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Understanding the viral load during the synthesis and after rebinding of virus imprinted particles *via* real-time quantitative PCR†

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In the present study, virus imprinted particles have been synthesized for recognizing and specifically binding viruses. These materials may be used for biomimetic sensing schemes and for selective removal of virus particles. Virus imprinting procedures require careful optimization of the synthesis route for obtaining selective and efficiently binding imprinted materials. A remaining limitation has been a facile method for the quantification of the viral load during the imprinting process. Herein, human adenovirus (AdV) was selected as a model virus facilitating the development and application of a rapid virus quantification method based on a molecular biological approach. A real-time quantitative polymerase chain reaction, a.k.a., the qPCR method was developed for monitoring the AdV viral load during the synthesis of AdV imprinted particles, and subsequent rebinding studies. The developed analytical strategy allows the direct, rapid, and sensitive quantification of human adenovirus type 5 concentrations during synthesis and application of AdV imprinted polymers (AdV-MIPs) with a broad dynamic range suitable for both application scenarios. In addition, it was demonstrated by gel electrophoresis analysis that viruses indeed bind to the beads even after several washing steps.

Introduction

Adenoviruses (AdVs) are ubiquitous, and are widely found in the environment.[1] These ds-DNA viruses cause a wide spectrum of illnesses including acute symptomatic and persistent asymptomatic infections. The main target concerning the human adenovirus is the respiratory tract. It is transmitted *via* droplets of respiratory or ocular secretions, and causes acute follicular conjunctivitis, epidemic keratoconjunctivitis, and – albeit less frequently – cystitis and gastroenteritis.[2–4] AdV is increasingly recognized as a significant viral pathogen, especially if already immunocompromised patients are infected. In such scenarios, accurate and timely diagnosis are of utmost importance.[5,6] Conventionally, AdVs are detected using either electron microscopy,[7] antigen-based assays[8] or virus isolation in cell cultures.[9] These methods are frequently associated with low sensitivity and extended time periods to perform the assay.[10] To overcome these problems, real-time quantitative polymerase chain reaction (qPCR) has been introduced as a rapid, sensitive, and reproducible analytical approach for virus quantification.[11]

Complementarily, in 2012, Cumbo *et al.*[12] published a surface imprinting approach for the production of biomimetic materials with virus recognition properties. In this study, binding of the virus to imprinted and control particles was analyzed *via* visual inspection and an ELISA test, yet it was of limited sensitivity and without quantification. Herein, we present a significantly more sensitive and quantitative strategy for analyzing virus imprinted particles based on real-time quantitative polymerase chain reaction, a.k.a., qPCR.

A major advantage of qPCR *vs.* ELISA tests and conventional PCR is its significantly enhanced sensitivity. In general, real-time PCR is considered to be approx. 1000-times more sensitive than ELISA, and 10–100-times more sensitive than conventional PCR.[13–15] High assay sensitivity is of particular relevance, if the virus titer is low, as usually encountered during the detection in mixed infections, or for the analysis of newly introduced infections of vector-transmitted diseases. Furthermore, qPCR provides an unsurpassed linear range, and allows for the simultaneous analysis of up to 96 samples.[16] Moreover, ELISA assays require specific labelling of the primary antibodies for each test. Last but not least, cross-reactivities may occur with the secondary antibody leading to non-specific signals.[17,18] In contrast, PCR primers are more universal and tailorable, and may be readily produced for any virus

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of interest. Likewise, conventional PCR reagents are subject to a reduced shelf life, limited stability, and significant production costs.[19]

Due to the limitations of PCR reagents mentioned above, cheap, robust, and potentially reusable artificial/synthetic receptors mimicking the recognition properties of antibodies would be beneficial for a variety of biomedical and environmental applications in virus diagnosis and removal and are of substantial interest.[20]

In recent years, molecular imprinting strategies have matured towards materials with recognition abilities comparable to their natural analogues even for large biomolecules such as proteins, as well as whole cells and even virus particles. Consequently, molecularly imprinted synthetic receptors for amino acids and proteins,[20,21] environmental pollutants,[22] as well as drugs and food constituents have successfully been generated.[23,24] Applications of these biomimetic recognition materials have been shown for product purification, monitoring of toxic pollutants in food or the environment, and rapid and reliable chemical analysis and diagnostics.[25–28]

Concerning the template species, imprinting voluminous biomolecular targets such as viruses remains challenging (Fig. 1) due to their intrinsic properties including a fragile architecture, large dimensions, and limited stability in organic solvents.[29]

Currently, in the field of medicine, healthcare, and biotechnology, the separation and removal of viruses from cell-culture media and cell debris represent a highly relevant topic. In particular, inefficient processing/removal results in significantly increased efforts during the development of gene therapy treatments, medical diagnostics, and vaccines.[30] In addition, since human pathogenic viruses were used, the future application of the developed VIPs in human diagnostics is evident.

To date, during virus imprinting, the viral load was determined *via* time-consuming plaque assays[31] or expensive sandwich ELISA tests.[12] In the present study, we demonstrate that qPCR is a suitable method for determining viral loads during virus imprinting for the example of AdV selected as a model virus. A rapid and sensitive method for quantifying viral loads of AdV during the imprinting process and also during the application of thus obtained materials for individual and multiple rebinding studies was established providing a suitable strategy for addressing this vital aspect in virus imprinting. Furthermore, the pronounced binding between virus imprinted beads and template virus particles even after several washing cycles was demonstrated *via* gel electrophoresis. The synthesis strategy for virus imprinted polymer particles is reported in detail elsewhere.[32]

Results and discussion

Quantitative polymerase chain reaction (qPCR)

The real-time quantitative polymerase chain reaction is an effective tool for the detection and quantification of DNA and RNA. This technique is highly sensitive, and even low copy numbers of viral targets are detectable. In the specific case of ds-DNA adenovirus, no additional step of the reverse transcription of viral RNA into DNA is required, *i.e.*, viral DNA can be directly amplified by DNA polymerase.[33–35] There are different methods to amplify and detect PCR products such as oligonucleotides[36] and fluorescently labelled primers.[37] However, due to the complexity of these approaches, commercially available methods are nowadays predominantly used. For example, fluorogenic probes are usually based on fluorescent resonance energy transfer (FRET) systems including, *e.g.*, TaqMan, mole-

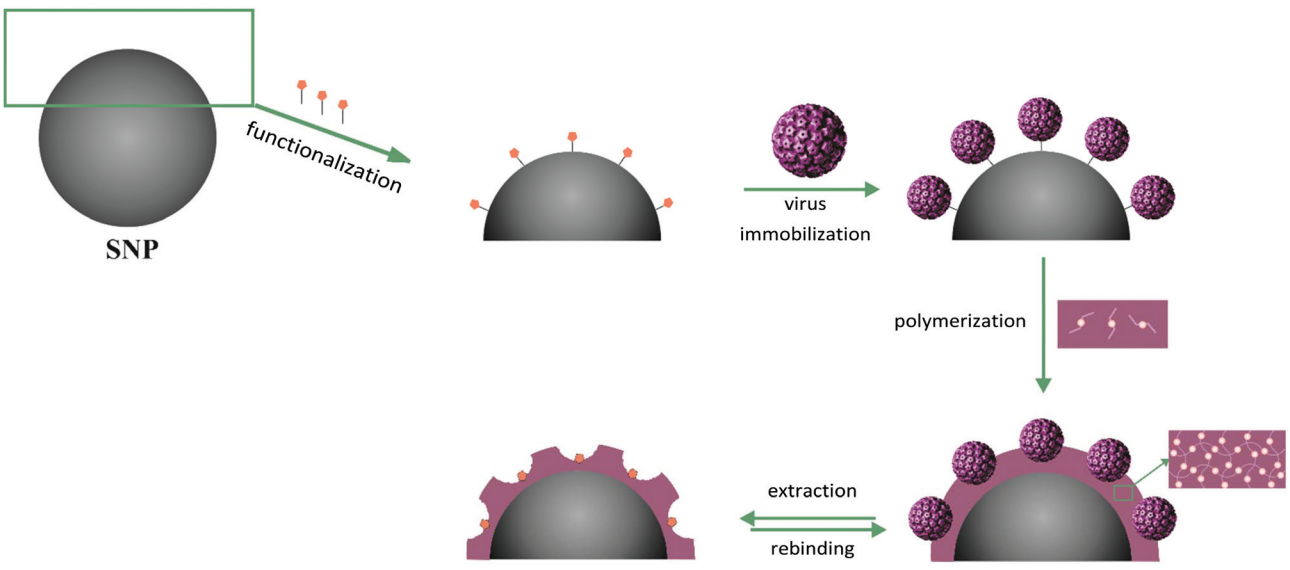

Fig. 1 Schematic of virus imprinting. Starting with the surface functionalization of silica micrometer-sized particles (SPs), the virus is immobilized at the particle surface. The imprinting process is initiated by the co-polymerization of selected organosilanes. Finally, the virus is removed, and a surface-imprinted hybrid core–shell particle is obtained.

cular beacons, and SYBR Green I, which is a ds-DNA-binding dye.[33] Due to its high sensitivity and specificity, the TaqMan approach was selected in the present study for the detection and determination of PCR products.

In order to determine the sensitivity and linear range of the AdV qPCR assay, 10-fold serial dilutions of a concentrated stock solution of AdV were prepared. The DNA of each sample was purified by using a QIAamp® DNA Mini Kit, and the purified DNA samples were applied as targets during the qPCR measurements. The amplification of the AdV substrate was then correlated with the input amount of AdV (see Fig. S-1†). When plotting the AdV input against the obtained C_t (Fig. 2), the correlation was linear across four orders of magnitude, *i.e.*, ranging from 1×10^3–1×10^6 IU AdV. Lower amounts (1×10^2–10^1 IU) of AdV were still detected, however, the C_t values were considered to be outside the linear range of the assay with unreasonably high standard deviations. At the high concentration end, 1×10^7 IU AdV could not be analyzed due to the DNA elution step, and hence, a 10-fold dilution of the DNA was used during isolation.

Subsequently, the ability of the assay to quantify complete viral particles was tested. The potential ability to quantify viral DNA without any DNA purification step derives from the fact that the capsid of the viruses is cracked at high temperatures, and the viral DNA is released.[38] Hence, in the present study, the sample was added directly, *i.e.*, without the DNA isolation step into a PCR tube, and the DNA was amplified. When using AdV without the prior DNA isolation step as an input for the assay, a linear correlation between the input virus dilution and C_t values was again obtained across seven 10-fold serial dilutions of the initial virus stock. Similar to the results obtained with the purified DNA, higher dilutions of the viral supernatant were still detectable (38–40C_t), yet considered to be outside the linear range of the assay (see Fig. S-2†).

When determining the AdV load in the supernatant of virus imprinting experiments, the same procedure has been applied. When frequently using the qPCR assay, executing the assay without DNA isolation is beneficial in terms of time and cost, as the AdV load is directly determined in the sample of interest. It was found that the qPCR assay was highly reproducible with a standard deviation of max. 0.42C_t values across different assays for each dilution.

For determining the intra-run reproducibility, three aliquots from each sample (1×10^7 and 1×10^1) were analyzed in the same run. The coefficient of variation (CV) was 1.23% in the AdV content (data not shown). The inter-run variation was evaluated for 6 individual samples by determining the AdV content during 3 independent experiments. As a result, an average CV of 0.93% was determined. It is hypothesized that due to the loss of DNA during the extraction and purification steps, the coefficient of variation (1.46%) was lower when using AdV directly (Fig. 2).

Actually, this rather minute loss appears negligible. In turn, the time-saving aspect and cost-effectiveness are indeed considered a major aspect; 96 samples – *i.e.*, 48 in duplicate – may be amplified simultaneously within less than two hours. Furthermore, such rapid quantification routines could certainly be adapted to other viruses.[32] Nevertheless, considering studies in real-world samples such as urine or blood, one may resort to the qPCR method with prior DNA pre-isolation steps, thereby avoiding any of these limitations.

qPCR during virus imprinting and rebinding experiments

Virus imprinted particles were prepared using a similar strategy reported by Shahgaldian *et al.*[39] The imprinting procedure (detailed synthesis strategy reported elsewhere[32]) is divided into three main steps: (i) covalent anchoring of the viral particles (AdV) at the surface of functionalized silica micrometer-sized particles, (ii) growth of an ultra-thin polysiloxane layer, and (iii) removal of the template virus resulting in surface binding sites at thus obtained core–shell hybrid particles.

To monitor the progress of the first step (i) during the imprinting process, samples were collected from the supernatant reaction solution at regular intervals, and the amount of remaining AdV was determined *via* qPCR. Each experiment was repeated three times. The results were normalized as the percentage of the virus initially added (*i.e.*, at $t = 0$). As shown in Fig. 3, thus obtained particles reveal particularly pro-

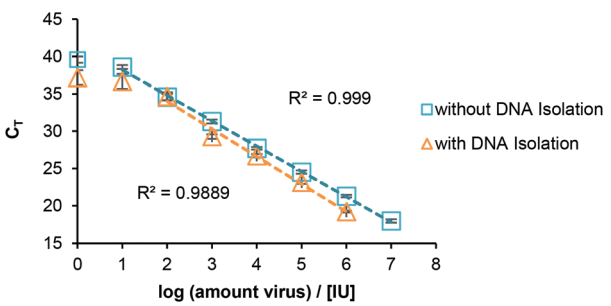

Fig. 2 Linear calibration function for AdV standards with the DNA isolation step (orange triangles) and AdV without prior DNA isolation (blue squares). The threshold cycle (C_t) of each sample was plotted against the logarithm of the number of IU of AdV. Each dilution was assayed in triplicate.

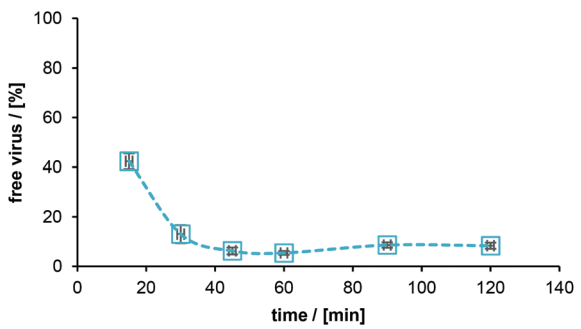

Fig. 3 Quantification of AdV in the supernatant during the first step of the imprinting procedure. To monitor the progress of the imprinting process, samples were collected from the reaction supernatant at regular intervals, and the amount of remaining AdV was determined using the qPCR. Each experiment was repeated three times. The results were normalized as percentage of the virus initially added (*i.e.*, at $t = 0$).

nounced kinetic binding characteristics. After an incubation time of 45 min, the remaining target concentration was nearly constant indicating that the binding kinetics is indeed rather fast. This is of particular importance for the practical utility of virus imprinted particles as rapid adsorption and detection of viruses are an advantage *vs.* more time-consuming strategies and are beneficial in a wide variety of application scenarios.[8,9] In fact when optimizing the rebinding process, equilibrium rebinding after 30 min has been achieved (data not shown[32]).

After the second step of the imprinting procedure, the obtained measurements did not show any virus in the supernatant. Furthermore, thus obtained virus imprinted beads were analyzed after the third step of the imprinting procedure regarding their selectivity *vs.* non-imprinted control particles (NIPs), and also investigated for their reusability. A standardized contact time with AdV of 30 min was selected. After rebinding, the particles were regenerated by applying a mixture of 1 M HCl and Triton X-100 (0.01% v/v). As Fig. 4 indicates, the virus imprinted beads not only show exceptional selectivity against the corresponding NIPs, but also are at least usable three times after regeneration with only a minimal loss in binding capacity.

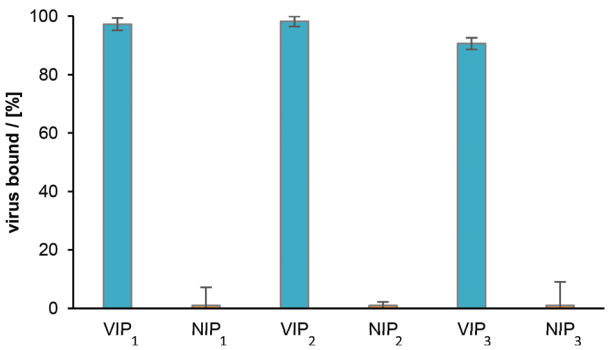

Fig. 4 Comparison of VIP and NIP, and multiple rebinding after regeneration of VIP/NIP. Multiple use of VIP/NIP with regeneration after binding using 1 M HCl and Triton X-100 (0.01% v/v). Values are normalized as percentage to the initially added amount of virus.

Gel electrophoresis

Gel electrophoresis is used as a standard procedure for separating DNA by size (*i.e.*, length in base pairs). Hence, *e.g.*, during purification, one may accurately determine the length of a DNA segment *via* analysis using agarose gel in comparison with a DNA ladder. Therefore, virus imprinted micrometer-sized particles were incubated with virus particles during rebinding experiments, washed three times with PBS, and then suspended in PBS. Thereafter, the suspension was heated to 95 °C – as during the PCR cycles – while shaking for 10 min. This procedure ensures that the bound virus particles disintegrate by heat, and the contained DNA is released. After centrifugation, a sample of the supernatant was used for the qPCR studies. Likewise, samples were analysed after each

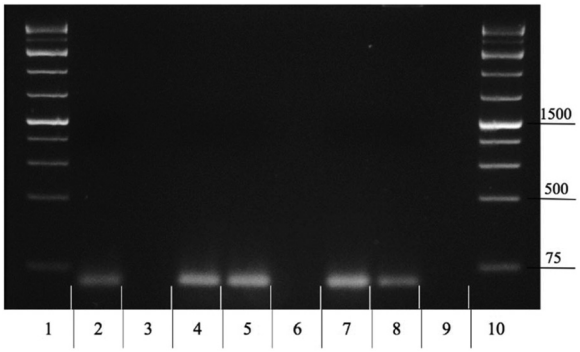

Fig. 5 Image of a gel electrophoresis in a 4% agarose gel. EtBr was added to the gel before electrophoresis and to the TAE buffer. Separation was performed at 100 V for 1.5 hours. The gel was exposed to UV light and the picture taken with a gel documentation system. Lane 1 ladder, lane 2 positive control (AdV 1.00 E4 IU), lane 3 negative (PBS), lanes 4 and 5 supernatants of washed and heated VIPs, lane 6 empty, lanes 7–9 supernatants of washing step 1–3 and lane 10 ladder.

washing step. However, the purpose of these measurements was not to demonstrate that virus can get removed by washing, rather that all viruses analysed after heating of the beads indeed originated from strongly bound adenovirus, *i.e.*, contributions by virus that was attached to the beads *via* weak interactions could be efficiently eliminated. The obtained PCR products were analyzed on a 4% agarose gel stained with ethidium bromide (Fig. 5). Lanes one and ten show the molecular weight markers. All other lanes show a band with the expected molecular weight of approx. 55 base pairs (bps). This is in accordance with the reference sequence of human adenovirus type 5, from which the primer and the probe were designed as well. The positive control in lane two containing 1.00 E4 IU AdV and the negative sample in lane three with only PBS also revealed the anticipated results. Lanes four and five contain the PCR products of the supernatants of VIPs, which were subject to incubation with virus solutions during rebinding experiments, washed, and heated. Both reveal a signal at the same height as the positive control. Hence, the viruses could be unambiguously determined by this approach as being indeed bound to the VIPs. Furthermore, it was confirmed that all the determined virus DNA resulted from virus particles attached to beads, as confirmed by the results obtained in lanes seven to nine. These lanes show the PBS supernatants after washing the beads that were incubated with virus solutions during rebinding studies.

Since lane nine, which represents the third washing step, did not show a band, no more virus could get washed from the beads and all the viruses evident from the bands in lanes four and five have to result from virus that was bound to the particles.

Using this approach, it could be finally confirmed that the adenovirus really bound to the particles, *i.e.*, first during the imprinting procedure, and then during the rebinding experiments. It was furthermore confirmed that the AdV did not get lost, *e.g.*, *via* adhesion to the walls of the reaction chamber during synthesis or rebinding.

Experimental section

Chemicals and materials

6× Orange DNA Loading Dye, O'GeneRuler 1 kb Plus DNA Ladder, TaqMan® Gene Expression Master Mix (5 mL), TaqMan® Primers & Probe and Distilled Water DNase/RNase Free were purchased from ThermoFisherScientific; EDTA was purchased from SERVA Electrophoresis GmbH; ethanol (99.5%) was purchased from VWR Chemicals and QIAamp® DNA Mini Kit (50) from QIAGEN GmbH; glacial acetic acid (≥99.7%) and Trizma® base (99.8%) were purchased from Sigma-Aldrich and sodium hydroxide (NaOH) from Fluka Chemika; Agarose low EEO and ethidium bromide solution 0.07% (5 mL) were purchased from AppliChem. Adenovirus, *i.e.*, human (Mastadenovirus) type 5 was originally purchased from ATCC® VR-5 and was used in a stock solution of 10^7 IU per 5 μL.

DNA isolation

For the DNA purification, a QIAamp® DNA Mini Kit (50) was used. The total DNA was isolated in accordance with the manufacturer's instructions.

TaqMan primer pair and probe

Prerequisites for a successful qPCR include the design of optimal primer pairs, use of appropriate primer concentration, as well as the correct storage of primer and probe solutions. The specific PCR primer pair and probe were designed in agreement with the viral sequence of the NCBI's (National Center for Biotechnology Information, USA) non-redundant protein sequence databases (Reference Sequence: AC_000008.1) based on a hexon of the AdV capsid protein using the primer designing software Primer Express® Software v3.0.1 (Applied Biosystems, Foster City, CA). Afterwards, to ensure the specificity prior to practical application, an *in silico* analysis was performed using the NCBI BLAST + program in order to compare homologous sequences and exclude unspecific amplification, respectively.

The viral titers were determined by quantitative real-time PCR (qPCR) using a StepOnePlus Real-Time PCR-System from Applied Biosystems and the Software StepOne™ v2.3. Primers were designed for the amplification of a 55 bp fragment (forward primer 5′-GTCCATGGGCGCACTCA-3′, reverse primer 5′-GGCGGAGTTGGCGTAGAGA-TAG-3′). The detection of the PCR product was performed using 5′-6-FAM-ACCTGGGCCAAAAC-MGB-3′ as the detection probe.

Rapid determination of adenovirus titers

For the quantification of viral titers in imprinting experiments, a standard curve (1×10^2–1×10^7 IU) was established using an adenovirus stock solution. Aliquots (5 μl) of the adenovirus stock solution or samples containing adenovirus were directly added to the PCR master mix (15 μl) without a DNA isolation procedure. For the amplification of viral DNA, the TaqMan Gene Expression Master Mix (Thermo Fisher) was used as recommended by the supplier. After an initial denaturation at 95 °C for 10 min, 40 cycles (95 °C for 15 s, 60 °C for 45 s) were performed. The efficiency of the qPCR was calculated by the following equation: $E\% = (10^{-1/\text{slope}} - 1) \times 100$.

Virus imprinting

Virus imprinted particles were prepared and analyzed as reported elsewhere in detail.[32]

Rebinding studies

The selectivity of the produced imprinted particles was examined by rebinding assays. Thereby, the unbound virus remaining in the supernatant was quantified by qPCR. All binding assays were performed using the non-imprinted particles as controls. For a typical binding assay, the target was removed from the imprinted beads by removal solution (1 M HCl and 0.01% v/v Triton X-100), washed and then suspended in PBS (final concentration: 6 mg mL^{-1}). Subsequently, 480 μg of these beads were transferred in a 0.5 μL Eppendorf tube, mixed with 77 μg BSA and virus solution (1×10^5–1×10^6 IU). The final volume was adjusted to 105 μL with PBS and the reaction was gently mixed for 30 minutes at room temperature. Afterwards, the tube was briefly centrifuged at 9000 rpm and 5 μl of the supernatants were removed for the virus quantification by the qPCR. During comparative studies, it could be shown that the amount of BSA did not adversely affect the standard curve.

Gel electrophoresis

For the preparation of a 4% agarose gel, 4 g agarose was poured into an Erlenmeyer flask along with 100 mL of 1× TAE buffer. Then it was microwaved for 1–3 min until the agarose was dissolved completely. The agarose solution was cooled down for 5 min, and 3 μL EtBr was added. The agarose was poured slowly into a gel tray with the well comb in place, and the gel was solidified at room temperature for approx. 30 min. To load the samples and run the agarose gel, one volume of 6× DNA loading was added to five volumes of each sample and mixed well. The box was filled with 1× TAE buffer mixed with EtBr (*i.e.*, 3 μL per 100 mL buffer). Then the gel was run using a Bio-Rad 1000/500 Constant Voltage Power Supply at 100 V for approx. 1.5 h. The results were visualized using a fluorescence gel documentation system after irradiation with ultraviolet light.

Conclusions

In summary, the present study shows an optimized method for rapid qPCR measurements for DNA of adenovirus, and demonstrates its utility especially without prior DNA isolation steps. This is a substantial advantage concerning time and costs *vs.* conventional procedures. Furthermore, the precision and reproducibility of the measurement are in fact increased in contrast to the analysis after DNA isolation, as no material loss is encountered. Due to the fact that 96 samples may be simultaneously analyzed, rapid screening in a variety of application

scenarios can be envisaged within short analysis time, which is in contrast to alternative methods such as SDS-PAGE.

Additionally, it was demonstrated using the qPCR that virus imprinted particles reveal exceptional selectivity *vs.* non-imprinted control particles with rapid binding kinetics, and that such imprinted beads readily rebind the target virus after several regeneration steps. Finally, it was demonstrated that a three-step-procedure, *i.e.*, heating of rebound particles, followed by qPCR measurement and gel electrophoresis of the qPCR amplification product provides unambiguous analytical results on the bound virions.

In particular, the combination of the qPCR and molecular imprinting strategies is an innovative and novel approach yielding biomimetic binding materials useful in a wide variety of application scenarios. The qPCR not only enables multiplexed (*i.e.*, highly parallelized) measurements but may also be performed for several targets simultaneously. This is beneficial for future selectivity studies in, *e.g.*, virus mixtures, and provides time and cost savings *vs.* conventional ELISA approaches.

Author contributions

The study was conceived by HS and BM, and executed by MG and SK. The manuscript was written by contributions from all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There is no conflict of interest.

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