Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A label-free immunoassay for Flavivirus detection by the Reflective Phantom Interface technology

Giovanni Tagliabue [a, 1], Valentina Faoro [b, 1], Serena Rizzo [c], Daniele Sblattero [c], Andrea Saccani [d], Gabriele Riccio [a], Tommaso Bellini [a, e], Matteo Salina [a], Marco Buscaglia [a, e, **], Alessandro Marcello [b, *]

[a] Proxentia S.r.l., 20139 Milano, Italy
[b] Molecular Virology Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy
[c] Dipartimento di Scienze della Vita, Università degli Studi di Trieste, 34100 Trieste, Italy
[d] Euroclone S.p.A, Italy
[e] Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, 20090 Segrate, Italy

ARTICLE INFO

Article history:
Received 15 April 2017
Accepted 3 May 2017
Available online xxx

Keywords:
Flavivirus
TBEV
WNV
NS1
Immunoassay
Label-free optical biosensor
Reflective Phantom Interface
Point-of-care
Protein microarray

ABSTRACT

Flaviviruses are widespread and cause clinically relevant arboviral diseases that impact locally and as imported travel-related infections. Direct detection of viraemia is limited, being typically undetectable at onset of symptoms. Therefore, diagnosis is primarily based on serology, which is complicated by high cross-reactivity across different species. The overlapping geographical distribution of the vectors in areas with a weak healthcare system, the increase of international travel and the similarity of symptoms highlight the need for rapid and reliable multi-parametric diagnostic tests in point-of-care formats. To this end we developed a bi-parametric serological microarray using recombinant NS1 proteins from Tick-borne encephalitis virus and West Nile virus coupled to a low-cost, label-free detection device based on the Reflective Phantom Interface (RPI) principle. Specific sequential detection of antibodies in solution demonstrates the feasibility of the approach for the surveillance and diagnosis of Flaviviruses.

1. Introduction

The genus *Flavivirus* of the family *Flaviviridae* consists of a number of viruses that are transmitted to humans by arthropod vectors [1]. Members of this genus include widespread pathogens of great importance for human health delivered by arthropods such as Dengue virus, Zika virus, Yellow fever virus, Japanese Encephalitis virus, West Nile virus (WNV) and Tick-borne encephalitis virus (TBEV). Flaviviruses are highly endemic in the tropical and sub-tropical areas of the world representing a growing risk for travellers and a constant threat to native populations due to the mobility of the vector and the animal reservoirs [2–4].

WNV mainly affects birds, but humans and horses become infected as incidental dead-end hosts causing disease [5]. Of all the mosquito-borne flaviviruses, WNV has the most widespread geographical distribution and the largest vector and host range [6]. The United States experienced a dramatic outbreak of WNV in 1999, which rapidly spread across the country becoming endemic with 44,000 reported human cases and 1911 deaths (CDC ArboNET 1999–2015). WNV infection is generally asymptomatic with about 20% of patients experiencing mild illness with fever and less than 1% developing serious neurological illness such as encephalitis or meningitis. No vaccines or drugs are available for humans and treatment is only supportive for patients with neuro-invasive WNV.

TBEV is the most prevalent autochthonous Flavivirus in Europe [7]. Incidence of TBEV in humans overlaps with seasonal peaks of feeding activity of the ticks in spring. TBEV is maintained in nature by ticks feeding on small mammals of the forest, while humans are occasional dead-end hosts, who become infected by a tick bite or by consumption of raw milk from infected goats. Approximately

* Corresponding author. Molecular Virology Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Padriciano 99, 34149 Trieste, Italy.
** Corresponding author. Proxentia S.r.l., 20139 Milano, Italy.
E-mail address: marcello@icgeb.org (A. Marcello).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2017.05.025
0006-291X/

5000–12,000 cases of TBE are reported in Europe each year [8]. As for WNV, TBEV infection is generally mild, but in some individuals the infection causes damage to the central nervous system, which could be fatal particularly in elderly people. Fatality rates are about 1–2% for the Western TBEV subtype, but much higher for the Eastern subtypes. A prophylactic vaccine derived from inactivated Western TBEV virions is available but no drugs are licensed for the treatment of TBEV.

WNV and TBEV distribution overlaps in Europe and both viruses are a risk in blood transfusions. Serology of these viruses is complicated by the vaccination for TBEV and by the cross-reactivity of the envelope E antigens. Both problems are overcome by the use of the non-structural glycoprotein NS1 as viral antigen for serology [9]. NS1 has a molecular weight of 46–55 kDa, depending on the glycosylation state, and can be found in different oligomeric states and at various cellular locations for specific functions. Intracellular NS1 is required for virus replication, while secreted NS1, which is highly immunogenic, plays several roles in pathogenesis. Protein microarrays coated with NS1 have been shown to discriminate well among immune sera from infection with various Flaviviruses and from vaccination leading to the development of a syndrome-based multi-parametric diagnostic test [10]. However, this study did not include TBEV NS1 in the array.

The development of virological diagnostic tests based on point-of-care testing (POCT) is gaining momentum, particularly following the recent dramatic outbreaks of viruses such as Ebola and Zika. POCT could in fact be the only option in resource-limited settings or during an outbreak when centralized diagnostic facilities are lacking [11,12]. POCT ranges from lateral-flow based mono-parametric strips to more sophisticated devices capable of multi-parametric analysis and data processing/communication [13,14]. Typical requirements of a POCT system are ease-of-use, short time-to-result and portability. To this aim, label-free detection methods can offer intrinsic advantages relative to more standard colorimetric or fluorescence approaches, because they provide real-time measurement of molecular binding without the need of additional reagents or washing steps [15,16]. In this work, we took advantage of a recently developed multi-spot immunoassay based on a novel optical label-free detection method called Reflective Phantom Interface (RPI). In this method, molecular binding is measured by the analysis of images of the light reflected by an interface with a very low reflectivity in aqueous solutions. Ligands interacting with receptors spotted on this surface induce an increase of reflected intensity that provides the readout of the assay without any kind of secondary labelling [17]. Most interesting, the analysis can be performed by a simple device composed by a smartphone with a portable cradle add-on containing only the disposable cartridge and a few optical components, hence devoiding costly electronics or microfluidic components [18]. Assays based on RPI detection were previously demonstrated for the detection of diagnostic biomarkers [17,19], DNA single-base mismatch [20,21] and viral infection in crude plant extracts [22]. Here we show that the RPI technology could be applied to a bi-parametric assay for the simultaneously detection of antibodies against WNV and TBEV NS1 in human serum providing a proof of principle for its application as a multi-parametric diagnostic assay for Flavivirus infections.

2. Materials and methods

2.1. Reflective Phantom Interface method

The principle of the RPI technology relies on the measurement of the intensity of light reflected by a planar interface with very low reflectivity. The binding of molecules in solution on specific probes immobilized on the sensing surface is directly quantified from the increase of reflected intensity without the need of fluorescent or colorimetric markers. The condition of weak reflectivity can be achieved by using a plastic material with refractive index similar to that of water [17] or by a glass prism with an optimized anti-reflective coating [19]. In this work, we exploited the glass RPI substrate embedded in a plastic 1-cm cuvette. RPI chip for anti-NS1 antibody detection were produced by ProXentia (Italy). The glass slides were coated with a multifunctional copolymer, which provided the reactive groups suitable for immobilizing the probes and simultaneously prevented nonspecific adsorption [23]. Viral and control proteins were immobilized in spots of 150–200 μm diameter by a non-contact dispensing system (sciFLEXARRAYER S3; Scienion AG, Germany). The chip surface was illuminated by the collimated light of a LED source with wavelength centered at 450 nm and the image of the reflected light was acquired by a CCD camera. The measured brightness of each spot was converted into the surface density of molecules at the interface as described previously [19]. The measured increase of surface density on the spots provided a quantitative and real-time measurement of the amount of captured target molecules, which depends on their concentration in the sample solution.

2.2. Viral antigens and antibodies

TBEV strain Neudoerfl NS1 (TBEV-NS1) and WNV strain NY99 NS1 (WNV-NS1) recombinant proteins from human cell lines were obtained from the Native Antigen Company (product codes TBEV-NS1-100 batch #15052110 and WNV-NS1-100 batch #248215, respectively). The IgG2ak monoclonals anti-Langat virus NS1 clone 5D9 [24] (TBEV-Ab) and anti-WNV NS1 clone 22-NS1 [25,26] (WNV-Ab) were obtained from BEI Resources (product codes #NR-40304 and #NR-10145, respectively). As a negative control, we used either bovine serum albumin (BSA) or recombinant his-tagged eritropoietin (EPO). An irrelevant anti-mouse monoclonal antibody was used as binding control. Certified HIV/HBV/HCV free human serum AB from Euroclone (ECS0219D) was used for some experiments.

3. Results

The sensing surface of the RPI glass slide was spotted with recombinant TBEV NS1 and WNV NS1 at a concentration of 0.5 g/l and 0.25 g/l and BSA or recombinant EPO at 0.5 g/l in PBS (Phosphate-buffered saline solution pH 7.4) using about 100 nL per spot. The prism was inserted into a standard 1-cm plastic cuvette and immersed in the incubation buffer (150 mM NaCl, 1% BSA and 50 mM Tris-HCl pH 7.6). After thermal equilibration, increasing concentrations of antibodies were added to the solution in the cuvette under continuous stirring to provide efficient mixing. The binding of the antibodies with the immobilized probes was quantified by the analysis of the RPI images, acquired by a simple optical set-up fabricated on an optical breadboard. The sensing surface was illuminated by the LED and the reflected light was acquired by the CCD camera (Fig. 1a and b). The device enables to analyze about 70 spots of different probes on the same RPI chip. In this work, each type of spot was present in multiple copies, from 6 to 15 (Fig. 1c), and the signal from the spots of the same kind was averaged in order to increase the signal-to-noise ratio. Upon the addition of the target antibodies, the brightness of the corresponding probe spots started increasing with time. As represented in Fig. 1d, the rate of this curve is related to the kinetics of the molecular recognition process and the asymptotic value provides the amount of bound molecules at equilibrium. In practice, the signal of each spot was converted into the amount of molecules adhering on the surface as described in Ref. [19] and the resulting binding curve was fitted

Please cite this article in press as: G. Tagliabue, et al., A label-free immunoassay for Flavivirus detection by the Reflective Phantom Interface technology, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.05.025

with an exponential growth function to extract the amplitude and rate.

In order to characterize the interaction parameters with TBEV-NS1 and WNV-NS1 antigens, increasing concentrations of antibodies were added in the measuring cuvette. Alternated additions of TBEV-Ab and WNV-Ab were performed to evaluate the possible cross interaction between the two types of antigens. Fig. 2 reports the behavior of the surface density measured on different spots as a function of time. A marked change of slope is observed only when the concentration of the corresponding antibody is increased, hence demonstrating negligible cross interaction. Moreover, the binding is observed only on TBEV-NS1 and WNV-NS1 spots, whereas no signal is measured on the surface regions outside the spot or on the control spots of BSA, both represented by black lines in the figure. This confirms the negligible amount of non-specific interaction on the sensing surface. Fig. 2 also shows larger amplitudes of the binding curves for WNV-NS1 spots relative to TBEV-NS1. This indicates a larger availability of antigen recognition sites on the spot surface. Similarly, for both interactions, larger spotting concentrations of antigen yielded larger values of surface density, although the scaling is not proportional – i.e. a two-fold increase of spotting concentration yields a smaller increase of surface density. This indicates that the spotted surface is close to a full monolayer of antigen molecules. The inset of Fig. 2 reports an enlarged view of the binding curves at the smallest concentrations, from 0.5 ng/ml to 23 ng/ml, This figure indicates that the spots of WNV-NS1 yielded a detectable change of slope relative to the baseline signal at antibody concentrations as low as 0.5 ng/ml.

The amplitude and the rate of each binding curve measured after the addition of the antibody to a final concentration c was extracted from the fitting with a exponential growth function. Fig. 3a reports a set of data measured for WNV-Ab and the fitting curves. The extracted asymptotic value of the exponential functions σ_{eq} is reported in Fig. 3b (blue circles). These data are fitted with a sigmoidal curve given by

$$\sigma_{eq} = \frac{\sigma_0}{1 + \frac{K_d}{c}} \quad (1)$$

from which the value of the saturation surface density σ_0 and of the dissociation constant K_d is obtained. Analogously, the rates of the binding curves provide information on the kinetic rate constants for association, k_{on} and dissociation, k_{off}, where $K_d = k_{off}/k_{on}$. As previously described [19], a rather robust approach to obtain the kinetic parameters from the binding curves at low concentrations is based on the analysis of the slope of the initial linear increase after the sample addition, $\Gamma_i = \sigma_0 k_{on} c$. The values of Γ_i extracted from the data of Fig. 3a are reported in Fig. 3c. From the linear fit of this values we extracted the rate constant k_{on}, whereas k_{off} was estimated as $k_{off} = k_{on} K_d$. The interaction parameters obtained from different experiments are reported in Table 1. The dissociation constants are all in the range 10–100 pM. For WNV-NS1, similar values are observed when using trehalose buffer or PBS buffer in the spotting solution. In contrast, a higher affinity is obtained with TBEV-NS1 spotted with buffer without trehalose. A similar trend is also observed for the maximum surface density σ_0 estimated at high concentration. The maximum values of σ_0 reported in Table 1 correspond to about 10^9 antibodies on a surface of 1 mm^2, or about 10^8 antibodies per spot. A smaller value of surface density is measured on TBEV-NS1 spotted with trehalose added to the PBS buffer. In general, all the considered interactions display rather high affinities (low values of K_D), which are primarily ascribed to the fast association kinetics, as indicated by k_{on} values in the range $2 \cdot 10^7$ - $6 \cdot 10^7$ $s^{-1}M^{-1}$.

The molecular recognition performance of WNV-Ab and TBEV-Ab antibodies was studied also in human serum. RPI sensing

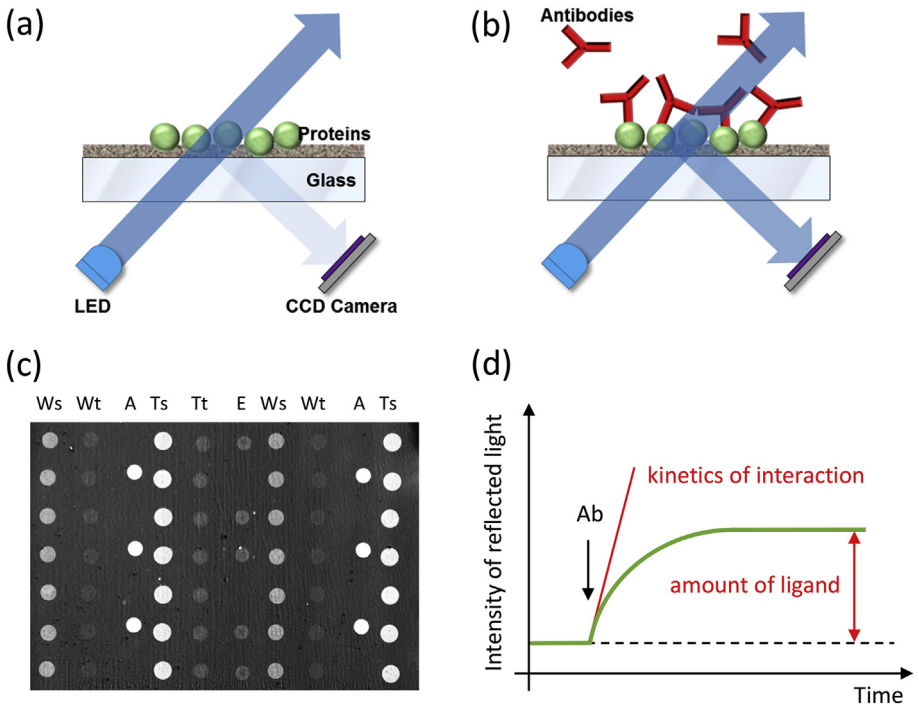

Fig. 1. Design of the RPI method. (a) Schematic representation of the RPI sensing surface and the optical set-up. Recombinant viral proteins are covalently immobilized on the surface of a glass RPI chip. The analysis of the image of the reflected light enables a quantification of the molecular layer. (b) The reflected light intensity increases upon binding of antibodies to the surface immobilized proteins. (c) RPI image of the surface spotted with WNV-NS1 in buffer with trehalose (Wt) or PBS buffer (Ws), TBEV-NS1 in buffer with trehalose (Tt) or PBS buffer (Ts), anti-mouse antibody (A – positive control protein) and EPO (E – negative control protein). The spot diameter is between 150 mm and 200 mm. (d) Schematic drawing of the binding curve and the parameters extracted from the analysis.

Please cite this article in press as: G. Tagliabue, et al., A label-free immunoassay for Flavivirus detection by the Reflective Phantom Interface technology, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.05.025

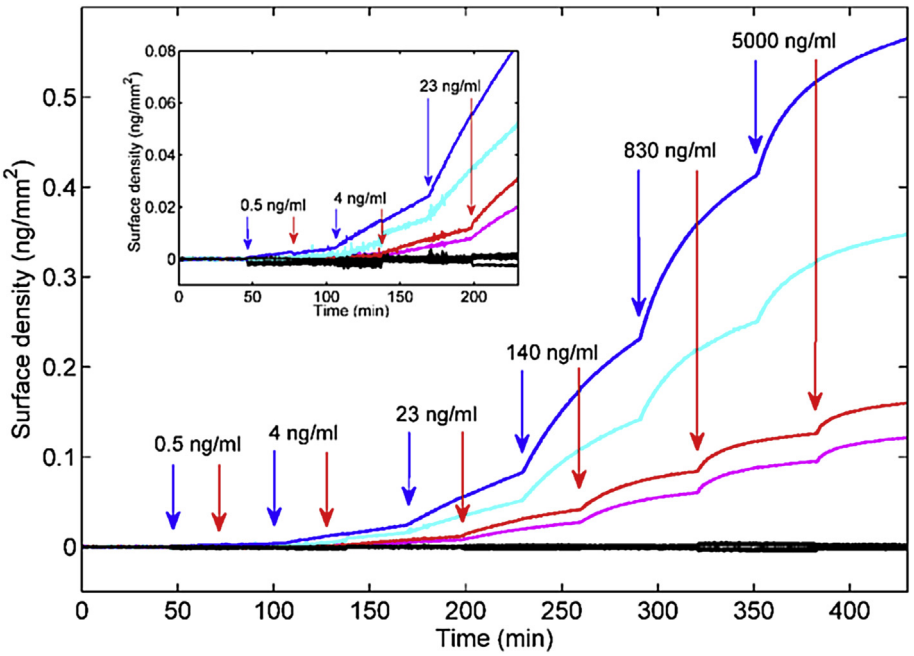

Fig. 2. Binding curves of specific antibodies to NS1 antigens. Increasing concentrations of WNV-Ab and TBEV-Ab are added alternately in the measuring cuvette containing the RPI chip spotted with WNV-NS1 and TBEV-NS1 at 0.5 g/l (blue: WNV; red: TBEV) and 0.25 g/l (cyan: WNV; magenta: TBEV) in the presence of trehalose. The black lines report the signal obtained from BSA and from circular areas surrounding the spots. The curves represent the behavior averaged on all spots and circles of the same kind. Antibodies were added one after the other as indicated by the arrows (blue: WNV-Ab; red: TBEV-Ab). The approximate final concentration reached in the cuvette after each addition is reported. The corresponding precise values are 0.54, 3.71, 22.7, 136.9, 826.6, 5005 ng/ml for WNV-Ab and 0.57 3.89 22.5139.7831.8 4965 ng/ml for TBEV-Ab.
Inset: enlarged view of the early time points of the curves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chips spotted with WNV-NS1,TBEV-NS1 and EPO as negative control were pre-incubated with 50% serum and then increasing concentrations of antibodies were added to the solution in the cuvette. Fig. 4a and b report the behavior observed adding WNV-Ab and TBEV-AB, respectively. Several differences are present relative to the experiments performed in buffer. In general, the amplitudes of the binding curves are significantly smaller. Through the analysis described above, this effect is ascribed to larger values of the k_D, which in the presence of serum is found to be in the range 0.5–2 nM, as reported in Table 2. The estimated surface density σ_0 at high concentrations is somewhat smaller when the antigens were spotted with trehalose but maintain values similar to those observed in buffer. For what concerns the kinetics of the antibody-antigen interaction, in serum the observed values of k_{on} were remarkably smaller, in the range $0.2 \cdot 10^7\ s^{-1}M^{-1} - 2 \cdot 10^7\ s^{-1}M^{-1}$. This suggests that the smaller strength of the interaction in serum can be primarily ascribed to the slower kinetics for association.

4. Discussion

In recent years, we are witnessing a transition in healthcare systems worldwide to decentralize the practice of healthcare outside of traditional settings. This transition is due to several factors including dramatic outbreaks of pathogenic viruses, which have exerted pressure to bring diagnosis and care directly to those in need. Indeed, POCT may represent a realistic solution to overcome the diagnostic gap in resource-limited countries and for implementing quick and capillary surveillance of large geographic areas for nascent infectious disease outbreaks [12]. Another important transition is towards the development of automatic multi-parametric syndrome-based assays for a quick anamnestic response. Therefore, efforts are being conducted to develop new concept devices that include these two demands in a user-friendly robust format with a good adherence to the principles of ASSURED (Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment free, or minimal, and Deliverable to end users) [11].

In this context, here we demonstrated the feasibility of the RPI-based test to detect the presence of antibodies towards the NS1 protein of Tick-borne encephalitis virus and West Nile virus. The detection system is based on a low-cost, label-free technology that has the potential of combining accuracy, rapidity and ease-of-use. The measurement is performed directly, without the need of washing steps or the addition of reagents. The time-to-result is limited by the intrinsic kinetic of the molecular recognition process, and therefore depends on the concentration to be detected. As shown in the inset of Fig. 2, in the case of WNV-Ab detection in buffer, about one hour can be required to clearly discriminate 0.5 ng/ml from the background, whereas a few minutes are enough to detect 23 ng/ml.

There is limited data available on the precise quantification of antigen specific antibodies at the serum level. Human antibody serum levels were quantified in immunized samples showing anti-tetanus toxoid specific levels variable from an average concentration of 5 μg/ml [27] up to 20 mg/ml [28]. Similar results have been obtained with anti pneumcoccal specific antibodies. Keeping in mind that usually serum samples are assayed at a typical 1:100 up to 1:1000 dilution the specific antibody serum concentration is compatible with the detection limit of the test. Although no direct antibody quantification data are available for WNV infection the analysis of the frequency of circulating B cells post immunization [29] is in keeping with studies showing that concentration of specific antibodies are proportional to the frequency of specific memory B cells, pointing to a serum antibody titers compatible with the assay also for WNV and other flaviviruses [27].

The data reported in Fig. 4 indicate that the sensitivity decreases when the detection is performed in 50% human serum, where

Please cite this article in press as: G. Tagliabue, et al., A label-free immunoassay for Flavivirus detection by the Reflective Phantom Interface technology, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.05.025

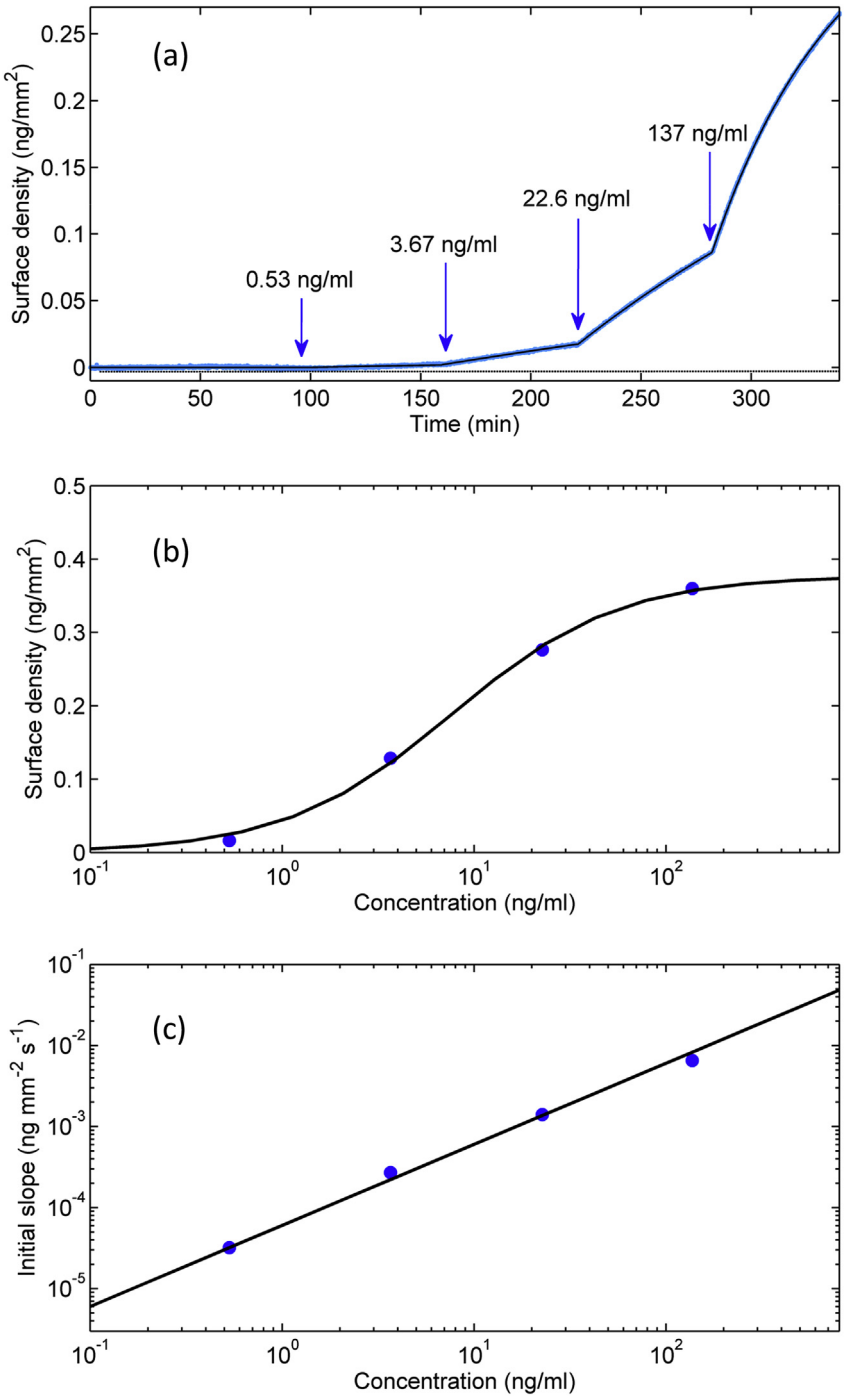

Fig. 3. Extraction of the interaction parameters from the binding curves. (a) The figure shows the binding curve (blue) obtained for WNV-NS1 spotted at 0.5 g/l in the presence of trehalose upon addition of WNV-Ab at the indicated concentrations (arrows). The black curve superimposed to the data represents the fitting with exponential growth functions, from which the rate and the amplitude of the binding is extracted. (b) Equilibrium surface density (blue dots) obtained from the data of panel (a) and fit with the pseudo-first order binding model of Equation (1) (black curve), from which the equilibrium dissociation constant K_D is obtained. (c) Slope of the binding curve immediately after the concentration increase (blue dots) and linear fit, from which the value of the kinetic constant for binding k_{on} is obtained. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher concentrations than those observed in buffer are required to achieve similar amplitudes of response. To note, however, that serum is normally diluted at least 1:100 for standard immunological assays and these data represent an extreme situation with a 1:2 dilution. Comparing the interaction parameters reported in Table 1 (buffer) and 2 (serum), the weaker signal response is ascribed to the larger K_D in serum, which in turn is primarily due to the smaller k_{on}. A possible origin of this difference can be the presence of weak competing interactions between the antibodies and some serum component. It must be noted that the tested antibodies were developed in mice, while natural human antibodies targeting WNV-NS1 or TBEV-NS1 could provide different interactions with serum. A complete characterization of the detection performance of the proposed label-free array will require further tests to be

Please cite this article in press as: G. Tagliabue, et al., A label-free immunoassay for Flavivirus detection by the Reflective Phantom Interface technology, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.05.025

Table 1
Binding parameters obtained in buffer.

Antibody	Spotting protocol	Surface density (ng/mm^2)	K_D (pM)	k_{off} (10^{-3} s^{-1})	k_{on} (10^7 s^{-1}M^{-1})
TBEV	Trehalose 0.25 g/l	0.06	104	5.1	5.6
	Trehalose 0.5 g/l	0.09	100	5.4	5.8
	PBS	0.24	22	1.2	5.5
WNV	Trehalose 0.25 g/l	0.20	55	1.7	3.2
	Trehalose 0.5 g/l	0.28	56	1.5	2.9
	PBS	0.25	77	1.8	2.3

Table 2
Binding parameters obtained in serum.

Antibody	Spotting protocol[a]	Surface density (ng/mm^2)	K_D (pM)	k_{off} (10^{-3} s^{-1})	k_{on} (10^7 s^{-1}M^{-1})
TBEV	trehalose	0.06	1730	18	1.04
	PBS	0.28	990	2.6	0.26
WNV	trehalose	0.03	1480	24	1.62
	PBS	0.38	593	3.2	0.54

[a] Antigens are immobilized using a spotting concentration of 0.5 g/l.

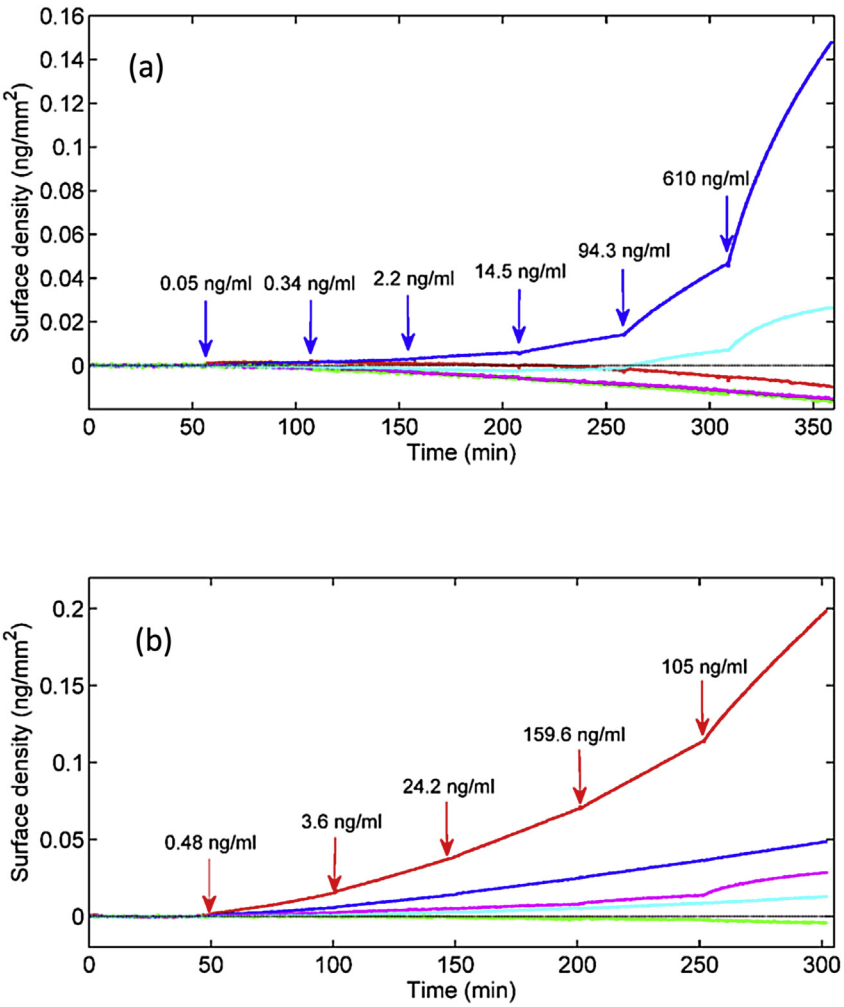

Fig. 4. Binding curves on WNV-NS1 and TBEV-NS1 spots in serum. After equilibration with 50% serum, increasing amounts of WNV-Ab (a) or TBEV-Ab (b) are added in cuvette. The time of addition and the corresponding concentrations are indicated by arrows. The curves correspond to the RPI signal measured on spots of WNV-NS1 with PBS buffer (blue), WNV-NS1 with trehalose (cyan), TBEV-NS1 with PBS buffer (red), TBEV-NS1 with trehalose (magenta), EPO (green). The curves represent the behavior averaged on all spots of the same kind. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: G. Tagliabue, et al., A label-free immunoassay for Flavivirus detection by the Reflective Phantom Interface technology, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.05.025

performed on real infected human blood samples.

An additional feature observed for some of the measured binding curves in serum is the drift of the signal as a function of time. This effect becomes clear on the spots not involved in the specific interaction with the target antibody in solution, i.e. red, magenta and green curve in Fig. 4a and blue, cyan and green curve in Fig. 4b. These drifts could be ascribed to the instability of the non-specific adhesion of serum components on the surface, either within the spotted regions or on the surrounding areas, and at the end limits the accuracy of the analysis for long measuring times. However, these data shows that the drift of the signal can be considered linear for time intervals of at least one hour, and therefore they can be easily compensated by considering the behavior of the baseline signal acquired before the addition of the sample.

To conclude, in this work we propose the use of the RPI technology applied to the serology of Flavivirus infections. We demonstrate that a protein array of the NS1 viral antigens allows the specific and simultaneous detection of antibodies to TBEV and WNV in solution. Such an assay could be easily convertible into a POCT format for multi-parametric syndrome-based diagnostics possibly taking advantage of the widely available smartphone technology [18]. Such promising data for sensitivity and specificity need to be confirmed on human samples from infected patients and robustness could only be assessed in a field trial.

Acknowledgements

Work by AM and DS is supported by grants from the Regione FVG of Italy (FLAVIPOC, SEVARE) and by Beneficentia Stiftung. ProXentia s.r.l. acknowledges the financial support from the European Union Horizon 2020 Programme, SME Instrument call, through the STEFY project (grant agreement no. 718855).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.05.025.

References

[1] B.D. Lindenbach, H.J. Thiel, C.M. Rice, Flaviviridae: the viruses and their replication, in: D.M. Knipe, P.M. Howley, D.E. Griffin, R.A. Lamb, M.A. Martin (Eds.), Fields Virology, Lippincott, Williams & Wilkins, Philadelphia, 2007, pp. 1101–1152.

[2] M.M. Baba, M. Ikusemoran, Is the absence or intermittent YF vaccination the major contributor to its persistent outbreaks in eastern Africa? Biochem. Biophys. Res. Commun. (2017).

[3] M. Baba, C.H. Logue, B. Oderinde, H. Abdulmaleek, J. Williams, J. Lewis, T.R. Laws, R. Hewson, A. Marcello, D.A.P, Evidence of arbovirus co-infection in suspected febrile malaria and typhoid patients in Nigeria, J. Infect. Dev. Ctries. 7 (2013) 51–59.

[4] A. Valderrama, Y. Diaz, S. Lopez-Verges, Interaction of Flavivirus with their mosquito vectors and their impact on the human health in the Americas, Biochem. Biophys. Res. Commun. (2017).

[5] S.L. Rossi, T.M. Ross, J.D. Evans, West Nile virus, Clin. Lab. Med. 30 (2010) 47–65.

[6] H. Artsob, D.J. Gubler, D.A. Enria, M.A. Morales, M. Pupo, M.L. Bunning, J.P. Dudley, West Nile virus in the new world: trends in the spread and proliferation of West Nile virus in the western hemisphere, Zoonoses Public Health 56 (2009) 357–369.

[7] T. Carletti, M.K. Zakaria, A. Marcello, The host cell response to tick-borne encephalitis virus, Biochem. Biophys. Res. Commun. (2017).

[8] L. Lindquist, O. Vapalahti, Tick-borne encephalitis, Lancet 371 (2008) 1861–1871.

[9] D.A. Muller, P.R. Young, The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker, Antivir. Res. 98 (2013) 192–208.

[10] N.B. Cleton, G.J. Godeke, J. Reimerink, M.F. Beersma, H.R. Doorn, L. Franco, M. Goeijenbier, M.A. Jimenez-Clavero, B.W. Johnson, M. Niedrig, A. Papa, V. Sambri, A. Tami, Z.I. Velasco-Salas, M.P. Koopmans, C.B. Reusken, Spot the difference-development of a syndrome based protein microarray for specific serological detection of multiple flavivirus infections in travelers, PLoS Negl. Trop. Dis. 9 (2015) e0003580.

[11] D. Mabey, R.W. Peeling, A. Ustianowski, M.D. Perkins, Diagnostics for the developing world, Nat. Rev. Microbiol. 2 (2004) 231–240.

[12] M. Baba, N. Vidergar, A. Marcello, Virological point-of-care testing for the developing world, Future Virol. 9 (2014) 595–603.

[13] P. Yager, T. Edwards, E. Fu, K. Helton, K. Nelson, M.R. Tam, B.H. Weigl, Microfluidic diagnostic technologies for global public health, Nature 442 (2006) 412–418.

[14] A. Marcello, D. Sblattero, C. Cioarec, P. Maiuri, P. Melpignano, A deep-blue OLED-based biochip for protein microarray fluorescence detection, Biosens. Bioelectron. 46 (2013) 44–47.

[15] G. Gauglitz, Point-of-care platforms, Annu. Rev. Anal. Chem. (Palo Alto Calif) 7 (2014) 297–315.

[16] G. Zanchetta, R. Lanfranco, F. Giavazzi, T. Bellini, M. Buscaglia, Emerging applications of label-free optical biosensors, Nanophotonics (2017), http://dx.doi.org/10.1515/nanoph-2016-0158.

[17] F. Giavazzi, M. Salina, R. Cerbino, M. Bassi, D. Prosperi, E. Ceccarello, F. Damin, L. Sola, M. Rusnati, M. Chiari, B. Chini, T. Bellini, M. Buscaglia, Multispot, label-free biodetection at a phantom plastic-water interface, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 9350–9355.

[18] F. Giavazzi, M. Salina, E. Ceccarello, A. Ilacqua, F. Damin, L. Sola, M. Chiari, B. Chini, R. Cerbino, T. Bellini, M. Buscaglia, A fast and simple label-free immunoassay based on a smartphone, Biosens. Bioelectron. 58 (2014) 395–402.

[19] M. Salina, F. Giavazzi, R. Lanfranco, E. Ceccarello, L. Sola, M. Chiari, B. Chini, R. Cerbino, T. Bellini, M. Buscaglia, Multi-spot, label-free immunoassay on reflectionless glass, Biosens. Bioelectron. 74 (2015) 539–545.

[20] G. Nava, E. Ceccarello, F. Giavazzi, M. Salina, F. Damin, M. Chiari, M. Buscaglia, T. Bellini, G. Zanchetta, Label-free detection of DNA single-base mismatches using a simple reflectance-based optical technique, Phys. Chem. Chem. Phys. 18 (2016) 13395–13402.

[21] C. Zilio, A. Bernardi, A. Palmioli, M. Salina, G. Tagliabue, M. Buscaglia, R. Consonni, M. Chiari, New “clickable” polymeric coating for glycan microarrays, Sens. Actuators B 215 (2015).

[22] M. Salina, F. Giavazzi, E. Ceccarello, F. Damin, M. Chiari, M. Ciuffo, G.P. Accotto, M. Buscaglia, Multi-spot, label-free detection of viral infection in complex media by a non-reflecting surface, Sens. Actuators B 223 (2016) 957–962.

[23] M. Cretich, G. Pirri, F. Damin, I. Solinas, M. Chiari, A new polymeric coating for protein microarrays, Anal. Biochem. 332 (2004) 67–74.

[24] L.C. Iacono-Connors, J.F. Smith, T.G. Ksiazek, C.L. Kelley, C.S. Schmaljohn, Characterization of Langat virus antigenic determinants defined by monoclonal antibodies to E, NS1 and preM and identification of a protective, non-neutralizing preM-specific monoclonal antibody, Virus Res. 43 (1996) 125–136.

[25] K.M. Chung, B.S. Thompson, D.H. Fremont, M.S. Diamond, Antibody recognition of cell surface-associated NS1 triggers Fc-gamma receptor-mediated phagocytosis and clearance of West Nile Virus-infected cells, J. Virol. 81 (2007) 9551–9555.

[26] K.M. Chung, G.E. Nybakken, B.S. Thompson, M.J. Engle, A. Marri, D.H. Fremont, M.S. Diamond, Antibodies against West Nile Virus nonstructural protein NS1 prevent lethal infection through Fc gamma receptor-dependent and -independent mechanisms, J. Virol. 80 (2006) 1340–1351.

[27] N.L. Bernasconi, E. Traggiai, A. Lanzavecchia, Maintenance of serological memory by polyclonal activation of human memory B cells, Science 298 (2002) 2199–2202.

[28] U. Schauer, F. Stemberg, C.H. Rieger, W. Buttner, M. Borte, S. Schubert, H. Mollers, F. Riedel, U. Herz, H. Renz, W. Herzog, Levels of antibodies specific to tetanus toxoid, Haemophilus influenzae type b, and pneumococcal capsular polysaccharide in healthy children and adults, Clin. Diagn. Lab. Immunol. 10 (2003) 202–207.

[29] K. Tsioris, N.T. Gupta, A.O. Ogunniyi, R.M. Zimnisky, F. Qian, Y. Yao, X. Wang, J.N. Stern, R. Chari, A.W. Briggs, C.R. Clouser, F. Vigneault, G.M. Church, M.N. Garcia, K.O. Murray, R.R. Montgomery, S.H. Kleinstein, J.C. Love, Neutralizing antibodies against West Nile virus identified directly from human B cells by single-cell analysis and next generation sequencing, Integr. Biol. (Camb) 7 (2015) 1587–1597.