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Cat flu: Broad spectrum polymeric antivirals

Aleksandra Synowiec[a,b], Irma Gryniuk[c], Magdalena Pachota[a,b], Łukasz Strzelec[b], Olga Roman[d], Katarzyna Kłysik-Trzciańska[e], Mateusz Zając[e], Inga Drebot[a], Katarzyna Gula[a], Aleksandra Andruchowicz[f], Zenon Rajfur[g], Krzysztof Szczubiałka[e], Maria Nowakowska[e,**], Krzysztof Pyrc[a,*]

[a] Virogenetics Laboratory of Virology, Malopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7a, 30-387, Krakow, Poland
[b] Microbiology Department, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387, Krakow, Poland
[c] Department of Cell Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387, Krakow, Poland
[d] Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387, Krakow, Poland
[e] Department of Physical Chemistry, Faculty of Chemistry, Jagiellonian University, Gronostajowa 2, 30-387, Krakow, Poland
[f] Veterinary Practice AMBUVET, Pod Fortem 2E, 31-302, Krakow, Poland
[g] Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Sciences, Jagiellonian University, Lojasiewicza 11, 30-348, Krakow, Poland

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ABSTRACT

Feline herpesvirus type 1 (FHV-1) and feline calicivirus (FCV) are considered as main causes of feline upper respiratory tract disease and the most common clinical manifestations include rhinotracheitis, conjunctivitis, and nasal/facial ulcerations. While the primary infection is relatively mild, secondary infections pose a threat to young or immunocompromised cats and may result in a fatal outcome. In this study, we made an effort to evaluate antiviral potency of poly(sodium 4-styrenesulfonates) (PSSNa) as potent FHV-1 and FCV inhibitors for topical use. Mechanistic studies showed that PSSNa exhibits a different mechanism of action depending on target species. While PSSNa acts directly on FHV-1 particles blocking their interaction with the host's cell and preventing the infection, the antiviral potency against FCV is based on inhibition at late stages of the viral replication cycle. Altogether, PSSNa polymers are promising drug candidates to be used in the treatment and prevention of the viral upper respiratory tract disease (URTD), regardless of the cause.

1. Introduction

Feline upper respiratory tract disease (URTD), colloquially termed as 'cat flu', is a relatively mild condition, manifested by oral or nasal discharges, lethargy, pyrexia, conjunctivitis and sneezing (Helps et al., 2005; Binns et al., 2000). The disease, however, may progress to the severe sequelae, with potentially fatal outcome (Bannasch and Foley, 2005). Feline herpesvirus type 1 (FHV-1) and feline calicivirus (FCV) are considered to be the two major causes of URTDs (Helps et al., 2005; Bannasch and Foley, 2005; Fernandez et al., 2017; Cohn, 2011).

Caliciviridae family encompasses non-enveloped, single-stranded, positive-sense RNA viruses (Fields BN et al., 2013) that can infect both humans and animals (Ohlinger et al., 1993; Bank-Wolf et al., 2010; Hurley et al., 2004). Feline calicivirus (FCV) (*Vesivirus* (Fields BN et al., 2013)) is prevalent worldwide in the domestic cat population (Radford et al., 2009), and is commonly associated with oral ulceration, salivation, chronic stomatitis, mild respiratory or conjunctival disorders and limping syndrome (Dawson et al., 1994; Reubel et al., 1992; TerWee et al., 1997). It has been, however, noted that some FCV isolates cause outbreaks with mortality rates reaching 40–60% (Hurley et al., 2004; Coyne et al., 2006; Pedersen et al., 2000; Reynolds et al., 2009; Schulz et al., 2011). No adequate antiviral treatment is available. Previous reports show that FCV infection may be inhibited *in vitro* (McDonagh et al., 2015; Wu et al., 2015; Fumian et al., 2018), but these findings were not confirmed in clinic.

Herpesviridae family encompasses enveloped, double-stranded DNA viruses associated with incurable, lifelong lasting herpetic infections. Feline herpesvirus type 1 (FHV-1, felid herpesvirus 1) is classified under the genus *Varicellovirus* and within the *Alphaherpesvirinae* subfamily (Davison et al., 2009). The virus is present in the domestic cat population worldwide, causing viral rhinotracheitis (FVR) and ocular disease (Gould, 2011; Hartley, 2010; Bistner et al., 1971; Stiles, 2003). It is

* Corresponding author.
** Corresponding author.
E-mail addresses: nowakows@chemia.uj.edu.pl (M. Nowakowska), k.a.pyrc@uj.edu.pl (K. Pyrc).
URL: http://virogenetics.info (K. Pyrc).

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estimated that 90% of cats are seropositive to this virus, whereas about 80% of infected animals establish lifelong latency and ~45% of these shed the virus (Gaskell and Povey, 1977; Maggs et al., 1999). The ocular herpetic disease is commonly manifested as acute conjunctivitis or chronic stromal keratitis and may lead to permanent eye damage or blindness (Gould, 2011; Hartley, 2010; Bistner et al., 1971; Stiles, 2003). Following the acute phase, the virus is transported retrogradely to sensory neurons, to establish incurable, life-long lasting latency in trigeminal ganglia. Recovery from latency results in recurrent disease (Gaskell and Povey, 1973, 1977; Nasisse et al., 1992). Treatment options for human herpetic infections often involve the use of specific nucleoside analogs (NA). These compounds are initially inactive and gain the function in infected cells due to phosphorylation by virus-encoded thymidine kinase (TK) and subsequently host GMP kinases (Miller and Miller, 1980, 1982) and act as effective viral polymerase inhibitors (Elion, 1983). While some of the compounds used in humans appear to be toxic in cats (Maggs, 2005; Maggs and Clarke, 2004; Nasisse et al., 1989), penciclovir (PCV) appears to be a potent and safe inhibitor of FHV-1 replication *in vitro* (Maggs and Clarke, 2004; Hussein et al., 2008; Groth et al., 2014); its prodrug famciclovir administered to cats is reported to be a safe and effective antiviral (Thomasy et al., 2011; Malik et al., 2009).

In this study, we evaluated the antiviral potency of poly(sodium 4-styrenesulfonates) (PSSNa) against FHV-1 and FCV. Obtained results show that these polymers inhibit replication of both viruses at nontoxic concentrations but their mode of action differs for the two tested pathogens. While PSSNa interacts with the FHV-1 virion and blocks its entry to the cell, it restricts FCV infection during later stages of the replication cycle. Our observations were confirmed with clinical strains. Additionally, what is important, PSSNa was previously used in clinic for other indications such as treatment of hyperkalemia (Mistry et al., 2016; Hollander-Rodriguez and Calvert, 2006; (FDA), 2017). However, given the above, we tested transdermal toxicity of concentrated polymers in a mouse model as it would be a beneficial route of administration in the treatment of URTD. As no toxicity was observed thus far, we believe that PSSNa is a safe and effective drug candidate to be used in the treatment of viral infections in domestic cats.

2. Materials and methods

2.1. Inhibitors

Standards of poly(sodium 4-styrenesulfonate) (PSSNa), M_w of 1.5, 5.4, 8, 19.3, 35, 46, 93.5, 200, 400, 780 and 1200 kDa ($PSSNa^{MwkDa}$), were purchased from Pressure Chemical. $PSSNa^{70kDa}$ and $PSSNa^{1000kDa}$ were purchased from Sigma-Aldrich, Poland. Stock solutions were prepared in distilled water (dH_2O) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) and stored at 4 °C.

2.2. Cell culture

Crandell Rees Feline Kidney cells (CrFK, ATCC: CCL-94) were maintained in Dulbecco-modified Eagle's medium (DMEM, high glucose, Life Technologies, Poland) supplemented with 3% heat-inactivated fetal bovine serum (FBS, Life Technologies, Poland) (3% DMEM). Medium was also supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (5 μg/ml) and ciprofloxacin (0.5 μg/ml). Cells were cultured at 37 °C in an atmosphere containing 5% CO_2 and humidity of 95%.

2.3. Viruses

Feline herpesvirus type 1 strain C-27 (FHV-1 C-27; ATCC: VR-636), feline calicivirus strain F9 (FCV F9; ATCC VR-782™) and clinical isolates FHV-1 K7 (Accession number: MK820647) and FCV K3 (Accession number: MK820648) were used. FHV-1 K7 and FCV K3 strains were obtained from cats with clinical signs of URTD. Briefly, oropharyngeal swabs were collected with virus transport plastic sticks (COPAN, Italy), resuspended in medium, filtered (0.2 μm) and overlaid on fully confluent CrFK cells. Plates were incubated up to 72 h and, if cytopathic effect (CPE) occurred, the supernatants were collected. Supernatants were further subjected to the plaque assay, and 24 h–48 h post-infection (p.i.) single plaques were collected. Viruses were typed by qPCR and sequencing of thymidine kinase gene fragment (FHV-1 K7) and major capsid protein, VP1 gene fragment (FCV K3).

Virus stocks and mock-infected samples were generated by inoculation of fully confluent CrFK cells with virus or control sample. 24 h (FCV) or 48 h (FHV-1) p.i. cells were lysed by two freeze-thaw cycles. Lysates were aliquoted and stored at −80 °C. Infectious samples were titrated according to the Reed and Muench protocol (Reed, 1938).

2.4. XTT cytotoxicity assay

CrFK cells were cultured on 96-well plate for 24 h. Subsequently, supernatants were discarded and 100 μl of fresh medium with polymer or with the mock sample was added to each well. After 48 h at 37 °C, cell viability was evaluated using XTT Viability Assay Kit (Biological Industries, Israel), according to the manufacturer's protocol. Following 2 h incubation at 37 °C the supernatants were transferred into the transparent 96-well plate and absorbance (λ = 480 nm) was measured. Obtained results were normalized to the absorbance of the control sample (untreated cells).

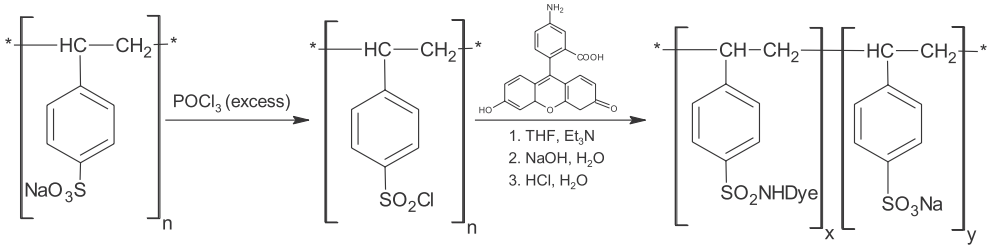
2.5. Quantitative real-time PCR (qPCR)

2.5 μl of isolated DNA or cDNA was amplified in a reaction of 10 μl containing 1 × Kapa Probe Fast qPCR MasterMix (Sigma-Aldrich, Poland), 100 nM of specific probe labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) and 450 nM of each sense and antisense primer. The sequences of oligonucleotides are listed in Supplementary Table 1.

Specific probe and primers were used to amplify 81 bp sequence fragment of the glycoprotein B (gB) gene of FHV-1 (Vogtlin et al., 2002) or 151 bp region of the FCV genome (nt 5321 to 5471) (Chander Y et al., 2007). The reaction was carried out in thermocycler (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad), according to the scheme: 3 min at 95 °C, followed by 39 cycles of 15 s at 95 °C and 30 s at 58 °C (FHV-1) or 51 °C (FCV). In order to quantify the nucleic acids, DNA standards were prepared as described previously (Pachota et al., 2017). For FCV linearized DNA standard was used as a template for *in vitro* transcription with T7 RiboMAX™ Express Large Scale RNA Production System (Promega). Transcribed RNA was DNase treated, and RNA was purified with lithium chloride (Invitrogen) precipitation. Subsequently, the concentration of RNA was assessed using a spectrophotometer, then the number of RNA copies per milliliter was calculated using the approximate molecular weight of ribonucleotide (340 g/mol) and Avogadro's constant. Eight 10-fold serial dilutions were used as qPCR template to develop a standard curve.

2.6. Plaque assay

CrFK cells were seeded in 24-well plates 24 h prior to the inoculation. At the time of the infection, 80–90% confluent cells were overlaid with 250 μl of serial (10 fold) dilutions of infectious samples. Cells were incubated for 1 h at 37 °C, washed once with PBS and overlaid with 0.5 ml of DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies, Poland), penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% methylcellulose (FHV-1; Sigma-Aldrich, Poland) or 0.6% UltraPure™ Low Melting Point Agarose (FCV; Thermo Scientific, Poland). 72 h (FHV-1) or 24 h (FCV) p.i. For visualization of FHV-1 plaques cells were incubated with dH_2O:methanol (1:1) with 0.1% crystal violet (20 min, room temperature) and washed once with



Scheme 1. Synthesis of fluorescently labeled PSSNa^{70kDa}.

dH_2O. For visualization of FCV plaques, cells were incubated with 1 ml/well of 4% paraformaldehyde (PFA) in dH_2O for at least 6 h at room temperature. Next cells were washed, incubated in dH_2O:methanol (1:1) with 0.1% crystal violet for 20 min at room temperature, and washed once with dH_2O.

2.7. Virus replication assay

Fully confluent CrFK cells were seeded on 96-wells plates 24 h prior to the inoculation. At the moment of infection, the supernatant was discarded and 20 µl of fresh medium supplemented with the polymer was added. Plates were incubated for 30 min at 37 °C and subsequently medium with polymer was discarded and 50 µl of polymer solution or control sample in 3% DMEM with mock or virus (800 $TCID_{50}$/ml) was added. Plates were incubated for 1.5 h (FCV) or 2 h (FHV-1) at 37 °C, supernatants were discarded and cells were washed twice with PBS. Finally, 100 µl of polymer solution or control sample in 3% DMEM was added to each well and cells were incubated for 18 h (FCV) or 48 h (FHV-1). After that time virus was quantified by qPCR and plaque assay.

2.8. Mechanism of action

Functional assays aimed to reveal the mechanism of polymer action were carried out as described below.

2.8.1. Assay I. Inactivation of virions

This assay allows determining whether the tested compound affects directly the viral particle. Briefly, concentrated virus suspension was incubated at 22 °C with polymers for 1 h. Subsequently, samples were diluted to decrease polymer concentration below the active range and virus titer is evaluated by plaque assay.

2.8.2. Assay II. Cell protection

This assay allows determining whether the polymer protects the cell from viral infection. CrFK cells were seeded on 96-wells plates 24 h prior to the inoculation. Fully confluent cells were incubated in the presence or absence of polymer for 1 h at 37 °C. Subsequently, plates were washed twice with PBS, then fresh medium containing mock or virus (400 $TCID_{50}$/ml) was added to each well and plates were incubated for 1.5 h or 2 h at 37 °C for FCV and FHV-1, respectively. Subsequently, plates were washed twice with PBS, overlaid with fresh medium and incubated for 18 h (FCV) or 48 h (FHV-1) at 37 °C. After that time, supernatants were collected, and the virus was quantified by qPCR and plaque assay.

2.8.3. Assay III. Virus attachment

This assay allows determining if polymers are able to block the attachment of virus particles to cellular receptors. CrFK cells were seeded on 96-wells plates 24 h prior to the inoculation. Fully confluent CrFK cells were cooled at 4 °C for 20 min. Subsequently, cells were overlaid with ice-cold medium containing mock or virus (400 $TCID_{50}$/ml) with or without the polymer. Plates were incubated for 1 h at 4 °C. While intracellular transport at 4 °C is hampered, viral adsorption to cellular receptors is possible. After the incubation, cells were washed twice with ice-cold PBS, fresh medium was added to each well and cells were incubated for 18 h (FCV) or 48 h (FHV-1) at 37 °C. After incubation, the virus was quantified in the supernatants by qPCR and plaque assay.

2.8.4. Assay IV. Inhibition of virus replication, assembly, and egress

This assay allows determining whether polymer inhibits the virus replication, assembly, or release. CrFK cells were seeded on 96-wells plates 24 h prior to the inoculation. Confluent CrFK cells were overlaid with fresh medium containing mock or virus (400 $TCID_{50}$/ml), and plates were incubated for 1.5 h (FCV) or 2 h (FHV-1) at 37 °C. Following incubation, cells were washed twice with PBS and overlaid with fresh medium containing the polymer. Plates were incubated for 18 h (FCV) or 48 h (FHV-1) at 37 °C. After incubation, supernatants were collected, and cells were washed twice with PBS, lysed with two freeze-thaw cycles and collected. The virus was quantified in supernatants and cell lysates by qPCR and plaque assay.

2.9. Synthesis of fluorescently labeled PSSNa^{70kDa}

Fluorescently labeled PSSNa (PSSNa$^{70kDa-F}$) was synthesized as described previously (Sohn et al., 1996), the details are described in Supplementary Materials and Methods. The synthesis is schematically presented in Scheme 1.

2.10. Synergistic effect

Synergistic effect of antiviral drugs was assessed as previously described (Benzekri et al., 2018), with some modifications. All dilutions were prepared using DMEM medium. Virus replication assay on fully confluent CrFK cells was performed. After 48 h the supernatants were collected, and virus yields and titers were assessed by qPCR and plaque assay, respectively. The synergy was evaluated by calculating the combination index (CI) (Benzekri et al., 2018).

2.11. Statistical analysis

Statistical analyses were performed with GraphPad Prism 7: one-way ANOVA with Tukey HSD *post hoc* test or non-parametric Kruskal-Wallis one-way variance analysis with Dunn's *post hoc* test. Data are presented as the mean ± SD (standard deviation), mean ± SEM (standard error of the mean) or medians ± interquartile range (when non-parametric tests were applied) from at least three independent experiments. p-value < 0.05 was considered as statistically significant.

3. Results

3.1. PSSNa inhibits FHV-1 and FCV replication in CrFK cell line at non-toxic concentration

XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide) assay was performed on CrFK cells to examine the cytotoxicity of eleven PSSNa polymers that differed in molecular weight (M_w). The assay relies on the ability of viable cells to convert XTT into

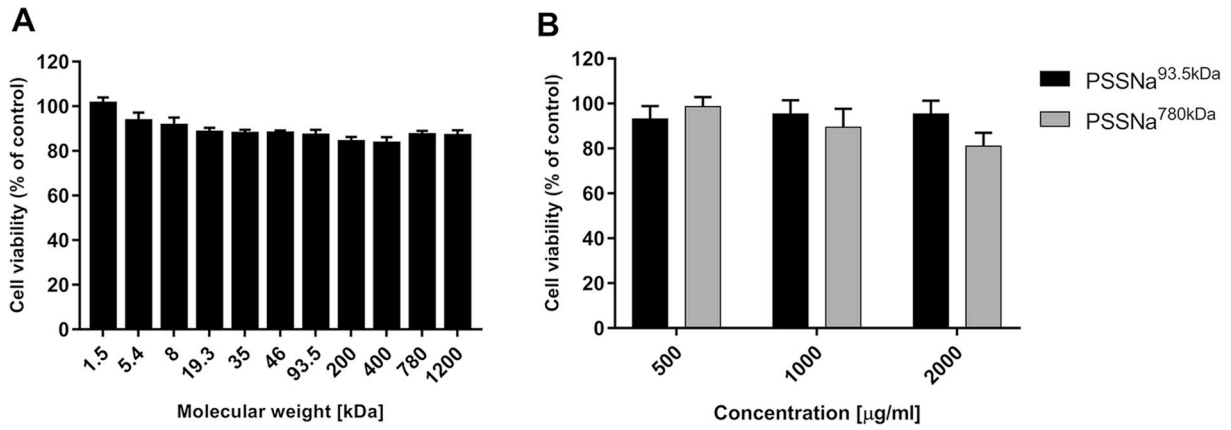


Fig. 1. Cytotoxicity of PSSNa polymers in CrFK cells. Cell viability was determined using XTT assay. PSSNa polymers (200 μg/ml) of different M_w [kDa] were used (**A**), for $PSSNa^{93.5kDa}$ and $PSSNa^{780kDa}$ higher concentrations of 500, 1000 and 2000 μg/ml were tested (**B**). The data are presented as the percentage of the reference, untreated sample for which viability was set to 100%. Results are presented as mean ± SEM from three independent experiments.

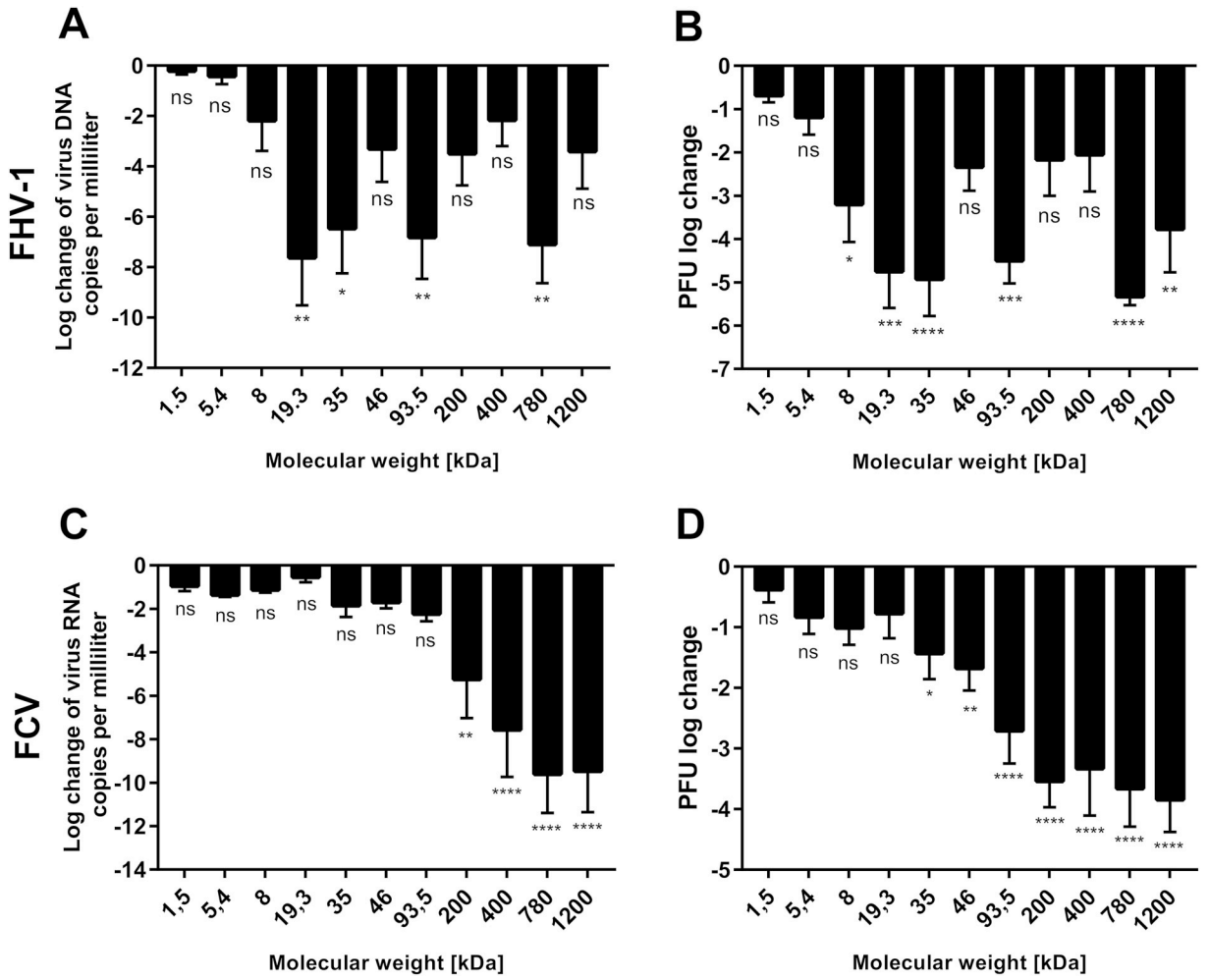


Fig. 2. PSSNa inhibits FCV and FHV-1 replication at non-toxic concentration. Experiments were carried out for each PSSNa polymer of different M_w at a concentration of 200 μg/ml (FCV) or 20 μg/ml (FHV-1). Inhibition of viral infection was assessed by qPCR and presented as log change of virus RNA/DNA copies per milliliter (**A, C**) or plaque assay and presented as PFU log change (**B, D**). Results were normalized to the values of untreated, infected cells and are presented as mean ± SEM from three independent experiments. To determine the significance of differences between compared groups, one-way ANOVA with post hoc Tukey HSD test was used. Values statistically significant are indicated by asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$, values statistically non-significant ($p > 0.05$) are indicated by "ns".

the colored derivative, which correlates with cell viability. No cytotoxicity was observed following the 48 h of incubation at tested concentrations (Fig. 1).

Furthermore, the dermal toxicity of $PSSNa^{1000kDa}$ was tested *in vivo* (BALB/C mice model). Firstly, XTT assay was performed on CrFK cells to determine the highest non-toxic *in vitro* concentration of PEG_{400} that

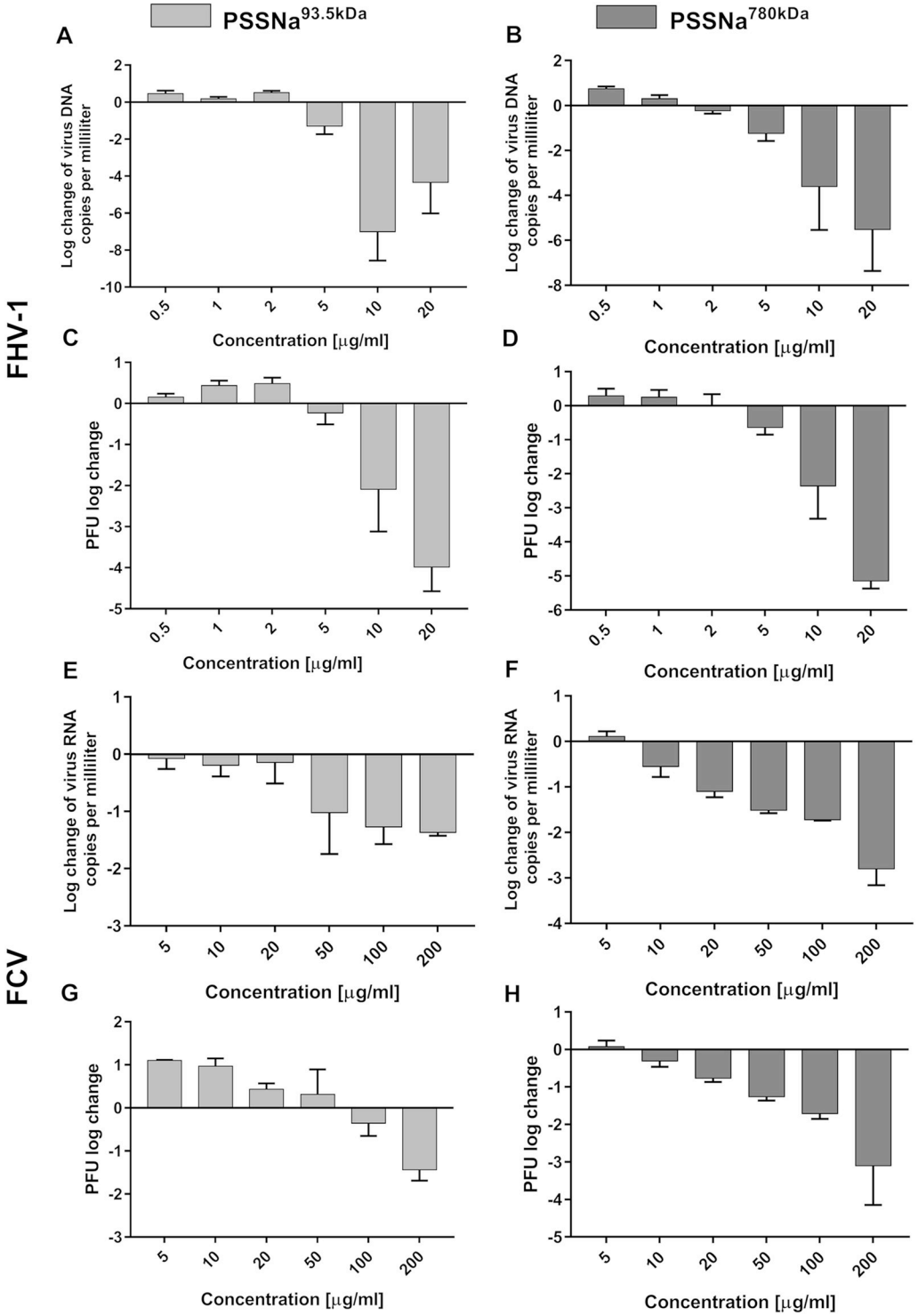


Fig. 3. Antiviral activity of PSSNa against FCV and FHV-1 is dose dependent. Virus replication assay was carried out for each PSSNa polymer. Inhibition of FCV and FHV-1 infection by PSSNa polymers was evaluated using qPCR and presented as log change of virus RNA/DNA copies per milliliter (**A, B, E, F**) or plaque assay and presented as PFU (plaque forming unit) log change (**C, D, G, H**). Results were normalized to the values of untreated, infected cells and are presented as mean ± SEM from three independent experiments.

was 30 mg/ml (Supplementary Fig. 1A). Subsequently, antiviral activity of the hydrogel containing PEG_{400} (30 mg/ml) and $PSSNa^{1000kDa}$ (200 μg/ml) against FCV and FHV-1 was confirmed *in vitro* (Supplementary Figs. 1B–E). The hydrogel with polymers or saline were administered daily for 7 days and no toxicity was observed at 50, 75, and 100 mg/ml. Mice were monitored each day and skin redness, lesions or other alterations were not observed during the experiment. Necropsy did not reveal any alteration of the internal organs and the biochemical blood analysis did not reveal any abnormalities (***data not shown***). The details regarding the methodology of dermal toxicity studies are given in supplementary materials and methods.

To examine antiviral potency of PSSNa Virus Replication Assay was performed for each polymer at 20 μg/ml for FHV-1 and at 200 μg/ml for FCV. Obtained results are presented in Fig. 2 and indicate that almost

Table 1
Determined IC_{50} values.

Target species	Polymer	IC_{50} ± SD [μg/ml]	
		qPCR	Plaque Assay
FHV-1 C-27	$PSSNa^{93.5kDa}$	2.25 ± 1.01	5.74 ± 1.32
	$PSSNa^{780kDa}$	2.28 ± 1.01	5.06 ± 1.33
FCV F9	$PSSNa^{93.5kDa}$	42.75 ± 2.46	49.51 ± 0.14
	$PSSNa^{780kDa}$	9.72 ± 1.05	10.47 ± 1.47

Table 2
Elemental composition of the non-labeled and labeled $PSSNa^{70kDa}$.

Polymer	Elemental composition [%]			
	N	C	H	S
$PSSNa^{70kDa}$	–	37.69	3.778	14.50
$PSSNa^{70kDa-F}$	0.29	37.93	4.895	15.54

all PSSNa polymers with M_w above 8 kDa block FHV-1 replication at 20 μg/ml, but there is no relationship between the M_w and the antiviral activity (Fig. 2A and B). On the other hand, the correlation between the M_w and the antiviral activity against FCV was observed, as PSSNa with higher M_w inhibited FCV replication more efficiently (Fig. 2C and D).

For further studies, $PSSNa^{93.5kDa}$ and $PSSNa^{780kDa}$ were selected due to their high antiviral activity and significant difference in their M_w. The IC_{50} values for $PSSNa^{93.5kDa}$ and $PSSNa^{780kDa}$ qPCR and plaque assays were used. The results are presented in Fig. 3 and calculated IC_{50} values are listed in Table 1.

3.2. Internalization of PSSNa to the cell

To determine whether PSSNa polymers are internalized to the cell, fluorescently labeled PSSNa with M_w = 70 kDa ($PSSNa^{70kDa-F}$) was synthesized. Elemental analysis revealed the presence of nitrogen in the obtained polymer that unambiguously confirmed the substitution of $PSSNa^{70kDa}$ with fluoresceinamine. The elemental composition of the polymer before and after substitution is presented in Table 2. In the FTIR spectra of $PSSNa^{70kDa-F}$ (Nicolet iS10 FT-IR spectrometer equipped with an ATR accessory (SMART iTX); Supplementary Fig. 2), a weak band at 1318 cm^{-1} appeared that is characteristic for sulfonamides (-SO_2-NH-). In comparison with the spectrum of $PSSNa^{70kDa}$, an additional band appeared at 947 cm^{-1} in the spectrum of $PSSNa^{70kDa-F}$. The electronic absorption spectra recorded in the visible spectral region for the labeled polymers resemble that of fluoresceinamine in water (Supplementary Fig. 3).

Localization of polymer after the incubation was analyzed with confocal microscopy after 2, 6 and 24 h. Presented images show that $PSSNa^{70kDa-F}$ is slowly absorbed and accumulated in the cell

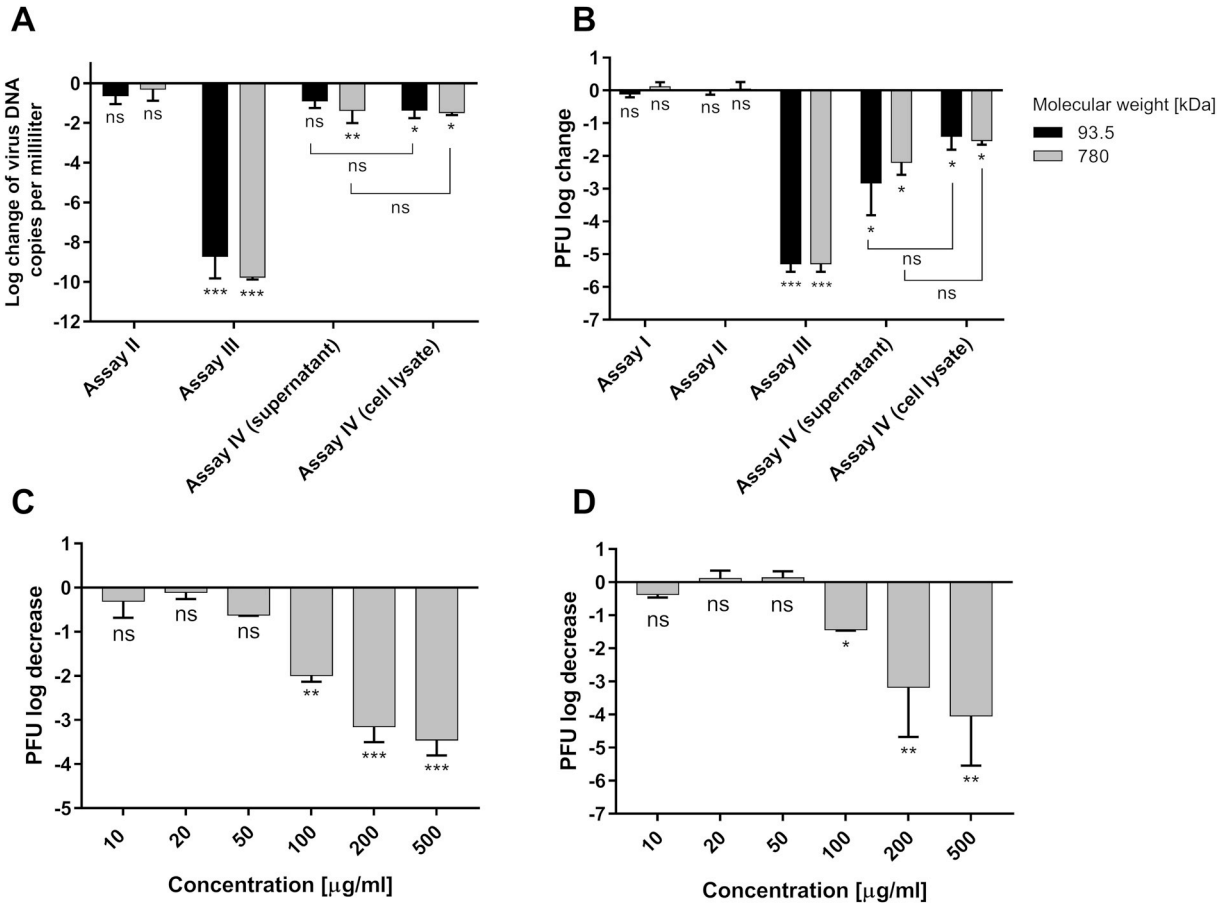


Fig. 4. PSSNa polymers inhibit FHV-1 replication mainly at early stages of the infection. Inhibition of viral replication in CrFK cells by PSSNa polymers at a concentration of 20 μg/ml was evaluated with qPCR and presented as log change of virus DNA copies per milliliter (**A**) or plaque assay and presented as PFU (plaque forming unit) log change (**B**). Assay I: inactivation; Assay II: cell protection; Assay III: virus attachment; Assay IV: late stages. The decrease in virus titers in Assay I (Inactivation) for increasing concentrations of $PSSNa^{93.5kDa}$ (**C**) and $PSSNa^{780kDa}$ (**D**) was presented. Results were normalized to the values of untreated, infected cells and are presented as mean ± SEM from three independent experiments. To determine the significance of differences between compared groups, one-way ANOVA with post hoc Tukey HSD test was used. Values statistically significant are indicated by asterisks: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001, values statistically nonsignificant (p > 0.05) are indicated by "ns".

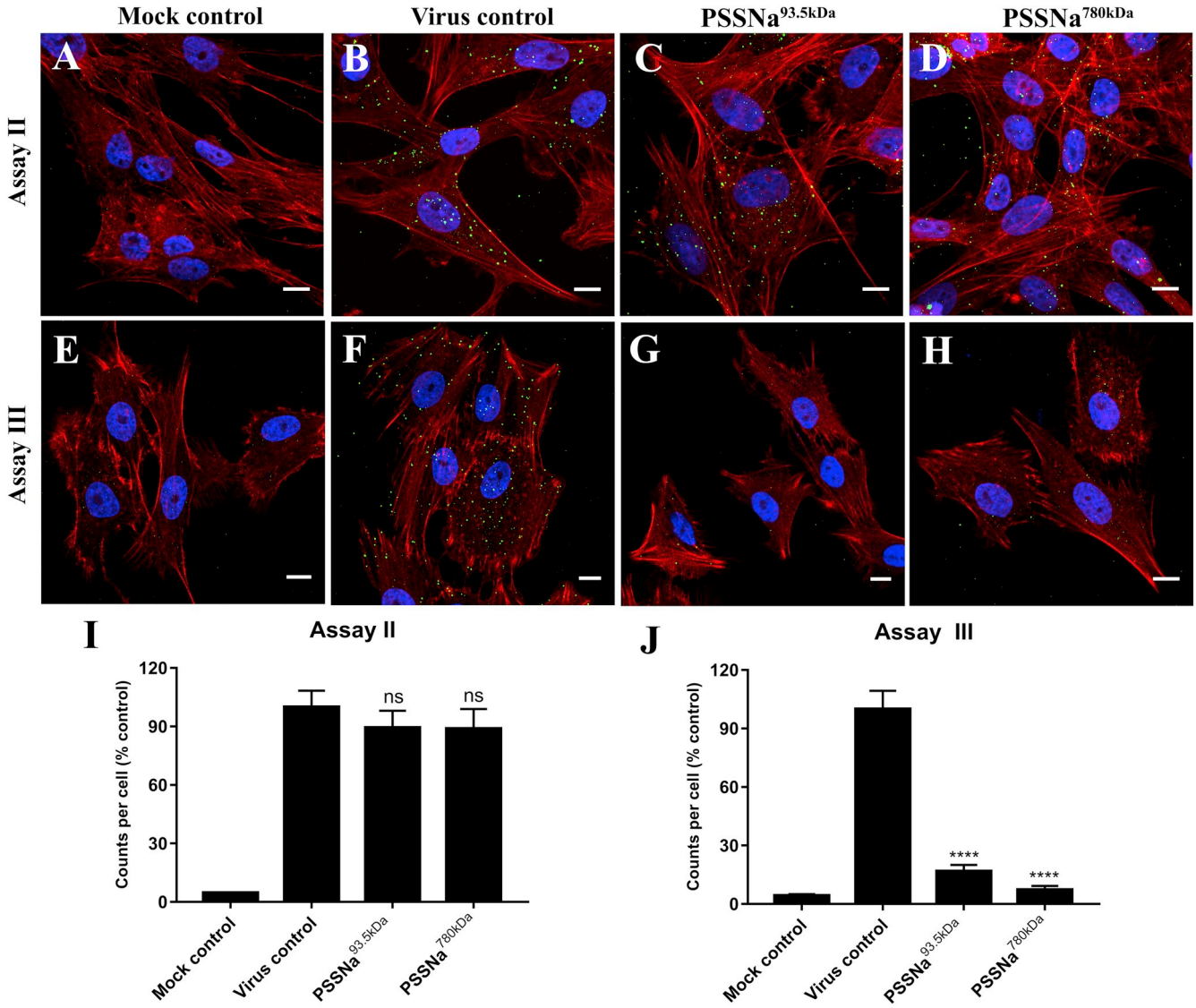


Fig. 5. PSSNa inhibits FHV-1 adhesion to the cell surface. Two functional assays: Cell Protection Assay (Assay II) and Attachment Assay (Assay III) were performed. CrFK cells were incubated in media supplemented with 200 μg/ml or in the absence of PSSNa polymer. (**A, E**) mock-infected cells (**B, F**) virus-infected cells (**C, G**) virus-infected cells in the presence of $PSSNa^{93.5kDa}$ (**D, H**) virus-infected cells in the presence of $PSSNa^{780kDa}$. Immunostaining was performed as described in the Supplementary information section. Blue color denotes nuclei, red f-actin, and green FHV-1 virions. Representative images are shown as maximum projections of XY stacks. Scale bar: 10 μm. The quantitative analysis of images was performed. The number of counts per cell was assessed using ImageJ FiJi and is presented as % of the virus control mean (**I, J**). The data is presented as mean ± SEM from two independent experiments and was calculated from at least 10 different cells. To determine the significance of differences between compared groups, one-way ANOVA with post hoc Tukey HSD test was used. Values statistically significant are indicated by asterisks: ****$p < 0.001$, values statistically nonsignificant ($p > 0.05$) are indicated by "ns".

(Supplementary Fig. 4).

3.3. PSSNa inhibits FHV-1 binding to the cell by direct interaction with virions

To determine, whether PSSNa affects FHV-1 attachment to the cell surface virus, *Assay III* was performed. Plaque assay and qPCR showed complete inhibition of FHV-1 replication by both PSSNa (Fig. 4A and B). *Assay II* was performed to determine whether PSSNa polymers interact with the cell and may protect it from viral infection. No significant decrease in virus yields or titers was detected (Fig. 4A and B).

To confirm that PSSNa interacts with FHV-1 and blocks its adhesion to the cells, virions bound to the cell were visualized with confocal microscopy (Fig. 5A–H). It is clearly visible that virus–cell binding is abolished in the presence of polymers. However, pre-incubation of cells with the polymer did not affect the number of virions bound to the cell surface. Further, to ensure the unbiased results, virions were scored and numerical results are presented in Fig. 5I and J.

The hypothesis that the polymer binds to virus particles and blocks their interaction with cellular receptors was also tested. Inactivation of virions assay (*Assay I*) was performed using $PSSNa^{93.5kDa}$ and $PSSNa^{780kDa}$ at six different concentrations (10, 20, 50, 100, 200 and 500 μg/ml). Virus stock was pre-incubated in the presence of polymer and the virus' infectivity was tested afterward using the plaque assay. Results are presented in Fig. 4C and D. Significant decrease in viral yield was observed for concentrations exceeding 50 μg/ml. Further, a surface binding assay was performed to find out if FHV-1 virion binding to the surface coated with the polymer occurs. Confocal visualization of viruses on the surfaces coated with fluorescently labeled $PSSNa^{70kDa-F}$ allowed to determine that virions bind to the polymer-coated surface more effectively than to non-coated surfaces (Fig. 6A). To confirm the obtained results, virions bound to the surfaces were scored and

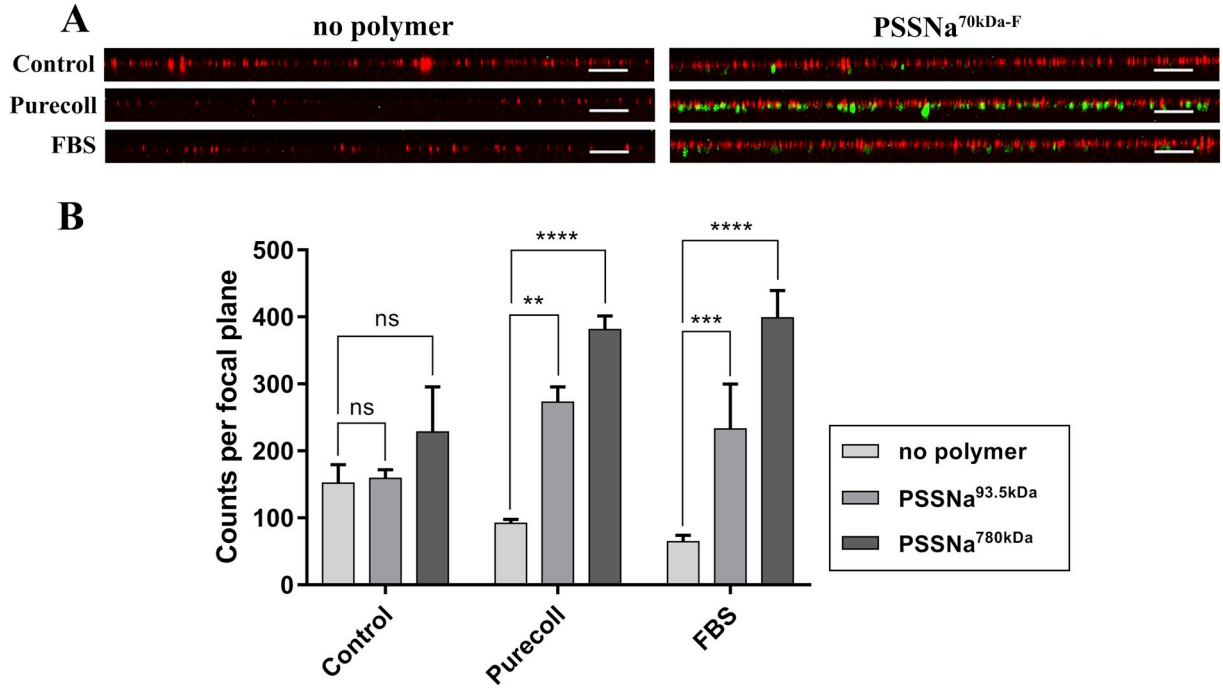


Fig. 6. FHV-1 virions interact with PSSNa polymers. In surface binding assay coverslips were coated with fluorescently labeled PSSNa polymer and nonspecific binding sites were blocked with bovine collagen solution (Purecoll) or fetal bovine serum (FBS) or not blocked (control). The red color denotes FHV-1 virions, while the green color corresponds to the PSSNa$^{kDa70-F}$. Selected images are presented as maximum projections of XZ stacks. Scale bar: 10 µm. To determine if the increase in the number of bound viruses is statistically significant, the number of virions was counted from 12 different focal planes obtained in two independent experiments and the results are presented in panel (**B**). Kruskal-Wallis one-way variance analysis with Dunn's post hoc test was applied. The data are presented as medians ± interquartile range. Values statistically significant are indicated by asterisks (****, $p < 0.0001$, ***, $p < 0.001$, **, $p < 0.01$), values statistically nonsignificant ($p > 0.05$) are indicated by "ns".

numerical results are presented in Fig. 6B.

It was shown that the polymer interacts directly with the viral particle and blocks its attachment and entry to the cell. It is worth to note that also in *Assay IV* that aimed to determine whether polymers may interfere with virus replication, assembly or egress, mild, but significant decrease in virus replication was noted (Fig. 4A and B). However, an additional mechanism of action at later stages of infection was excluded after performing the experiment where samples were collected right after one round of FHV-1 replication cycle ~16 h (***data not shown***).

3.4. PSSNa^{780kDa} inhibits FCV replication and, to a lower extent, also early stages of the infection

Mechanism of PSSNa action was also delineated for FCV. First, we made an effort to determine whether viral particles are able to attach to the cell surface in the presence of polymer (*Assay III*). Pre-cooled cells were infected with FCV (400 $TCID_{50}$/ml) in the presence of PSSNa$^{93.5kDa}$ or PSSNa^{780kDa} at 200, 500 and 1000 µg/ml and incubated for 1 h at 4 °C. 18 h p.i. virus yields and titers were assessed. PSSNa^{780kDa} but not PSSNa$^{93.5kDa}$ inhibited FCV infection at 500 µg/ml and higher (Fig. 7A and B). *Assay II* revealed that pre-incubation of cells with PSSNa polymers did not affect the FCV infection (Fig. 7C and D), showing that the polymers do not protect the cells from the infection.

Confocal analysis confirmed these observations, as FCV adhesion to the cells in the presence of PSSNa^{780kDa} was limited, while no alteration in virus adhesion was visible for PSSNa$^{93.5kDa}$ (Fig. 8A–H). To confirm these observations, virions bound to the cell surface were scored and numerical results are presented in Fig. 8I and J.

The surface binding assay was performed, where FCV virion binding to the surface coated with the polymer was evaluated. Unfortunately, we were not able to visualize the PSSNa$^{70kDa-F}$ polymer, as FCV does not bind to PSSNa of M_w as low as that of PSSNa$^{70kDa-F}$, while fluorescently labeled PSSNa of higher M_w was not water-soluble. Nonetheless, confocal visualization of viruses on the surfaces coated with PSSNa^{780kDa} allowed to determine that also FCV virions bind to the polymer-coated surface more effectively than to non-coated surfaces (Fig. 9A). Further, to ensure the unbiased results, virions bound to the surfaces were scored and numerical results are presented in Fig. 9B.

We observed that only PSSNa of high M_w blocks FCV adhesion to the cell, but the antiviral effect of PSSNa of lower M_w was also observed. *Assay IV* was performed to determine whether PSSNa interferes with viral replication, assembly, and egress of FCV. Significant inhibition was observed for both tested polymers, as presented in Fig. 7C and D. As virus titers and yields were decreased in supernatants and in cell lysates, we believe that the polymers inhibit virus replication stage.

3.5. Clinical strains

To ensure that the inhibition is not limited to cell culture-adapted strains, we recovered clinical isolates for both species from the clinical material collected in fall of 2018 (FHV-1 K7 and FCV K3) from cats with symptoms of URTD and tested PSSNa activity against isolated viruses. CrFK cells were incubated in the presence of PSSNa$^{93.5kDa}$ and PSSNa^{780kDa} polymers at 20, 200, and 500 µg/ml (FHV-1 K7); or 200, 500, and 1000 µg/ml (FCV K3) through the whole infection process. Viral titers and yields were evaluated after the infection. Obtained results, presented in Fig. 10 A, B, indicate that FHV-1 K7 was fully inhibited by both PSSNa polymers. Inhibition of FCV K3 virus was weaker but still significant and PSSNa^{780kDa} restricted viral infection more efficiently (Fig. 10 C, D).

3.6. Synergistic effect of PSSNa^{780kDa} and ACV

To determine whether PSSNa and nucleoside analogs used in the treatment of herpetic infections show synergistic, additive or

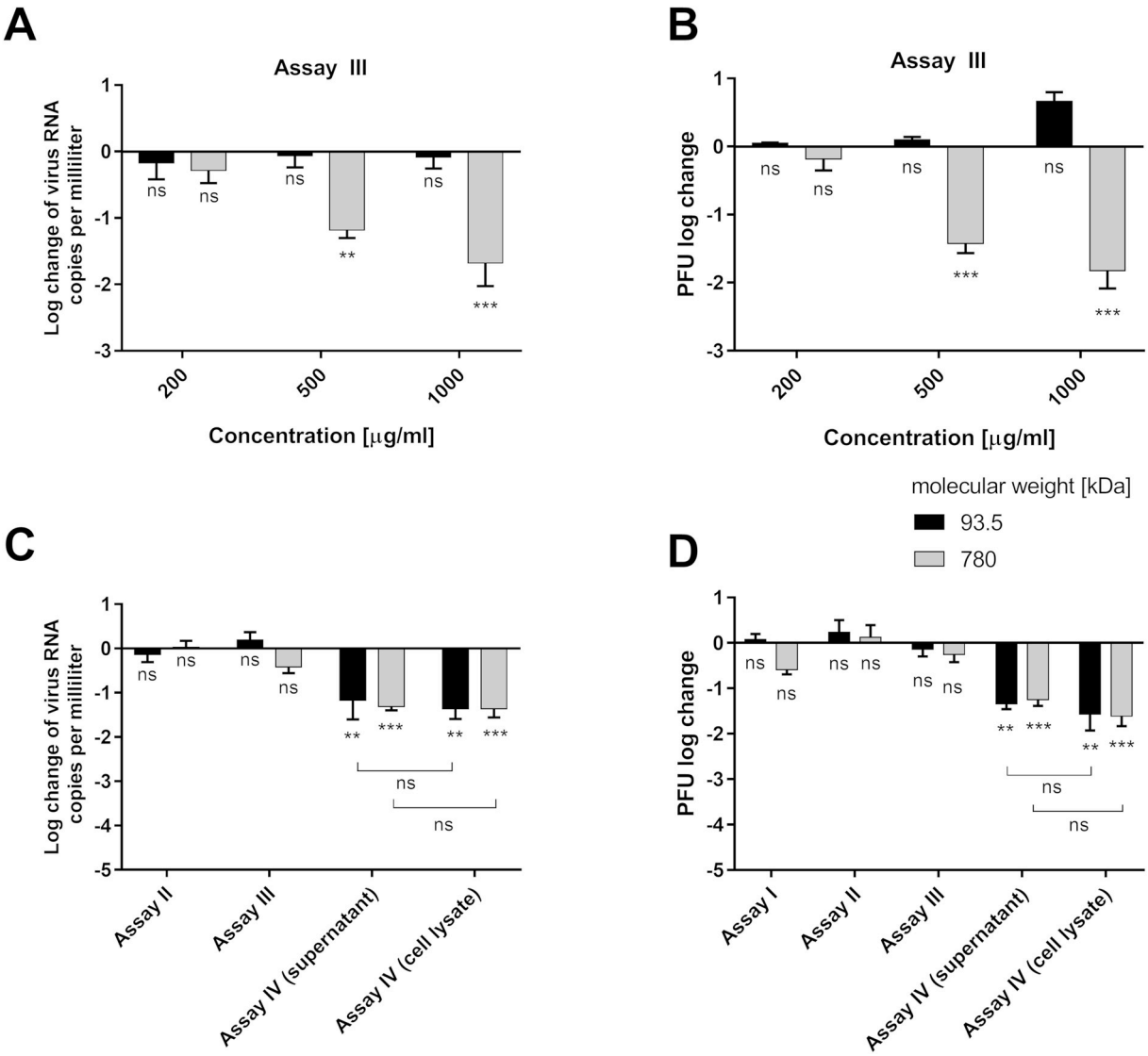


Fig. 7. PSSNa polymers inhibit FCV replication and adhesion. Inhibition of viral replication in CrFK cells by PSSNa polymers was evaluated with qPCR and presented as log change of virus DNA copies per milliliter (**A, C**) or plaque assay and presented as PFU (plaque forming unit) log change (**B, D**). Assay I: Inactivation; Assay II: Cell Protection; Assay III: Virus Attachment; Assay IV: Late Stages. The decrease in virus titers in Assay III for increasing concentrations of $PSSNa^{93.5kDa}$ and $PSSNa^{780kDa}$ (**A**, **B**) was presented. Results were normalized to the values of untreated, infected cells and are presented as mean ± SEM from three independent experiments. To determine the significance of differences between compared groups, one-way ANOVA with post hoc Tukey HSD test was used. Values statistically significant are indicated by asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$, values statistically nonsignificant ($p > 0.05$) are indicated by "ns".

antagonistic effect, combined drug effect assay was performed. Referring to literature, IC_{50} value for ACV varies from 13.5 to 56 μg/ml (Maggs and Clarke, 2004; van der Meulen et al., 2006) and from 0.3 to 32.9 μg/ml for PCV (Maggs and Clarke, 2004; Hussein et al., 2008; Williams et al., 2004). First, XTT assay was performed to exclude drug-related toxicity. Second, virus replication assay was performed. Calculated IC_{50} for FHV-1 C-27 at 400 $TCID_{50}$/ml equaled to 12.1 ± 1.37 μg/ml for ACV and 1.37 ± 1.11 μg/ml for PCV, as determined by qPCR (Supplementary Fig. 5).

Subsequently, the synergistic effect of ACV/PCV and $PSSNa^{780kDa}$ was investigated. As shown in Table 3, calculated CI values were **0.92** for $PSSNa^{780kDa}$/ACV and **0.46** for $PSSNa^{780kDa}$/PCV. Obtained results indicate that ACV and PCV exhibit synergistic effect while combined with $PSSNa^{780kDa}$, and that this effect is more pronounced for PCV, which is used in the treatment of FHV-1 infection.

4. Discussion

Feline upper respiratory tract disease (URTD) is a common disease, caused mainly by feline herpesvirus type 1 (FHV-1) and/or feline calicivirus (FCV), which may have fatal sequelae if not managed appropriately. Available prophylactic measures – a trivalent vaccine (FVRCP) containing feline herpesvirus (FHV), feline calicivirus (FCV) and feline panleukopenia virus (FPV) antigens is not fully protective and the exposure still leads to the infection and virus transmission (Scott and Geissinger, 1999). Current FHV-1 therapy is based on virostatic agents, mainly nucleoside analogs (NA) and concomitant antibiotic therapy to manage secondary bacterial infections. No antiviral therapy is available for FCV.

In order to identify the active compounds, we screened a wide library of polymers. Some compounds were active against other herpesviruses, including dextran substituted with GTMAC (Pachota et al., 2017), poly(sodium 4-styrenesulfonates) (PSSNa) (Herold et al., 2000) and cationic block polymers (Pachota et al., 2019). Unexpectedly,

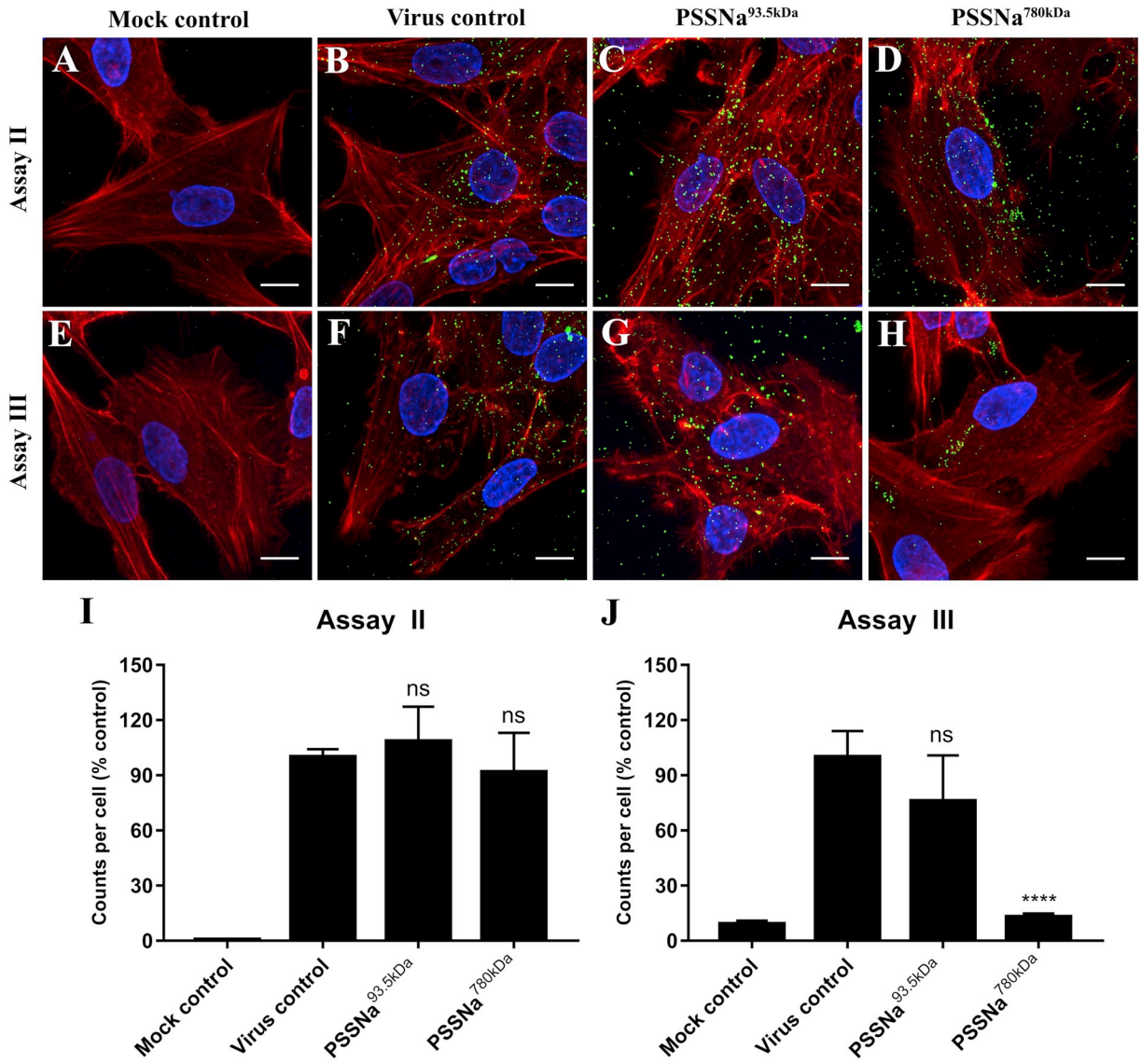


Fig. 8. PSSNa inhibits viral adhesion to the cell surface. Two functional assays: Cell Protection Assay (Assay II) and Attachment Assay (Assay III) were performed. CrFK cells were incubated in media supplemented with 200 μg/ml (Assay II), 1000 μg/ml (Assay III) or in the absence of PSSNa polymer. (**A, E**) mock-infected cells (**B, F**) virus-infected cells (**C, G**) virus-infected cells in the presence of $PSSNa^{93.5kDa}$ (**D, H**) virus-infected cells in the presence of $PSSNa^{780kDa}$. Immunostaining was performed as described in the Supplementary information section. Blue color denotes nuclei, red f-actin, and green FCV virions. Representative images are shown as maximum projections of XY stacks. Scale bar: 10 μm. The quantitative analysis of images was performed. The number of counts per cell was assessed using ImageJ FiJi and is presented as % of the virus control mean (**I, J**). The data are presented as mean ± SEM from two independent experiments and were calculated from at least 10 different cells. To determine the significance of differences between compared groups, one-way ANOVA with post hoc Tukey HSD test was used. Values statistically significant are indicated by asterisks: ****$p < 0.001$, values statistically nonsignificant ($p > 0.05$) are indicated by "ns".

dextran derivatives and cationic block polymers did not exhibit anti-FHV activity (***data not shown***), but PSSNa effectively inhibited viral replication and was selected for further studies. Surprisingly, PSSNa exhibited antiviral potency also against FCV and to the authors' knowledge it is the first report where one compound is able to inhibit two viral agents associated with feline URTD. What is more, the polymers were able to abolish replication of laboratory (FHV-1 C-27, FCV F9) and clinical strains (FHV-1 K7, FCV K3).

To select the optimal compound, we tested a series of polymers varying in M_w. In this study, we show that PSSNa exhibit promising antiviral activity against FHV-1 and FCV. While no association between polymer M_w and antiviral activity was noted for FHV-1, such association was observed for FCV.

To resolve the mechanism of PSSNa action we performed an array of functional assays. While pre-incubation of the polymer with cells did not affect virus attachment and entry, pre-incubation of FHV-1 virions with PSSNa reduced the number of virions attached to the cells and consequently limited virus replication. Subsequently, we confirmed that PSSNa interacts with FHV-1 virions and blocks their attachment to the cells. Here, the inhibitory effect was not dependent on the polymer size and we believe that the polymer binds to the well-defined GAG-binding site on the viral surface and blocks its attachment. The local charge distribution seems to be more important here, while the polymeric chain conformation is less relevant. Of note, if the polymer was present at later stages of the infection, it also abolished replication of the virus, but this resulted from an inhibition of multi-cycle infection and virus transmission instead of an additional mechanism of action. PSSNa is a sulfonated anionic derivative of polystyrene. It is known for its broad-spectrum antimicrobial activity, as it was reported to inhibit bacteria and viruses (Herold et al., 2000; Anderson et al., 2000; Christensen et al., 2001; Simoes et al., 2002). Considering that antimicrobial potency of PSSNa is mainly observed during early stages of

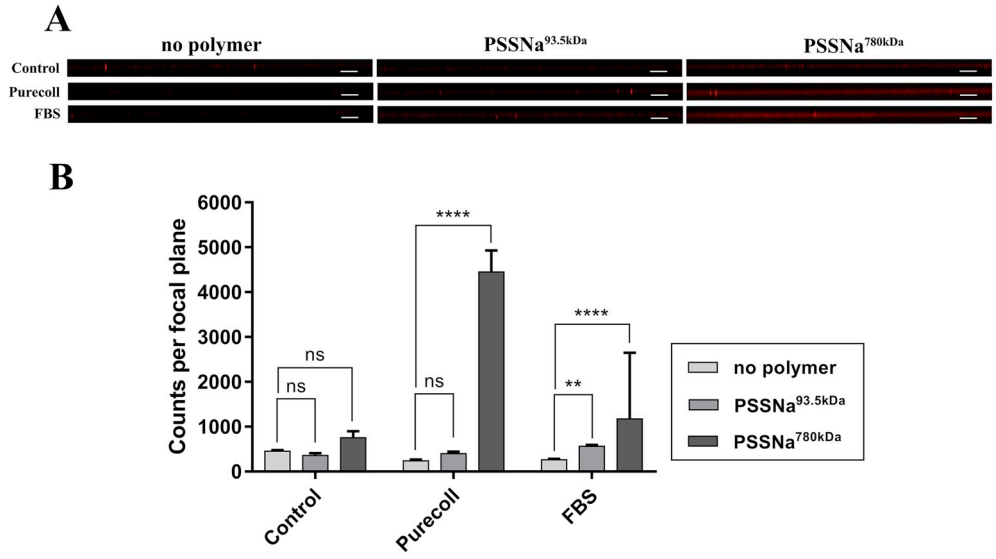


Fig. 9. FCV interacts with high molecular weight PSSNa. In surface binding assay coverslips were coated with $PSSNa^{93.5kDa}$ and $PSSNa^{780kDa}$ polymer and nonspecific binding sites were blocked with bovine collagen solution (Purecoll) or fetal bovine serum (FBS) or not blocked (control). The red color denotes FCV virions. Selected images are presented as maximum projections of XZ stacks (**A**). Scale bar: 10 µm. To determine if the increase in the number of bound viruses is statistically significant, the number of virions was counted in 12 different focal planes obtained in two independent experiments and the results are presented in panel (**B**). Kruskal-Wallis one-way variance analysis with Dunn's post hoc test was applied. The data are presented as medians ± interquartile range. Values statistically significant are indicated by asterisks (****, $p < 0.0001$, **, $p < 0.01$), values statistically nonsignificant ($p > 0.05$) are indicated by "ns".

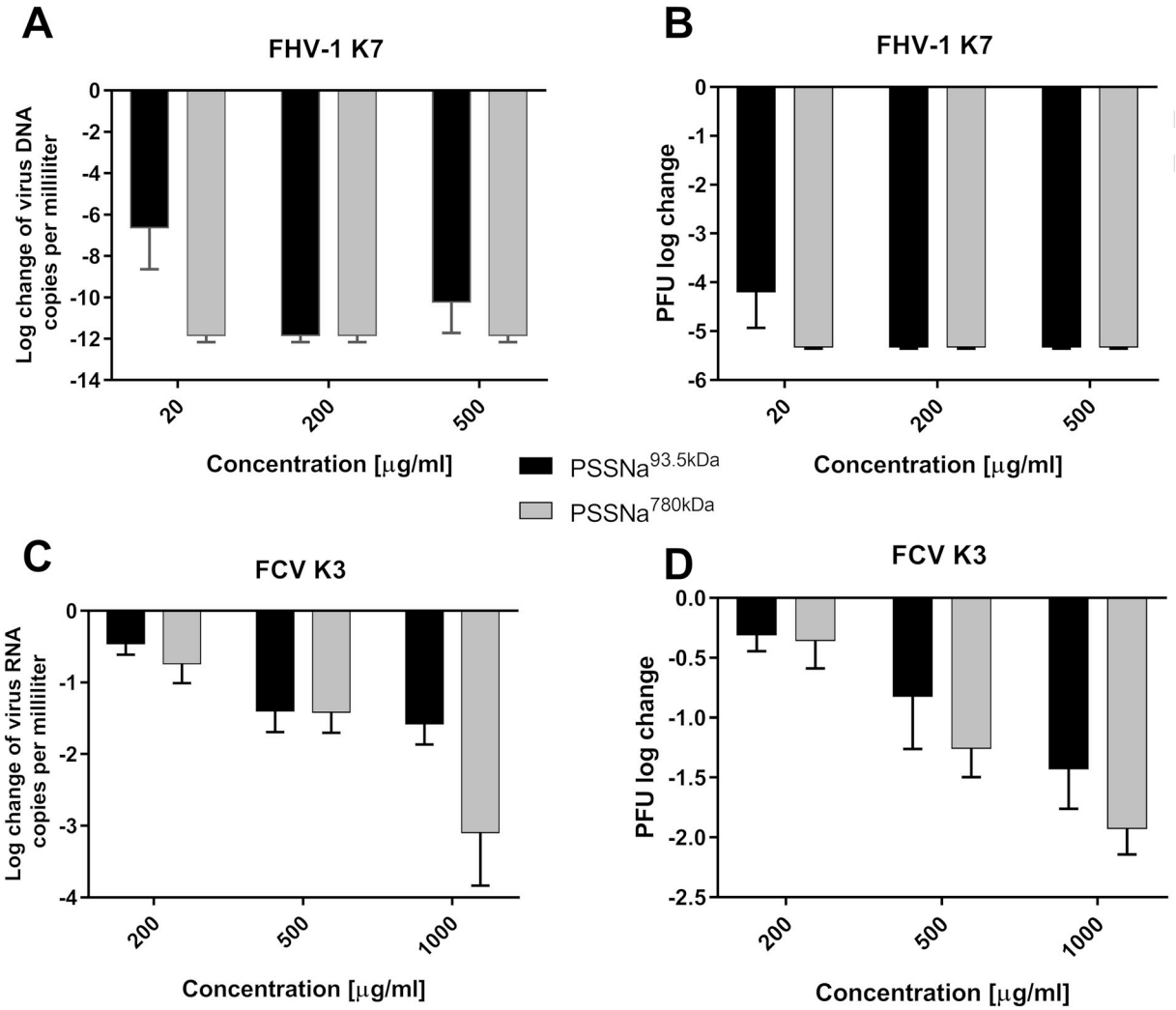


Fig. 10. Antiviral activity of PSSNa against clinical strains of FCV and FHV-1 is dose dependent. Virus replication assay was performed as described. Two veterinary strains FCV K3 (**A, B**) and FHV-1 K7 (**C, D**) were used, antiviral activity was evaluated with qPCR and presented as log change of virus RNA/DNA copies per milliliter (**A, C**) or plaque assay and presented as PFU (plaque forming unit) log change (**B, D**). Experiments were carried out for two PSSNa polymers of different M_w ($PSSNa^{93.5kDa}$ and $PSSNa^{780kDa}$) at 200, 500 and 1000 µg/ml for FCV K3 or at 20, 200 and 500 µg/ml for FHV K7. Results were normalized to the values of untreated, infected cells are presented as mean ± SEM from three independent experiments.

Table 3
ACV, PCV and PSSNa^{780kDa} effect on FHV-1 replication.

	IC_{50} [μg/ml]			CI
ACV	**ACV (PSSNa^{780kDa})**	**PSSNa^{780kDa}**	**PSSNa^{780kDa} (ACV)**	
12.1	2.96	2.45	1.64	0.92
PCV	**PCV (PSSNa^{780kDa})**	**PSSNa^{780kDa}**	**PSSNa^{780kDa} (PCV)**	
1.37	0.31	2.45	0.55	0.46

IC_{50} – concentration required to obtain 50% of inhibitory concentration in presence of compound [μg/ml], **IC_{50} "Compound X (Compound Y)"** – concentration required to obtain 50% of inhibitory concentration in presence of compound X [μg/ml] when the concentration of compound Y is constant and equal to $\frac{IC_{50}}{2}$, **CI** – combination index.

the infection, it was proposed that it mimics negatively charged glycosaminoglycans (GAGs; e.g., heparan sulfate) and binds to virions or microbes at the GAGs binding sites, consequently blocking their attachment to the cell (Ito et al., 1989; Baba et al., 1988; Mohan et al., 1992; Zacharopoulos and Phillips, 1997). Considering this property it was surprising to observe that PSSNa also inhibits FCV infection, as it was reported that FCV does not require GAGs to enter the cell (Stuart and Brown, 2007). Functional assays allowed us to determine that the mechanism of polymer action was somewhat different in this case. Polymers inhibited the viral replication and we assume that they directly or indirectly affect essential steps of the viral replication cycle, as viral enzymes or their cellular partners. However, further studies are necessary to elucidate the detailed mechanism of action. Interestingly, polymers with higher Mw were more potent against FCV due to their ability to inhibit also the attachment of viruses to susceptible cells. Intriguingly, FCV does not bind to GAGs and they do not have a well-defined binding site that may be blocked with the negatively charged PSSNa, as it was proposed for FHV-1. We believe that in this case, PSSNa binds to the surface of the virion and forms a protective shell that sterically blocks its interaction with the cell. To do so, it must be of a certain size and must adopt certain 3D conformation, what may explain the lack of antiviral effect for shorter polymers.

Importantly, PSSNa polymers are already clinically approved drugs in the treatment of hyperkalemia in humans (Mistry et al., 2016; Hollander-Rodriguez and Calvert, 2006; (FDA), 2017). PSSNa is administered orally or as an enema ((FDA), 2017). The recommended doses are 15–60 g orally for the adult hyperkalemia patient up to four times a day (Mistry et al., 2016; (FDA), 2017). To determine if PSSNa polymers are not toxic, dermal toxicity was evaluated *in vivo* in a mouse model. The obtained results indicate that PSSNa polymer hydrogel at concentrations up to 100 mg/ml (higher were not tested) is not toxic while administered on the skin and that PSSNa polymer retains its antiviral properties in the PEG hydrogel. We believe that PSSNa-PEG hydrogel may be administered topically as a cream on the affected areas of eyes and skin or as an aerosol in the treatment of mouth ulcers and sore throat.

Synergistic activity for FHV-1 was evaluated. Although the mode of action of acyclovir and penciclovir is different than PSSNa polymers, we observed the synergistic activity. That can be expected considering that while PSSNa hinders virus entrance to the cell, the acyclovir and penciclovir affect viral replication in the host cells.

5. Conclusions

All things considered, here we report a ready-to-use, safe and effective treatment of the URTD in cats. PSSNa effectively inhibits FHV-1 and FCV *in vitro*, while no toxicity *in vitro* or *in vivo* is observed. The compound shows dual activity, as it blocks FHV-1 attachment sites and at the same time it interferes with FCV replication. We believe that PSSNa may be used topically e.g., as a hydrogel, to limit the transmission, mitigate the symptoms and protect from lethal sequelae.

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Author contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104563.

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