelled ligands, the binding reaction was turned from ternary into second order reducing the time needed for the formation of antibody–antigen complexes (Udenfriend et al. 1985). A further increase in sensitivity was achieved by using ^{125}I as radioactive label. The higher specific activity of the decay of ^{125}I to ^{125}Te combined the higher average energy (35 keV) and operating distance in (35 μm) of the emitted Auger electrons resulted in an increased emission of photons from the scintillator microbeads (Udenfriend et al. 1985, 1987). An additional advantage of the use of ^{125}I is that ligands can be labelled by a broad range of well-established and commercially available methods and kits for iodination, whereas the synthesis of ^{3}H-labelled ligands is usually more complex. Although it is possible to establish SPAs with other isotopes commonly used in life science applications (e.g. ^{14}C, ^{35}S), only few publications describe such an approach. The β particles emitted by these isotopes are too energetic resulting in increased path length in aqueous buffer and thereby in higher background signals due to unspecific activation of scintillator beads (Udenfriend et al. 1985; Carpenter et al. 2002). The SPA beads are generally available in two types: inorganic Yttrium silicate (YSi) or organic Polyvinyltoluene (PVT) containing diphenylanthracine as scintillator. Although YSi is one of the most efficient solid scintillators with an average counting efficiency of 60% compared to liquid-scintillation counting, the high density of YSi beads of $\approx$4 g cm^{-3} results in faster settling compared to the lower density PVT beads (1.05 g cm^{-3}) (Carpenter et al. 2002). The SPA approach in general offers a higher versatility as it allows to

determine association and dissociation kinetics of radioactive ligands to their target protein. However, although this feature might be highly desirable for drug discovery applications, it is not essentially needed for the quantification of cGMP in biological samples.

The commercially available cGMP SPA kits are based on PVT microspheres coated with anti-rabbit antibodies and primary rabbit anti-cGMP antibodies raised against 2′-O-succinyl derivative of cGMP as described by Steiner and co-workers (Steiner et al. 1972a). cGMP is quantified by displacement of the radioactive cGMP tracer from the antibody resulting in reduced scintillation rates (Fig. 1). Due to its advantages compared to tritium labelled tracers, the available SPA kits are using ^{125}I-cGMP as ligand. However, as mentioned above for RIA, the use of this isotope as tracer reduces the shelf life of the assay kit. Beside the use of radioactive tracers, SPA has similar drawbacks than RIA, namely the labour-intensive sample processing prior to cGMP determination. Although both methods have similar sensitivity and specificity, SPA offers several advantages. As a homogenous assay format, SPA has no need for precipitation and washing steps strongly facilitating the assay and allowing its automation. The use of 96 well plates and scintillator beads emitting photons in the visible spectrum (e.g. 420 nm) permit signal detection with a standard luminescence counter albeit the possibility of signal quenching due to coloured compounds has to be taken into account. In summary, compared to RIA, the SPA approach offers higher versatility and sample throughput with matching sensitivity and specificity at comparable high costs.

cGMP Enzyme Linked Immunosorbent Assay (ELISA)

The development of specific antibodies to succinylated cGMP–protein conjugates (Steiner et al. 1972a, b; Richman et al. 1980) allowed the development of various cGMP RIA and SPA approaches for the detection of femtomolar amounts of cGMP (see above). To maximize the sensitivity, most assay kits used ^{125}I-cGMP as tracer as its specific activity is much higher compared to ^{3}H- or ^{14}C-cGMP. The high specific activity of ^{125}I reflects its relative short half-life of 59.4 days demanding newly synthesized ^{125}I-cGMP every few weeks. In addition, there are further drawbacks of radioactive assays in general: Handling with radioactive compounds demand special needs for storage, waste disposal, and lab hardware as well as staff training to work with radioactive samples. These obstacles led to the development of non-radioactive enzyme-linked immunosorbent assays to quantify cGMP levels with comparable sensitivity as RIA.

Various authors published non-radioactive ELISA approaches for the detection of cGMP. Virtually all methods are based on polyclonal rabbit anti-cGMP antibodies raised against succinylated cGMP coupled to bovine, human, or rabbit serum albumin (Yamamoto et al. 1982; Horton et al. 1992; Wellard et al. 2004) or keyhole limpet hemocyanin (Pradelles et al. 1989). Tsugawa and co-workers published a cGMP-ELISA based on a monoclonal antibody directed against succinylated cGMP coupled to human serum albumin (Tsugawa et al. 1991). Due to the small molecular

weight of cGMP, it is hardly possible to generate antibodies against different epitopes, which don't interfere with each others binding to the molecule. Since the independent binding of two antibodies is a prerequisite for direct sandwich ELISAs, all published enzyme-linked approaches are designed as competitive ELISA using only one specific anti-cGMP antibody. Different assay designs have been implemented. Some approaches used the initial immunogen (cGMP coupled to rabbit or human serum albumin) to coat microtitre plates (Tsugawa et al. 1991; Wellard et al. 2004). Upon reaching equilibrium of binding between plate-bound HSA-cGMP and free cGMP to the anti-cGMP antibody, the sample's cGMP concentration was reflected by the amount of plate-bound antibody. This primary antibody was subsequently quantified via commercial available secondary antibodies coupled to detection enzymes such as alkaline phosphatase (AP) or horse radish peroxidase (HRP). Other publications established the coating of surfaces such as microtitre plates (Pradelles and Grassi 1989; Horton et al. 1992) or polystyrene particles (Yamamoto et al. 1982) with secondary anti-rabbit antibodies to bind the primary anti-cGMP antibody. Subsequently, the sample's cGMP competes for binding to the primary antibody with cGMP-coupled detection enzymes such as acetylcholine esterase (Pradelles et al. 1989), HRP (Horton et al. 1992), or β-galactosidase (Yamamoto et al. 1982), allowing the quantification of cGMP via a colorimetric enzyme reaction. However, this assay design demands special cGMP tracer such as HRP–cGMP, which in turn determines the enzymatic detection reaction.

The approach to coat microtitre plates with the initial immunogen like HSA–cGMP offers a broader versatility as standard commercially available secondary anti-rabbit antibodies with different coupled detection systems can be used to detect the amount of bound primary anti-cGMP antibody. All published as well as commercially available assays are, independently from their design, able to detect low femtomolar amounts of cGMP in biological samples offering the same range of sensitivity as RIA or SPA. In contrast to these assays, ELISA, as an entirely non-radioactive approach, does not require any special facilities or waste disposal. Samples including crude cell lysates can be directly measured without any time consuming extraction and lyophilization procedure increasing the feasibility and sample throughput of this assay format compared to available RIA and SPA kits. Although ELISA demands various washing steps (in contrast to the homogenous assay formats such as SPA), it can be automated to some extent allowing the processing of a fair number of samples in manageable time. Moreover, the extensive wash steps impede any interference of coloured chemicals or sample components with the subsequent enzymatic detection. Depending of the detection system and the chromogenic substrate, data acquisition can be accomplished on standard filter-equipped 96-well plate readers. Although no radioactive tracers are used, the shelf life of some commercially available cGMP-ELISA is in the same range as described for RIA and SPA. Nevertheless, shelf life as well as price of cGMP-ELISAs can vary strongly between different manufacturers (see Table 1). In general, the price per kit is in the same range as for RIA or SPA. Taken together, the matching sensitivity and specificity for cGMP compared to RIA/SPA, the use of non-radioactive reagents

and standard filter-based plate readers, and the possibility to process a fair number of samples make cGMP-ELISAs a good choice for many labs.

Detection of cGMP via Luminescent Oxygen Channelling Assay (LOCI)

The luminescent oxygen channelling assay (LOCI) or ALPHAscreen was first described by Ullman and co-workers (Ullman et al. 1994). Generally, the assay is based on the same principles as the incipiently published scintillation proximity assay (Hart and Greenwald 1979a). Both approaches, SPA and LOCI, crosslink via an antigen–antibody interaction, two different kind of microspheres, whose proximity result in the emission of detectable photons (Hart and Greenwald 1979a; Ullman et al. 1996). However, in contrast to SPAs in which the close proximity of radioisotope and scintillator is the basis for the emission of photons, LOCI represents an entirely non-radioactive approach.

The LOCI technique is based on two species of coated beads of which one has dissolved in it a photosensitizer whereas the other contains the chemiluminescer. The photosensitizer beads are loaded with the dye Phthalocyanine that shows a strong absorption at wavelength of 680 nm allowing the use of commercially available 680 nm-solid-state lasers for excitation. Once activated, the dye generates a highly instable singlet oxygen molecule, which is able to diffuse approximately 200 nm before falling back into the ground state. The short half-life of the singlet oxygen ensures a low background signal of the LOCI approach, as the $^1O_2$ source has to be in very close proximity to the $^1O_2$ acceptor to generate chemiluminescence. The $^1O_2$ accepting particles contain the alkene Thioxene, which rapidly reacts with singlet oxygen molecules resulting in chemiluminescence at 390 nm (Ullman et al. 1996). This energy is instantaneously transferred to fluorophores (e.g. bisphenylethynylanthracene) dissolved in the same microsphere shifting the emission to a wavelength of 520–600 nm (Ullman et al. 1996; Gabriel et al. 2003). This emission (upon excitation at 680 nm) directly correlates with the amount of photosensitizer–chemiluminescer particle pairs and allow therefore the quantification of the antigen–antibody interaction. Both kind of particles are coated with a dextran-based hydrogel that strongly diminished unspecific interactions and prevents due to its hydrophilicity sedimentation of the particles in solution (Ullman et al. 1994, 1996).

With respect to the quantification of cGMP, Perkin Elmer commercializes the LOCI approach as ALPHAscreen. In this assay kit, the photosensitizer beads have bound streptavidin to their surface, whereas the chemiluminescent particles are coated (via protein A) with an anti-cGMP antibody. The ALPHAscreen is designed as a competitive assay: Unlabelled and biotin-tagged cGMP compete for the antibody-binding sites. In the absence of unlabelled cGMP, the biotinylated cGMP results in the maximum crosslink of both beads and therefore in a maximum signal. Increasing amounts of unlabelled cGMP result in the displacement of biotin-cGMP from the antibody-binding site and, as a consequence, in the dissociation of the $^1O_2$-donor–acceptor particle pairs. In the presence of 1 μM unlabelled cGMP, the laser-induced emission of light is virtually abolished.

ALPHAscreen is capable of detecting low femtomolar amounts of cGMP in samples suggesting that this approach offers a comparable sensitivity than RIA, SPA, or ELISA. ALPHAscreen, as a homogenous assay format, can be readily automated and allows the determination of cGMP in cell free and cellular assays in 96, 384, or 1,536 well plates. In contrast to RIA and SPA, cellular cGMP can be directly quantified in crude cell lysates with high sensitivity and specificity, thus allowing the processing of a large number of samples and rendering time consuming extraction procedures unnecessary. ALPHAscreen is not based on enzymatic detection systems making this assay format less vulnerable to compounds, which might have the potency to interfere with enzymatic read-out systems. Nevertheless, as the emitted light is in the visible spectrum the signal strength can be affected by colour quenching chemicals. Also it has to be taken into account that antioxidants or metal ions might be able to scavenge the singlet oxygen released from the photosensitizer beads. The relatively low excitation energy at a wavelength of 680 nm results in reduced background fluorescence; however, it demands a data acquisition system compatible with the required excitation and emission wavelengths. ALPHAscreen in principle is able to continuously monitor cGMP accumulation and/or degradation in assays using purified enzyme preparations or cell lysates. The assay kit is expensive as it has to be combined with an additional Protein A kit (see Table 1). Furthermore, the ALPHAscreen cGMP kit is only available for analysing 10,000 or 50,000 samples, what might be oversized for many laboratories. In contrast, the necessary Protein A kit is already available at a kit size of 500 samples. Nevertheless, the price of the kit is very competitive, if very large numbers of samples have to be screened. Due to its novelty compared to established assay formats, it is hard to estimate the feasibility of ALPHAscreen for the detection of cGMP apart from its use as HTS assay. If appropriate plate readers are present, the sensitivity of the assay and its capability to monitor and quantify cGMP directly in cell lysates are making ALPHAscreen an option compared to RIA, SPA, or ELISA.

Detection of cGMP by Enzyme Fragment Complementation Assays (EFC)

Enzyme fragment complementation (EFC) assays are based on the principle that various enzymes such as β-galactosidase, β-lactamase, luciferase, or dihydrofolatereductase can be genetically split into two independent expressible and catalytic inactive fragments (enzyme donor: ED, enzyme acceptor: EA) which can, however, recombine to form the holoenzyme and thereby regain catalytic activity (Remy and Michnick 1999; Rojo-Niersbach et al. 2000; Ozawa et al. 2001; Wehrman et al. 2002; Weber et al. 2007). EFC has been adapted to fit different assay strategies depending on the kind of interaction that needs to be investigated. In order to track protein–protein interactions, the proteins of interest can be genetically fused to the EA and the ED, respectively. Once the proteins interact the fused EA/ED pair form the holoenzyme allowing to follow the process of protein interactions via the regained catalytic activity. To avoid a possible impact of the enzyme fragments onto

the interaction of the fused proteins, the enzyme fragments have been engineered to show a rather low affinity for each other (Eglen 2002; Golla and Seethala 2002).

The commercially available cGMP detection kit 'HitHunter' from DiscoveRx is based on a slightly modified strategy. In contrast to the low affinity EA/ED pair described above, HitHunter takes advantage of a high affinity ($K_D \approx 1\,nM$) interaction of two engineered fragments of *E. Coli* β-galactosidase (Eglen 2002; Golla and Seethala 2002). The assay is based on the spontaneous recombination of the large catalytically inactive enzyme fragment (enzyme acceptor, EA) and the approximately 4 kDa small enzyme donor. In order to link enzyme complementation to cGMP concentrations, the small enzyme donor is chemically conjugated to cGMP. Although this ED-tagged cGMP is able to trigger the high affinity enzyme complementation with the EA, this process is blocked by the presence of anti-cGMP antibodies in the assay mixture neutralizing the available ED-cGMP (Eglen 2002; Golla and Seethala 2002; Weber et al. 2004). Increasing amounts of native cGMP produced either by guanylate cyclase activity assays or by the addition of cGMP containing samples to the assay mixture, result in a concentration dependent displacement of ED-cGMP from the anti-cGMP antibodies. Free ED-cGMP immediately binds to the EA fragment forming enzymatically active β-galactosidase, which in term catalyses the conversion of a chemiluminescent substrate, such as dioxetane (Weber et al. 2004). Thereby, the HitHunter EFC approach directly links cGMP concentration to the emission of chemiluminescence which can be quantified by standard scintillation readers (Weber et al. 2004). HitHunter is capable of detecting cGMP in biological samples at femtomolar amounts matching the sensitivity of the above-described ALPHAscreen. The manufacturer's protocol recommends the use of HitHunter with 96 or 384 well plates. However, the comparable HitHunter cAMP detection kit was successfully miniaturized to 1,536 and even 3,456 well plates (Weber et al. 2004, 2007).

The pros and cons of HitHunter reassemble most of the considerations mentioned above for ALPHAscreen. The assay directly detects cGMP in crude cell lysates as well as in cell free assays, dramatically reducing labour-intensive preprocessing of the sample, prior to measuring. HitHunter can be automated thus allowing the processing of a very large number of samples. The use of chemiluminescent substrate for the β-galactosidase read-out system enables the use of standard photomultiplier based data acquisition systems. Theoretically, as the addition of substrate is the last step in the assay protocol, any of the commercially available fluorogenic or chromogenic substrates for β-galactosidase might be used (see http://www.sigmaaldrich.com/catalog/search/TablePage/15845000), although the sensitivity of the assay could be compromised. Nevertheless, the free choice of substrate would allow adapting HitHunter to the detection hardware available in the respective lab, and if used in a 96 well format also chromogenic substrates might offer sufficient sensitivity. The assay kit is sold at a competitive price (see Table 1) and, in contrast to ALPHAscreen, in a kit size that matches the demands of many laboratories (800 test points). As HitHunter uses an enzymatic read-out system, one has to be aware that compounds able to inhibit β-galactosidase might affect the signal strength. A major advantage of the assay is that, in contrast to virtually

all other competition-based methods, the EFC offers a gain-of-signal read-out with increasing amounts of cGMP. Taken together, HitHunter represents a very promising approach for the detection of cGMP not only for HTS but also non-HTS applications, as it offers a non-radioactive assay format able to detect cGMP with high sensitivity and specificity even in crude cell lysates. Despite its capability to be miniaturized to 1,536 or even 3,456 well plates might not be of interest for many applications, the combination of a gain-of-signal read-out with the possibility to adapt the assay to pre-existing hardware by choosing different substrates makes HitHunter a very attractive choice for the detection of cGMP.

Detection of cGMP by Time Resolved Fluorescence (TRF)

Fluorescence techniques for the detection of protein–protein or protein ligand interactions have become increasingly important over the last decade. Various approaches have been developed including dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA), fluorescence polarization (FP), fluorescence resonance energy transfer (FRET), and homogenous time resolved fluorescence (HTRF) (Hemmila and Webb 1997; Trinquet and Mathis 2006). These assays have been published and commercialized for the detection of various ligands including cAMP. Although an adaptation would have been unproblematic, at present only HTRF has been described for the detection of cGMP.

HTRF is based on the radiation-less energy transfer of from a donor- to an acceptor-fluorophore via Foerster resonance energy transfer (FRET). Thereby, the energy from the excited FRET donor (e.g. via UV-light) is partially transferred to the FRET acceptor, which emits light of a lower wavelength. As FRET is very sensitive to the distance of both molecules, this approach can be used to detect the interaction of a labelled ligand (e.g. cGMP) with a labelled receptor (e.g. anti-cGMP antibody). However, one of the major drawbacks in the development of such an approach has been the background fluorescence of complex assay mixtures such as cell lysates or human sera, which resulted in serious limitations of sensitivity (Mathis 1993). Dilution of samples reduced the impact of the background fluorescence but in turn also decreased the sensitivity. As most background fluorescence is very short-lived ($\approx$4 ns; Trinquet and Mathis 2006), the use of rare earth chelates such as Europium (Eu), Terbium (Tb), or Samarium (Sm) as FRET donors represented a major progress in the development of HTRF. The extraordinary long fluorescence lifetimes of rare earth chelates of up to 1,000 µs combined with time resolved fluorescence detection allowed the temporal discrimination between background and label fluorescence (Mathis 1993, 1995, 1999). Later the thermodynamically more stable cryptates were developed as FRET donors (Mathis 1999; Bazin et al. 2001). Eu^{3+}-Cryptates (Eu^{3+}-tris-bipyridine) are stable in aqueous buffers and can be readily linked to biomolecules such as antibodies. The cryptate-molecule complexes the europium ion, shields it from interactions with signal-quenching buffer components, and transfers absorbed energy to the metal ion (Mathis 1995, 1999). In the case that

the excited europium cryptate is in close proximity to the FRET acceptor XL665 (a chemically stabilized derivative of the algae fluorophore allophycocyanin (Trinquet et al. 2001)), the energy can be partially transferred to this fluorophore and is subsequently emitted as a detectable photon. As the emission spectra of XL665 show virtually no overlap with the emission spectra of the europium–cryptate, both fluorophores can be easily discriminated.

The cGMP HTRF assay is designed as competitive immunoassay. An Europium–cryptate complex (FRET donor) is coupled to the anti-cGMP antibody, whereas the FRET acceptor XL665 is chemically conjugated to cGMP. Once interaction takes place, the antibody-conjugated europium–cryptate complex (activated by an UV-laser pulse) can transfer energy to the FRET-acceptor linked to cGMP. Due to the long lifetime of the activated cryptate, the background fluorescence has already died away when energy is transferred to the FRET acceptor, strongly increasing the signal-to-noise ratio. Subsequently, the FRET-activated cGMP–XL665 molecule emits fluorescence at a wavelength of 665 nm. In the absence of unlabelled cGMP, maximum signal can be detected. Increasing amounts of cGMP in the assay mixture displace the XL665–cGMP complex from the europium–cryptate–antibody complex and result in a concentration dependent decrease of the emission at 665 nm. The cGMP HTRF assay has been described to detect femtomolar amounts of cGMP in biological samples.

HTRF is able to detect cGMP in cell free assays as well as in crude cell lysates rendering the processing of samples prior to cGMP detection unnecessary. As homogenous assay, HTRF can be readily automated and miniaturized to 96-, 384-, or 1,536-well plates allowing the screening of very large numbers of samples. The sensitivity of HTRF for cGMP has been reported to be slightly lower than for other competition based assay formats (see Table 1). However, due to the novelty of the HTRF-based cGMP detection kit, no independent papers have been published yet corroborating the reported sensitivity. As a biophysical approach, HTRF is not as vulnerable to potential enzyme inhibitors as EFC-based assays like HitHunter are. Also antioxidants, which could affect the ALPHAscreen signalling, are unlikely to have an impact on HTRF. One of initial problems in the development of HTRF was the UV absorption of media or cell lysates quenching the available energy to excite the Europium–cryptate complex. This absorption-induced lack of energy would have reduced the cGMP–XL656 light emission resulting in false-positive readings. The parallel detection of the FRET-based fluorescence at 665 nm and a reference signal at 620 nm solved this problem. For this reason, data acquisition systems compatible with HTRF have to be capable of detecting both wavelengths in parallel and, in addition, to handle the delayed light emission after UV excitation. These pre-requisites indicate that HTRF requires more sophisticated plate readers compared to other competitive immunoassays, what might be the major drawback of the HTRF-based detection of cGMP. If compatible hardware is at hand, HTRF offers cGMP detection with high sensitivity and sample throughput for a reasonable price.

2.2.4 cGMP Immunofluorescence

The development of cGMP-specific antibodies (Steiner et al. 1969, 1972b) allowed the development of highly sensitive and reliable biochemical cGMP detection assays as described above. In parallel, these antibodies raised the possibility to detect and localize cGMP in tissues or cells by immuno histochemical (IHC) approaches (Bloom et al. 1972; Wedner et al. 1972). The detection of water-soluble molecules of low molecular weight such as cAMP or cGMP by IHC generally suffers from several drawbacks. One problem is due to the different derivatization of cyclic nucleotides to achieve immunogenic cGMP/cAMP–protein conjugates. Whereas specific antibodies were raised against conjugates of the 2′-succinylated derivatives of the corresponding cyclic nucleotides (Steiner et al. 1969, 1972a), the widely used IHC protocols are based on the formaldehyde or paraformaldehyde fixation of tissues, which result in different derivatizations of cGMP compared to the initial immunogen. Early publications describing IHC for the detection of cAMP omitted therefore any fixation step to avoid the formation of less immunogenic derivatives of cellular cAMP (Wedner et al. 1972). As the omission of tissue fixation resulted in the loss of most cellular cyclic nucleotides (Cumming et al. 1980; Ortez 1980), later publications applied a paraformaldehyde (de Vente and Steinbusch 1992) or formaldehyde fixation, which retained approximately 30% of cellular cGMP levels (de Vente et al. 1996). To overcome the problem of cGMP derivatization by formaldehyde, de Vente and co-workers raised specific antibodies against formaldehyde fixed cGMP–protein conjugates (de Vente et al. 1987). Although this approach offered some advantages, the feasibility and specificity of the commercially available and widely used antibodies against succinylated derivatives of cGMP for IHC in formaldehyde- and paraformaldehyde-fixed tissues was shown by different publications and methods (Mehlhorn et al. 2000; Bloch et al. 2001; Korkmaz et al. 2005; Tian and Yang 2006; Werkstrom et al. 2006) such as gas phase acetylation of tissue cAMP, which resulted in the same increase in sensitivity as observed in RIAs (Chan-Palay and Palay 1979; Rosenberg et al. 1979). Nowadays, the most widely applied IHC protocols are based on 3–4% formaldehyde (de Vente et al. 1996; Mehlhorn et al. 2000) or paraformaldehyde-fixed tissues (de Vente and Steinbusch 1992; Bloch et al. 2001; Tian and Yang 2006), permeabilization, and subsequent cGMP detection by commercial available antibodies raised against the succinylated derivatives of cGMP in a dilution range from 1:100 to 1:600. cGMP immunofluorescence is not comparable to any of the other biochemical approaches to detect cGMP, as the goal of IHC is not the precise quantification of cGMP with the highest possible sensitivity but its intracellular localization in tissue slides or cells. Thus cGMP IHC is able to offer unique information about the distribution of cGMP. To some extent, IHC is also able to detect alterations of tissue cGMP concentrations, however, this detection is of a more qualitative rather than quantitative character. cGMP IHC is a well-established method able to give valuable complementary information about the distribution of cGMP in tissues. Nevertheless, this method has to be accompanied by one of the above-mentioned cGMP detection assays if the accurate quantification of cGMP is of interest.

3 Summary

Four decades of cGMP research have left today's scientists in the beneficial situation to choose from a broad variety of established and commercialized methods to quantify cGMP derived from enzymatic assays or extracted from biological samples. All the methods presented here can be successfully used to quantify cGMP; nevertheless, the applied approach should be chosen carefully to meet the respective demands. The perfect cGMP detection assay should be: highly sensitive and specific, non-radioactive, cheap, easy to handle, able to directly detect cGMP in samples without any purification, offer high sample throughput, be robust against any kind of interference, and adaptable to any kind of standard plate reader. None of the described approaches is able to meet all of these requirements; however, it is easily possible to narrow down the choice based on the respective research aims and lab equipment.

The primary goal, the quantification of cGMP with sufficient sensitivity, is fulfilled by all presented assays with an exception of PDE-Glo. This PKA-based luminescent approach has been designed to detect the PDE-induced decrease of artificially high cGMP concentrations, making it unfeasible for the detection of low levels of cGMP in biological samples. Next, it should be taken into account that the use of radioactivity as detection system has some severe drawbacks, namely the half-life of some common isotopes, the disposal of waste, the need for special facilities, staff training, and hardware to quantify radioactive samples. To avoid these problems, and as there are various non-radioactive alternatives of matching or even higher sensitivity available, it might be worth to decide against radioactive approaches such as RIA or SPA. In addition, these assays demand labour-intensive sample purification, drastically reducing the sample throughput.

From this short list, the appropriate approach should be chosen pragmatically based on the respective needs e.g. estimated usage and sample throughput, price, and compatibility with existing data acquisition hardware. For the infrequent cGMP detection of a manageable amount of samples commercially available ELISAs represent a good choice. Although these kits are quite expensive compared to the amount of samples that can be processed, ELISAs offer high sensitivity, are easy to handle, allow the detection of cGMP in cell lysates without pre-purification and are compatible with most 96 well plate readers. If cGMP needs to be detected more frequently, commercial ELISAs can become an expensive choice and other methods might be taken into consideration. In the case that HPLC and or LC/MS hardware is at hand and established, the direct quantification of cGMP can be a good choice at a reasonable price. The sensitivity, especially when combined with MS is very good, and if sample injection and data processing is automated, a fair amount of samples can be analysed. Also, the approach of enzymatic cycling might be considered as cheap and sensitive alternative that can be adapted to many plate readers. However, as it might take some time to establish and optimize the assay conditions, the amount of samples should be high enough to justify the effort. Moreover, it should be considered that samples have to undergo a pre-purification procedure prior to the determination of cGMP levels.

Recently, three assay formats developed for high throughput screenings, namely ALPHAscreen (Perkin Elmer), HitHunter (DiscoveRx), and cGMP HTRF (Cisbio), have become commercially available. All three assays are able to detect femtomolar amounts of cGMP in cell lysates with high specificity rendering sample purification unnecessary. In addition, washing and precipitation steps are omitted resulting in easier handling and increased sample throughput. ALPHAscreen, HitHunter, and HTRF can be used in 96, 384, and 1,536 well plates and should meet most demands with respect to sensitivity and sample throughput. The question which of these assays is appropriate for the respective application is not easy to answer and depends presumably on the existing data acquisition hardware. Although available at a reasonable price and kit size, HTRF requires more sophisticated plate readers making this assay unfeasible for laboratories, not equipped with the appropriate hardware. The requirements for ALPHAscreen with respect to plate readers are lower. However, the available kit size of at least 10,000 test points (and the corresponding price, especially in combination with the necessary Protein A kit) might be oversized for many applications. In contrast, HitHunter is available at a smaller kit size (see Table 1) and generates a chemiluminescence signal that should be detectable with standard luminescence counters. Furthermore, it might be possible to adapt the assay to standard filter-based plate readers by using commercially available chromogenic β-galactosidase substrates, albeit the sensitivity of the assay could be reduced. The fact that HitHunter is the only competition-based immunoassay that offers a gain-of-signal read-out is an additional advantage making this assay a good choice for processing large numbers of samples. Nevertheless it has to be taken into account that although these novel assay formats represent a promising alternative to established methods, there are currently no independent publications available to support the potential feasibility of these assays for the biochemical detection of cGMP aside high throughput screening applications.

4 Concluding Remarks

During the last four decades, a broad range of assays have been conceived and established pushing the boundaries of cGMP detection with respect to sensitivity and sample throughput to the technical limit of the respective time. Some of the described approaches for the biochemical detection of cGMP are no longer used as they offer only limited sensitivity, are laborious, and allowed only the processing of a small number of samples. However, to the respective time, these assays represented state-of-the art approaches and are worth to be mentioned to describe the step-wise evolution of the biochemical cGMP detection systems from the first thin layer chromatography to actual high throughput approaches.

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