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(54) **ANIONICALLY MODIFIED N-SULFONIC POLYALLYLAMINE DERIVATIVE, PHARMACEUTICAL COMPOSITION COMPRISING THE N-SULFONIC POLYALLYLAMINE DERIVATIVE AS THE ACTIVE SUBSTANCE AND USE OF THE N-SULFONIC POLYALLYLAMINE DERIVATIVE FOR THE PRODUCTION OF A MEDICINE**

ANIONISCH MODIFIZIERTES N-SULFONISCHEM POLYALLYLAMINDERIVAT, PHARMAZEUTISCHE ZUSAMMENSETZUNGEN MIT DEM N-SULFONISCHEN POLYALLYLAMINDERIVAT ALS WIRKSTOFF UND VERWENDUNG DES N-SULFONISCHEN POLYALLYLAMINDERIVATS ZUR HERSTELLUNG EINES MEDIKAMENTS

DÉRIVÉ DE POLYALLYLAMINE N-SULFONIQUE MODIFIÉ ANIONIQUEMENT, COMPOSITION PHARMACEUTIQUE COMPRENANT LEDIT DÉRIVÉ POLYALLYLAMINE N-SULFONIQUE EN TANT QUE SUBSTANCE ACTIVE ET UTILISATION DUDIT DÉRIVÉ POUR LA PRODUCTION D'UN MÉDICAMENT

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(Cont. next page)

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Description

[0001] The invention relates to an anionically modified polyallylamine, an N-sulfonic polyallylamine derivative, application of the N-sulfonic polyallylamine derivative as a medicine, particularly for prevention and treatment of respiratory tract infections caused by human metapneumovirus (hMPV) and human rhinovirus (HRV), as well as infections with influenza A virus (IAV), and to a pharmaceutical composition comprising the N-sulfonic polyallylamine derivative and the application thereof.

[0002] The human metapneumovirus (hMPV), described for the first time in 2001, belongs to the *Paramyxoviridae* family, *Pneumovinae* subfamily, *Pneumovirus* genus. Similarly to the influenza virus or the human respiratory syncytial virus (hRSV), it causes respiratory tract diseases, though with a milder course. The hMPV is responsible for 7-8% of viral diseases of the respiratory tract among children and 2-3% among adults [1], attacking ciliated epithelial cells of the respiratory tract. A disease caused by the human metapneumovirus has influenza-like symptoms (rhinitis, cough, fever). The virus is widespread on all continents, and the highest frequency of its occurrence in observed in winter and spring. It is characterised by droplet infection [2]. More severe symptoms, including serious infections of the lower respiratory tract, are found mainly with children and infants under the age of five [1][2], with elderly people above the age of sixty and with persons having a reduced immunity level [2].

[0003] As the American Lung Association and the latest reviews on the subject report, no medicine inhibiting or preventing infections caused by the hMPV has been approved for use hitherto [1,2,3,4,5]. Till 2012, only ribavirin ((1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) - a nucleoside being a guanosine analogue - exhibiting a broad range of actions against various viruses (RNA, DNA) of the respiratory tract [2,4,5], and immunoglobulins [2,5] were used for treatment of very severe acute infections caused by the hMPV among patients with lung transplants. However, they were used only in critical cases, because of their undesirable effects and potential teratogenic activity [2,4].

[0004] Till now, a method for inhibition of hMPV replication by using peptides was disclosed, the peptides exhibiting a strong affinity to viral fusion protein FF, thus blocking the lifecycle of the human metapneumovirus already at the stage of its fusion to host cells (Patent Application No. US20040229219A1, *Method of inhibiting human metapneumovirus and human coronavirus in the prevention and treatment* of *severe acute respiratory syndrome (SARS),* 2004). Other patent documents relate to prevention and inhibition of the hMPV by using proper antibodies or recombined human metapneumoviruses (application as vaccines).

[0005] The human rhinovirus (HRV) belongs to the *Picornaviridae* family. These viruses do not have a glycoprotein envelope, and their genetic material is constituted by a single RNA strand with a positive polarity. HRV virions belong to the smallest known viruses, and their diameter does not exceed 30 nm. During the year, particularly in winter and spring, they cause infections of the upper respiratory tract with humans, appearing as the common cold. As the medical website Medscape reports, these viruses are the cause of up to 25-80% of all infection cases. In spite of the fact that they are mainly linked with the common cold, rhinoviruses also cause otitis media, sinusitis and inflammations of the lower respiratory tract, including bronchiolitis, bronchitis and pneumonia [7]. With children, they may cause wheezing and asthma exacerbations as well [7,8].

[0006] In spite of the fact that these viruses are the cause of so frequent infections of the upper respiratory tract with humans, development of an effective vaccine preventing these infections is not possible. The main problem is human rhinoviruses occur as a great many serotypes (more than 100 HRV serotypes are known [9]). Moreover, there are no known commercially available medicines at present which would inhibit replication of human rhinoviruses. Literature references indicate the possibility of using α-2b interferon - a glycoprotein with activity based on enhancing the immune response of the organism, or a recombined ICAM-1 protein, being a synthetic analogue of receptors on the surface of the host cells, used by viruses to attach to these cells [10]. Pleconaril and Pirodavir, two synthetic low-molecular compounds, seemed to be the most promising inhibitors of HRV replication [11]. The compounds attach to the hydrophobic part of the HRV virion capsid, precluding the subsequent liberation of the viral RNA, thus preventing initiation of the virus replication cycle. Unfortunately, because of their side effects, these substances were not accepted by the Food and Drug Administration (FDA) for oral common cold treatment [12]. Grassauer *et al.* proved in their paper that iota-carrageenan exhibits antirhinoviral activity, the compound belonging to polysaccharides containing sulfate groups. The authors suggest that iota-carrageenan could also find application in prevention and treatment of common colds caused by the rhinoviruses [13]; however, as a result of its strong propensity to form gels and the low solubility resulting from this, the usefulness of carrageenans as antirhinoviral agents seems to be limited.

[0007] The influenza A virus (IAV) causes respiratory tract infections with an acute and severe course. It is one of the most clinically significant pathogens of the respiratory tract. Influenza virus infections occur most often in winter (seasonal influenza, the highest incidence being in January-February [1]). The World Health Organisation (WHO) estimates that there are 3-5 million cases of influenza yearly, including 250-500 thousand fatal cases [14,15]. The most severe infections occur with children under the age of two, elderly people above the age of sixty-five and with persons having a reduced immunity level. The WHO recommends vaccination against influenza viruses as the most effective method for prevention of infection [16]. The emergence of new types of the virus may lead to the development of an epidemic or a pandemic.

The high variability of the virus, difficulties in rapidly obtaining an adequate number of vaccines before the epidemic wave and occasional insufficient effectiveness of the vaccine results in the fact that this disease still constitutes a significant medical and epidemiological problem.

[0008] The anti-influenza medicines currently used affect one of two stages of the replication cycle of the influenza virus. Namely, they disturb the stage of removal of the protein envelope of the virus after it penetrates the cell by blocking the ion channels of the M2 protein (Amantadine and Rimantadine), or they inhibit liberation of new virus molecules from the infected cell by affecting neuraminidase (NA), a viral envelope protein responsible, most of all, for the liberation of the newly formed influenza virus molecules from the infected cells (Zanamivir and Oseltamivir) [17-21]. The envelope of the influenza virus also contains haemagglutinin (HA) - a glycoprotein responsible, most of all, for the process of attachment and penetration of the interior of epithelial cells in the respiratory tract by the virions, and thus for the initiation of infections. Haemagglutinin-blocking anti-influenza medicines include high-molecular drugs - peptides and proteins, e.g. EB (entry blocker) peptide, attaching specifically to HA, preventing a repeated infection. NDFRSKT peptide exhibits a high antiviral activity and - similarly to the EB peptide - inhibits HA activity. The principle of operation of another peptide, FLUDASE, is different, as the inhibition occurs by a removal of the receptor (sialic acid residue) from the surface of the host cells, thus precluding attachment and cell penetration of the virions [21].

[0009] Unfortunately, high genetic variability leads to rapid adaptation of the pathogen to the environment and the emergence of strains resistant to the therapy. For example, M2 protein inhibitors are already ineffective and not used. Similarly, strains of the influenza virus resistant to hitherto used neuraminidase-blocking medicines, i.e. Oseltamivir and Zanamivir, have already emerged. In this connection, new neuraminidase inhibitors - Laninamivir, Favipiravir and Peramivir - have been introduced lately in Japan and South Korea. Laninamivir, administered only by inhalation, effectively inhibits infections caused by viruses resistant to Oseltamivir, while Peramivir, administered intravenously, is particularly useful in the treatment of patients who cannot take Zanamivir (e.g. patients affected with asthma) infected with an influenza virus strain resistant to Oseltamivir [21].

[0010] One may find information in literature that also carrageenans interact with particles of the influenza virus directly, precluding its absorption and cell penetration [20,22,23,24].

[0011] Document XP002751154 (Thomas Scientific, London, GB; AN 1987-237525) and patent application JP-A-S62 53665 disclose a polyallylamine derivative anionically modified by substitution of a hydrogen atom in the amine group with a sulfonic group (NSPAH), having a molecular weight (Mw) of 10,000, which is used as blood anticoagulant.

[0012] In the document XP002751155 Janagase Akira et al. "Manufacture of antithrombogenic organic polymers", (Chemical Abstracts Service, Columbus, Ohio, US, 9 July 1988 (1988-07-09) and patent application JP-A-S62 57562 disclose a sodium salt of an NSPAH, having a molecular weight of 10,000, which is used in the preparation of various anti-thrombogenic articles.

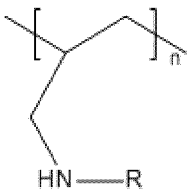
[0013] Patent CA-A-2868718 reveals a sodium salt of an NSPAH, prepared from a starting commercial "polylallylamine L" with about 175 repeating units.

[0014] In the article XP035076625 of Artyushenko, S. V. et al. "Study of interaction of polystyrene sulfonate with polymerization degree of 8 and polyallylamine with bilayer lipids membranes"; Biophysics (20120331), 57(2), pp. 179-180, describes interaction of polystyrene sulfonate (polymerization degree of 8, PSS-8) and polyallylamine (PAA, mol. mass 60 kDa) with viruses of the para- and orthomyxovirus family (on the examples of measles, parotiditis, and influenza viruses) suppresses their infectivity. The possible mechanism of virus-inhibiting action of these chemical compounds is damaging of interfacial antigenic proteins of para- and orthomyxoviruses.

[0015] Patent application US-A-2002/025919 discloses an antiviral compound comprises a linear non-carbohydrate polymer having a plurality of side chain groups, wherein at least one of the side chain groups has an anionic-or cationic-containing moiety bonded or linked thereto.

[0016] The goal of the invention was to develop a substance inhibiting replication of the human metapneumovirus (hMPV), the human rhinovirus (HRV) and the influenza A virus (IAV), which would find application in prevention or therapy of infections caused by these viruses in human organisms.

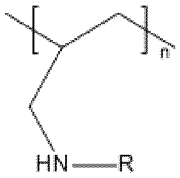
[0017] Surprisingly, it was discovered that this goal is achieved by a polyallylamine derivative anionically modified by substitution of a hydrogen atom in the amine group with a sulfonic group (NSPAH), having **Formula 1** presented below.



Formula 1 Anionically modified polyallylamine NSPAH

wherein each R is independently selected from $—SO_3^-$ and -H, at least one R is $—SO_3^-$ group, and n is an integer from 150 to 15000.

[0018] Thus, the invention relates to the anionically modified N-sulfonic polyallylamine derivative (NSPAH) with **Formula 1**



Formula 1

wherein each R is independently selected from $—SO_3^-$ and -H, at least one R is $—SO_3^-$ group, and n is an integer from 150 to 15000,

for use in therapy and prevention of infections caused by the human metapneumovirus hMPV, respiratory tract infections caused by the human rhinoviruses (HRV) and infections caused by the influenza A virus.

[0019] Preferably, the N-sulfonic polyallylamine derivative is in the form of a sodium salt.

[0020] The invention also relates to a pharmaceutical composition, characterised in that it contains the N-sulfonic polyallylamine derivative according to the invention as an active substance.

[0021] The invention also relates to the aforementioned pharmaceutical composition to be used as a medicine, particularly to be used in therapy and prevention of infections caused by the human metapneumoviruses hMPV, respiratory tract infections caused by the human rhinovirus (HRV) and infections caused by the influenza A virus.

[0022] Preferably, this composition is in the form of a solution or an aerosol administered to the upper respiratory tract.

[0023] The invention also relates to application of the N-sulfonic polyallylamine derivative according to the invention for production of a medicine to be used in therapy and prevention of infections caused by the human metapneumovirus hMPV, respiratory tract infections caused by the human rhinoviruses (HRV) and infections caused by the influenza A virus, while preferably, the N-sulfonic polyallylamine derivative according to the invention is used for production of a medicine having the form of a solution or an aerosol administered to the upper respiratory tract.

Brief description of the drawing

[0024]

Fig. 1 shows FTIR-ATR spectra of the polyallylamine before (PAH-15-0 - solid line) and after modification (NSPAH-15-30, NSPAH-15-95, NSPAH-65-75, NSPAH-65-89 - dashed lines).

Fig. 2a, 2b, 2c and 2d show the results of a cytotoxicity study of NSPAH-15-30, NSPAH-15-95, NSPAH-65-75 and NSPAH-65-89 polymers, respectively, carried out on the LLC-MK2 cell line by XTT and NR tests.

Fig. 3a, 3b, 3c and 3d show the results of a cytotoxicity study of NSPAH-15-30, NSPAH-15-95, NSPAH-65-75 and NSPAH-65-89 polymers, respectively, carried out on the MDCK cell line (Madin-Darby dog kidney cells) by XTT and NR tests.

Fig. 4 shows the inhibition of replication of the human metapneumovirus (hMPV) by the N-sulfonic polyallylamine derivatives according to the invention, depending on the concentration, the degree of substitution with sulfonic groups and the molecular mass of the studied polymers.

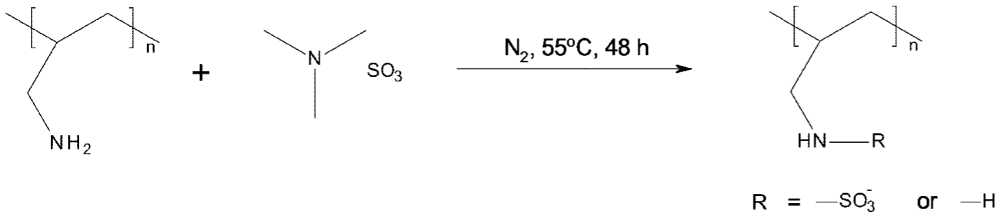
Fig. 5 showsthe inhibition of replication of the human rhinovirus (HRV) by the anionically modified polyallylamine derivatives, depending on the concentration and the molecular mass of the studied polymers.
Fig. 6 shows the inhibition of replication of the influenza A virus (IAV) by the N-sulfonic polyallylamine derivatives, depending on the concentration, the degree of substitution with sulfonic groups and the molecular mass of the studied polymers.

[0025] The subject of the invention is presented in more detail in the following embodiments:

Example 1.

Preparation and physico-chemical characterisation of the anionic polyallylamine derivative (NSPAH)

[0026] The reaction of the polyallylamine modification, shown in **Scheme 1,** was used earlier for preparation of an N-sulfonic chitosan derivative [6].



Scheme 1. Reaction of the N-sulfonic polyallylamine derivative (NSPAH) preparation.

[0027] 0.5 g of polyallylamine hydrochloride (PAH-15) with a molecular mass of Mw ~15 kDa or 2.45 ml of 20% polyallylamine hydrochloride solution (PAH-65) with a molecular mass of Mw ~65 kDa were dissolved in 25 ml of distilled water. 1.85 g of sodium carbonate was then added, and the mixture was stirred using a magnetic stirrer for 45 minutes in order to unlock the amine groups. During this time, the mixture was degassed by passing nitrogen through the system and afterwards a proper amount **(Table 1)** of sulfur trioxide-trimethylamine complex (STTC). The reaction was carried out for 48 hours at 55°C, with the mixture being stirred using a magnetic stirrer. After this time, the reaction mixture was cooled to room temperature and dialysed against water for 7 days. The obtained polymers were isolated from the solutions by freezing in a freeze dryer for 48 hours.
[0028] The structure of the anionically modified polyallylamines is confirmed by FTIR-ATR spectra (**Fig. 1**). Values of the degree of substitution are collected in **Table 1.**
[0029] In the FTIR-ATR spectra of the modified polyallylamines, bands at 631-662, 1044-1086 and 1211-1198 cm^{-1} occur, characteristic for stretching vibrations of sulfonic moieties present in the modified polyallylamine.
[0030] The degree of substitution (DS) with sulfonic groups was calculated based on the results of elemental analysis. The degree of substitution and zeta potential of the obtained polymers are presented in **Table 1.**

Table 1. Reaction conditions, composition and physico-chemical characteristics of the synthesised N-sulfonic polyallylamines.

Polymer	STTC/amine groups molar ratio	DS [%][a]	zeta potential [mV][b] PBS buffer pH=7.4	zeta potential [mV][c] medium 0% DMEM
—	—	—	—	-5.1±0.5
PAH-15-0	—	0	+26.4±1.4	+7.0±0.6
PAH-65-0	—	0	+4.3±0.5	—
NSPAH-15-95	5.0	95	-11.5±0.4	-15.2±0.6
NSPAH-56-98	5.0	98	-13.6±2.2	-24.4±1.6

(continued)

Polymer	STTC/amine groups molar ratio	DS [%][a]	zeta potential [mV][b] PBS buffer pH=7.4	zeta potential [mV][c] medium 0% DMEM
NSPAH-65-89	5.0	89	-16.7±0.8	-30.0±0.7

[a] The degree of substitution with sulfonic groups calculated based on elemental analysis. The DS is a percentage of amine groups substituted with the sulfonic group; therefore, e.g., DS = 30% means that in 30% of amine groups in allylamine units, one H atom was replaced with the SO_3^-moiety.
[b] polymer concentration 0.5 mg/ml, temperature 25°C; average of 5 measurements.
[c] polymer concentration 0.5 mg/ml, temperature 25°C; average of 5 measurements.

Example 2.

Cytotoxicity of the studied polymers

[0031] The cytotoxicity of anionically modified polyallylamines towards LLCMK2 cells (*Macaca mulatta* monkey kidney cells) and MDCK cells (dog kidney cells) **(Figs. 2, 3**) was examined.

[0032] The cytotoxicity was determined based on two tests. The first test consisted of a colorimetric test based on the ability of mitochondrial enzymes (succinate dehydrogenase) to reduce the XTT dye ((2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) to coloured Formazan salts. Determination of the number and condition of cells may be done based on the existence of a direct dependence between cell viability and the amount of dye formed, calculated from an absorbance measurement at the absorption maximum (450 nm) **(Figs. 2, 3**).

[0033] Evaluation of cell viability was also carried out using neutral red (NR). The test is based on the ability of neutral red to pass to the cytoplasm *via* passive transport. The dye accumulates in the lysosomes of living cells. The percentage of living cells is calculated after a lysis of cells and absorption measurements of the obtained solutions at 540 nm **(Figs. 2, 3**).

[0034] In the case of the LLC-MK2 line culture, the cells were grown for 6 days in a medium defined with DMEM without serum, but with an addition of trypsin, a medium containing the studied sulfonic polyallylamines in increasing concentrations. After this time, the cytotoxicity of the studied substances was determined according to the methods described above. Moreover, morphological changes in the cells in the presence of the polymers were observed using a phase contrast microscope. For the concentrations used, i.e. 5000, 2500, 1250 and 625 μg/ml, a cytotoxicity level of 50% was not achieved for any of the studied polymers. In other words, the studied sulfonic polyallylamines are not toxic for the LLC-MK2 cells. **Fig. 2a, 2b, 2c and 2d** show the results of a cytotoxicity study of NSPAH-15-30, NSPAH-15-95, NSPAH-65-75 and NSPAH-65-89 polymers, respectively, carried out on the LLC-MK2 cell line by XTT and NR tests. The results obtained from the measurements carried out were consistent with the observations concerning the lack of changes in cell morphology.

[0035] In the case of the MDCK line culture, the cells were incubated for 2 days in a medium defined with DMEM without serum containing the studied N-sulfonic polyallylamine derivatives in increasing concentrations. After this time, the cytotoxicity of the studied substances was determined using the methods described above. Moreover, morphological changes in the cells in the presence of the polymers were observed using a phase contrast microscope. For the concentration range of 625-5000 μg/ml, a cytotoxicity level of 50% was not achieved for any of the studied polymers. Therefore, it was proven that the studied N-sulfonic polyallylamine derivatives are not toxic for MDCK cells. **Fig. 3a, 3b, 3c and 3d** show the results of a cytotoxicity study of NSPAH-15-30, NSPAH-15-95, NSPAH-65-75 and NSPAH-65-89 polymers, respectively, carried out on the MDCK cell line by XTT and NR tests.

[0036] The results obtained from the measurements carried out were consistent with the observations concerning the lack of changes in cell morphology.

Example 3.

Influence of the studied polymers on inhibition of the cytopathic effect caused by replication of the human metapneumovirus (hMPV)

[0037] Inhibition of replication of the human metapneumovirus by sulfonic polyallylamines was examined. It was observed that the antiviral effect was stronger the higher the degree of substitution of the polyallylamine with sulfonic groups and the higher the molecular mass of the polymer was.

[0038] The experiment was carried out by infecting susceptible cells - the LLC-MK2 line - with the human metapneu-

movirus in the presence of the polymers in increasing concentrations. The cells were in a medium defined with DMEM without bovine serum, but with an addition of trypsin, for the entire time. After two hours of incubation at 37°C, the non-bound virions were washed out by triple rinsing of the cells with a PBS solution, and solutions of polymers with proper concentrations were then introduced. The infected cells were incubated at 37°C for 6 days.

[0039] After the assigned incubation time, morphological changes were observed using a phase contrast microscope. Inhibition of replication and no cytopathic effect (CPE) were observed with the polymer concentrations presented in **Table 2.**

Table 2. Values of the polymer concentrations, above which inhibition of hMPV replication and no CPE were observed.

Virus	Polymer	Minimum polymer concentration with which CPE was not observed [μg/ml]
hMPV	NSPAH-15-30	>2000
	NSPAH-15-95	1000
	NSPAH-65-75	500
	NSPAH-65-89	500

Example 4.

Study on the influence of sulfonic polyallylamines on hMPV replication in LLC-MK2 cells using RT-PCR real-time analysis.

[0040] The influence of sulfonic polyallylamines on hMPV replication was examined by a measurement of the number of RNA copies in the medium using real-time RT-qPCR analysis (Reverse Transcription Quantitative Polymerase Chain Reaction). In the study, the LLC-MK2 cells were incubated for 6 days in a medium defined with DMEM without bovine serum, but with an addition of trypsin. The infection was carried out in the presence of the polymer; after 2 hours of incubation of the cells with the virus, the medium was removed, and a fresh medium comprising polymers with proper concentrations was then introduced. The incubation was continued for 6 days. When the incubation was completed, total RNA was isolated from the cell supernatants. After the reverse transcription reaction, cDNAwas used as a matrix for the PCR.

[0041] The experiment was carried out using increasing concentrations of polymers. The obtained results are shown in **Fig. 4,** illustrating the inhibition of replication of the human metapneumovirus (hMPV) by the N-sulfonic polyallylamine derivatives, depending on the concentration, the degree of substitution with sulfonic groups and the molecular mass of the studied polymers. The number of viral RNA copies was expressed as the number of RNA copies in 1 ml of the sample.

[0042] Based on the presented dependencies, the values of concentrations of the N-sulfonic polyallylamines were determined, for which a 50% inhibition of replication of the human metapneumovirus (the so-called IC_{50}) occurred. These values are gathered together in **Table 3**.It was observed that sulfonic polyallylamines caused an inhibition of replication of the human metapneumovirus, while in the control samples (without the addition of the polymers), normal replication was found. A dependence of the antiviral effect vs. the degree of substitution with sulfonic groups and the molecular mass of the polyallylamines was proven. The studies carried out indicate that the effect is stronger the higher the molecular mass is and with the highest degree of substitution of the polymer.

Table 3. Values of IC_{50} determined based on the results of real-time PCR analysis (qRT-PCR).

Polymer	Polymer concentration for IC_{50} [μg/ml]
PAH-15-0	—
PAH-65-0	—
NSPAH-15-30	335.2±1.21
NSPAH-15-95	239.0±1.20
NSPAH-65-75	20.2±1.02
NSPAH-65-89	12.9±1.01

Example 5.

Influence of the studied polymers on inhibition of the cytopathic effect (CPE) caused by replication of the human rhinovirus (HRV)

[0043] Inhibition of rhinovirus replication by anionically modified polyallylamines with molecular masses of 15 kDa, 56 kDa and 65 kDa, and a high degree of substitution with sulfonic groups, 95%, 98% and 89%, respectively, was examined.

[0044] The experiment was carried out by infecting susceptible cells - the HeLa line - with the rhinovirus in the presence of the polymers in increasing concentrations. The cells were in a medium defined with DMEM without serum for the entire time. After two hours of incubation at 32°C, the non-bound virions were washed out by triple rinsing of the cells with a PBS solution, and solutions of polymers with proper concentrations were then introduced. The infected cells were incubated at 32°C for 2 days till the CPE occurred as a result of HRV infection.

[0045] After the assigned incubation time, morphological changes were observed using a phase contrast microscope. Inhibition of HRV replication, manifesting itself as a lack of the CPE, was observed in concentrations of the polymers equal to 1.0 mg/ml **(Table 4).**

Table 4. Values of the polymer concentrations, above which inhibition of HRV replication, manifesting itself as a lack of the CPE, was observed.

Virus	Polymer	Polymer concentration, above which the CPE was not observed [mg/ml]
HRV	NSPAH-15-95	1.0
	NSPAH-56-98	1.0
	NSPAH-65-89	1.0

Example 6.

Study on the influence of anionically modified polyallylamines on HRV replication in HeLa cells using RT-qPCR real-time analysis.

[0046] The influence of anionically modified polyallylamines on HRV replication was examined by a measurement of the number of RNA copies in the medium using real-time RT-qPCR analysis (Reverse Transcription Quantitative Polymerase Chain Reaction). In the study, the HeLa cells were incubated for 2 days in a medium defined with DMEM without bovine serum. The cells were infected with the HRV at a dose of $TCID_{50}$ = 400 in the presence of the polymers. After 2 hours of incubation at 32°C, the medium was removed, the cells were rinsed with a PBS solution three times, and a fresh medium comprising polymers with proper concentrations was then introduced. The incubation was continued for 2 days at 32°C, and RNA was then isolated from the cell supernatants. After the reverse transcription reaction, RNA was used as a matrix for the PCR.

[0047] The experiment was carried out using increasing concentrations of polymers. The obtained results are shown in Fig. 5. The decrease in the number of copies of viral RNA in the studied sample in relation to the control sample, LRV (Log Reduction Value), was determined using the following formula:
where:

c_i is the number of copies of viral RNA [copies/ml] in the studied sample for a given concentration of the studied polymer;
c_0 is the number of copies of viral RNA [copies/ml] in the control sample, i.e. without the polymer inhibitor.

[0048] **Fig. 5** shows the inhibition of replication of the human rhinovirus (HRV) by the anionically modified polyallylamine derivatives, depending on the concentration and the molecular mass of the studied polymers.

[0049] It was observed that sulfonic polyallylamines inhibit replication of the human rhinovirus very strongly (a dose of the polymers at a concentration of 100 □g/ml causes a decrease in the amount of the viral RNA in the sample to a non-measurable low value), while in the control samples (without the addition of the polymers), normal HRV replication was found.

[0050] Also, a dependence between the molecular mass of the N-sulfonic polyallylamines and their antiviral activity against the HRV was proven. The studies carried out indicate that the effect is stronger the higher the molecular mass of the N-sulfonic polyallylamine derivative is.

Example 7.

Influence of the studied polymers on the cytopathic effect caused by the influenza A virus (IAV).

[0051] Inhibition of replication of the influenza A virus by the N-sulfonic polyallylamine derivatives was examined. It was observed that the antiviral effect was stronger the higher the degree of substitution of the polyallylamine with sulfonic groups was and the higher the molecular mass of the polymer was. The experiment was carried out by infecting susceptible cells (MDCK) in the presence of increasing concentrations of the polymers. While infecting the cells with the IAV, they were in a medium defined with DMEM without bovine serum. After two hours of incubation at 37°C, the non-bound virions were washed out by triple rinsing of the cells with a PBS buffer, and solutions of polymers with proper concentrations were then introduced. The infected cells were incubated at 37°C for 2 days.

[0052] After the assigned incubation time, morphological changes were observed using a phase contrast microscope. Inhibition of IAV replication and a lack of the cytopathic effect were observed already at minimum concentrations of the polymers, which is presented in **Table 5.**

Table 5. Values of the polymer concentrations, above which inhibition of IAV replication and no CPE were observed.

Polymer	Minimum polymer concentration with which the CPE vanished [μg/ml]
NSPAH-15-30	1000
NSPAH-15-95	500
NSPAH-65-75	250
NSPAH-65-89	250

Example 8.

Study on the influence of N-sulfonic polyallylamine derivatives on IAV replication in MDCK cells using RT-PCR real-time analysis.

[0053] The influence of N-sulfonic polyallylamine derivatives on IAV replication was examined by a measurement of the number of RNA copies in the medium using real-time RT-qPCR analysis (Reverse Transcription Quantitative Polymerase Chain Reaction). In the study, the MDCK cells were incubated for 2 days in a medium defined with DMEM without bovine serum. The infection was carried out in the presence of the polymer; after 2 hours, the medium was removed, and a fresh medium comprising polymers with proper concentrations was then introduced. The incubation was continued for 2 days. When incubation was completed, total RNA was isolated from the cell supernatants. After the reverse transcription reaction, cDNA was used as a matrix for the PCR.

[0054] The experiment was carried out using increasing concentrations of polymers. The obtained results are shown in **Fig. 6** illustrating the inhibition of replication of the influenza A virus (IAV) by the N-sulfonic polyallylamine derivatives, depending on the concentration, the degree of substitution with sulfonic groups and the molecular mass of the studied polymers. The number of viral RNA copies was expressed as the number of RNA chains in 1 ml of the sample. Concentrations of the N-sulfonic polyallylamines, with which a 50% inhibition of replication of the influenza A virus (the so-called IC_{50}) was observed, are gathered in **Table 6.** It was observed that the N-sulfonic polyallylamine derivatives caused an inhibition of replication of the influenza A virus, while in the control samples (without the addition of the polymers), normal replication was found. A dependence of the antiviral effect *vs.* the degree of substitution with sulfonic groups and the molecular mass of the N-sulfonic polyallylamine derivatives was proven. The studies carried out indicate that the effect is stronger the higher the molecular mass is and with the highest the degree of substitution of the polymer. Non-modified polyallylamines (PAH-15 and PAH-65) did not exhibit antiviral properties, but at the same time they were highly toxic to MDCK and LLC-MK2 cell lines.

Table 6. Values of IC_{50} determined based on the results of real-time PCR analysis (qRT-PCR).

Polymer	Polymer concentration for IC_{50} [μg/ml]
PAH-15-0	—
PAH-65-0	—
NSPAH-15-30	53.4±1.7

(continued)

Polymer	Polymer concentration for IC_{50} [μg/ml]
NSPAH-15-95	4.5±2.1
NSPAH-65-75	0.5±1.2
NSPAH-65-89	0.6±1.1

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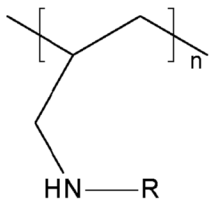
Claims

1. An anionically modified polyallylamine derivative (NSPAH) of the **Formula 1**



Formula 1

wherein

each R is independently selected from—SO_3^- and -H,
at least one R is —SO_3^- group, and
n is an integer from 150 to 15000,

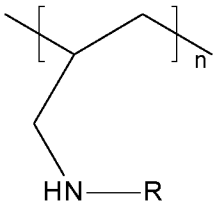
for use in therapy and prevention of infections caused by the human metapneumovirus hMPV, respiratory tract infections caused by the human rhinoviruses (HRV) and infections caused by the influenza A virus.

2. The anionically modified polyallylamine derivative for use according to claim 1, having the form of a sodium salt.

3. The anionically modified polyallylamine derivative for use according to claim 1 or 2, having the form of a solution or an aerosol administered to the upper respiratory tract.

Patentansprüche

1. Ein anionisch modifiziertes Polyallylaminderivat (NSPAH) der Formulierung 1



Formulierung 1

wobei

jedes R unabhängig gewählt wird aus —SO_3^- und —H,
mindestens ein R die Gruppe —SO_3^- ist und
n eine ganze Zahl zwischen 150 und 15000 ist,

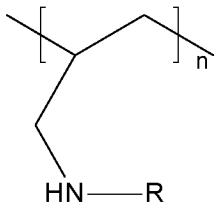
zur Verwendung in der Therapie und Prävention von Infektionen, die durch das humane Metapneumovirus hMPV verursacht werden, von Atemwegsinfektionen, die durch humane Rhinoviren (HRV) verursacht werden, und von Infektionen, die durch das Influenza-A-Virus verursacht werden.

2. Das anionisch modifizierte Polyallylaminderivat zur Verwendung gemäß Anspruch 1, in Form eines Natriumsalzes.

3. Das anionisch modifizierte Polyallylaminderivat zur Verwendung gemäß Anspruch 1 oder 2, in Form einer Lösung oder eines Aerosols, das in die oberen Atemwege verabreicht wird.

Revendications

1. Dérivé de polyallylamine modifié anioniquement (NSPAH) de la Formule 1 :



Formule 1

dans laquelle

chaque R est indépendamment choisi parmi $-SO_3^-$ et -H;
au moins un R est un groupe $-SO_3^-$; et
n est un entier de 150 à 15000,

pour une utilisation en thérapie et prévention des infections causées par le métapneumovirus humain hMPV, des infections des voies respiratoires causées par les rhinovirus humains (HRV) et des infections causées par le virus de la grippe A.

2. Dérivé de polyallylamine modifié anioniquement pour une utilisation selon la revendication 1, ayant la forme d'un sel de sodium.

3. Dérivé de polyallylamine modifié anioniquement pour une utilisation selon l'une des revendications 1 ou 2, ayant la forme d'une solution ou d'un aérosol administré aux voies respiratoires supérieures.

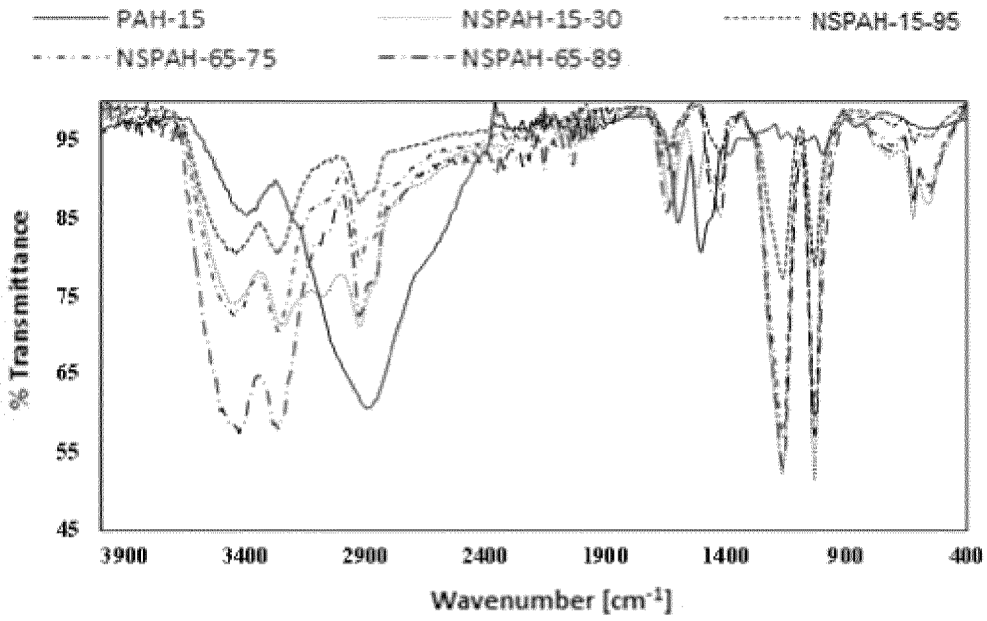

PAH-15
NSPAH-15-30
NSPAH-15-95
NSPAH-65-75
NSPAH-65-89
% Transmittance
95
85
75
65
55
45
3900
3400
2900
2400
1900
1400
900
400
Wavenumber [cm-1]

Fig. 1

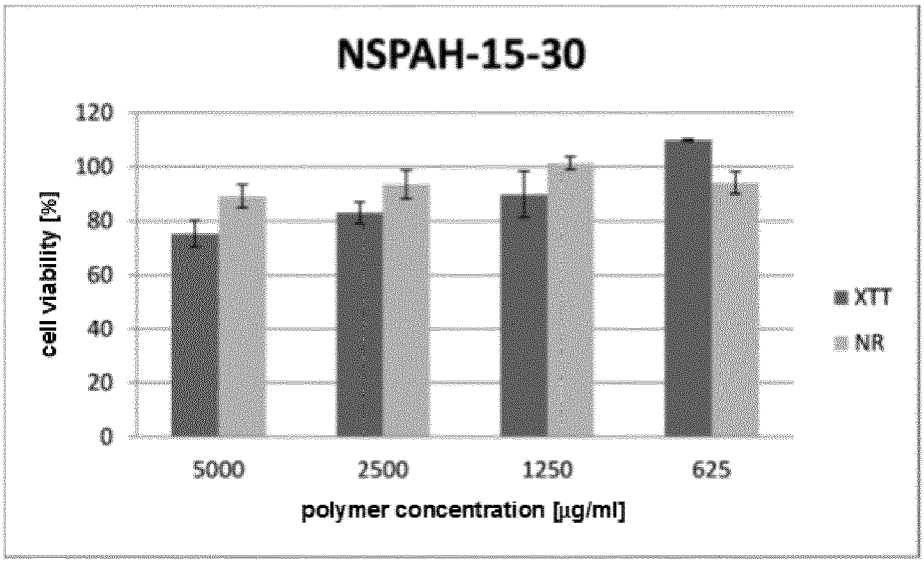

NSPAH-15-30
cell viability [%]
120
100
80
60
40
20
0
5000
2500
1250
625
polymer concentration [µg/ml]
XTT
NR

Fig. 2a

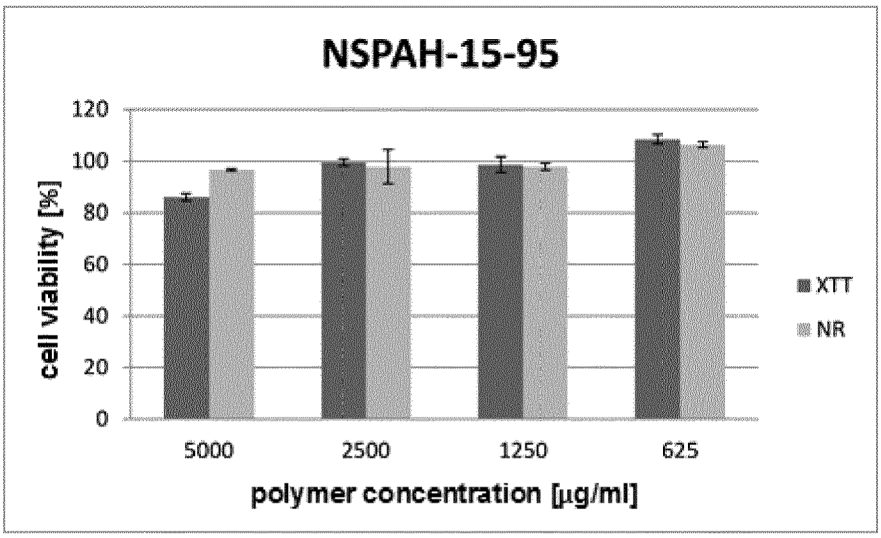
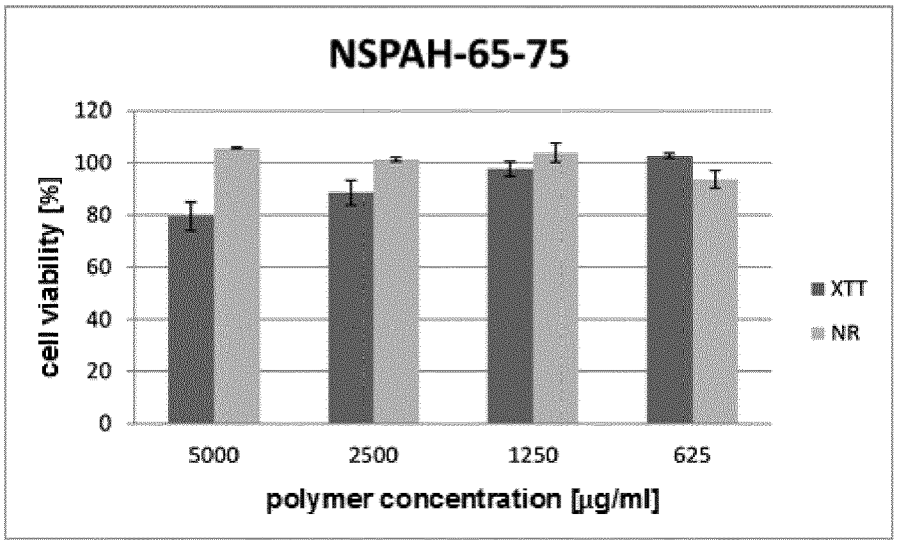

NSPAH-15-95
cell viability [%]
120
100
80
60
40
20
0
5000
2500
1250
625
polymer concentration [µg/ml]
XTT
NR

Fig. 2b


NSPAH-65-75
cell viability [%]
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100
80
60
40
20
0
5000
2500
1250
625
polymer concentration [µg/ml]
XTT
NR

Fig. 2c

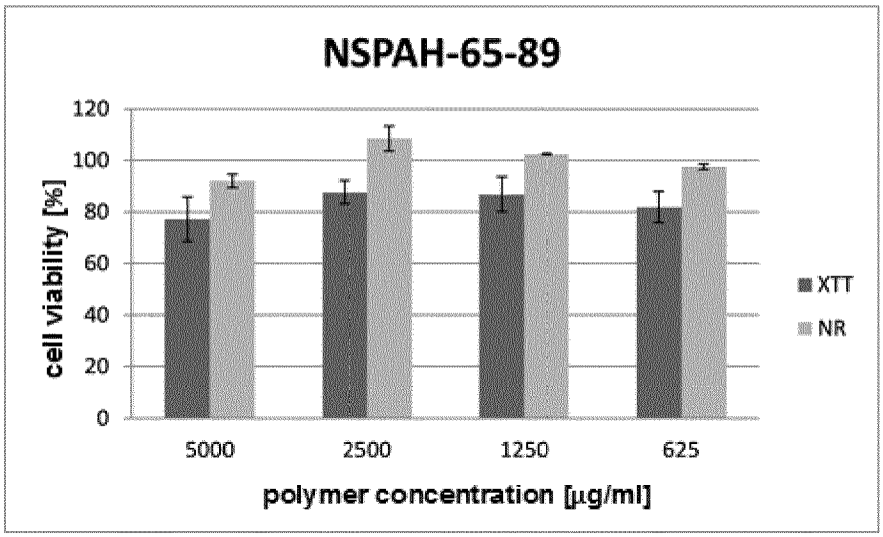
NSPAH-65-89
120
100
80
60
40
20
0
cell viability [%]
5000
2500
1250
625
polymer concentration [µg/ml]
XTT
NR

Fig. 2d

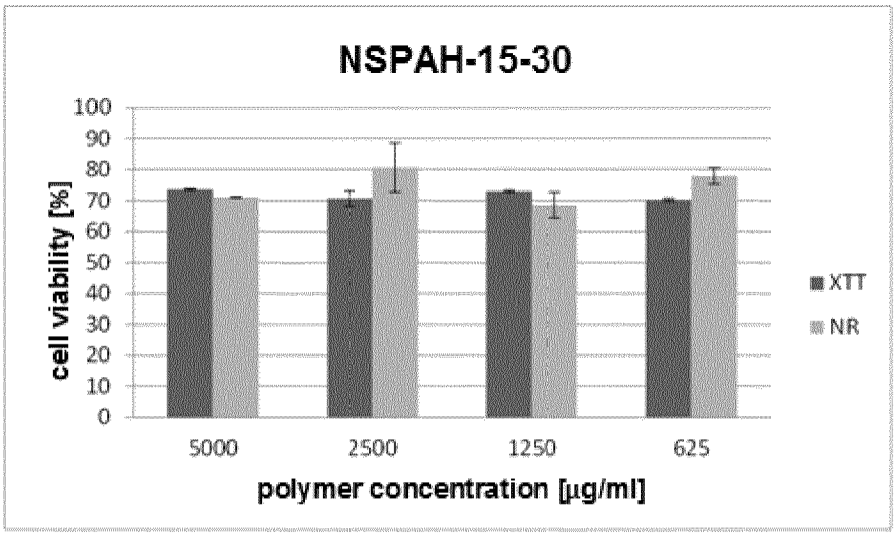
NSPAH-15-30
cell viability [%]
100
90
80
70
60
50
40
30
20
10
0
5000
2500
1250
625
polymer concentration [µg/ml]
XTT
NR

Fig. 3a

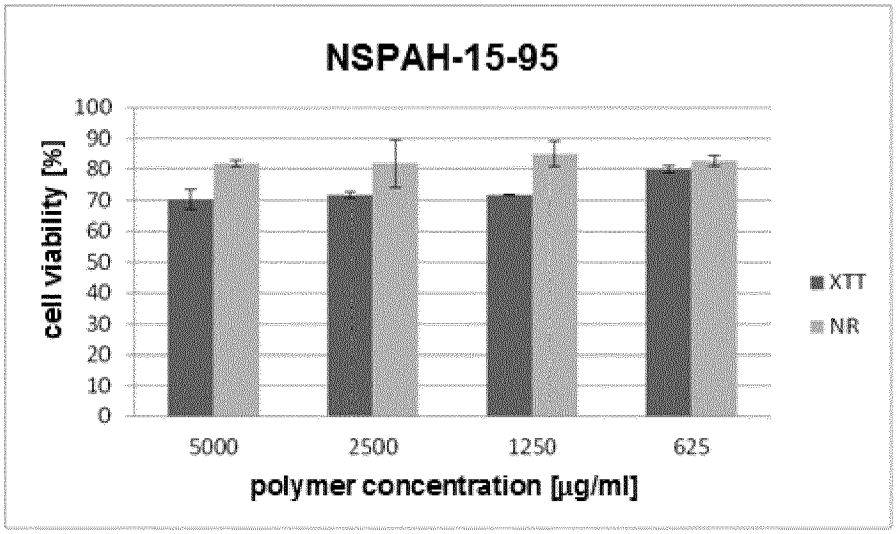
NSPAH-15-95
cell viability [%]
100
90
80
70
60
50
40
30
20
10
0
5000
2500
1250
625
polymer concentration [µg/ml]
XTT
NR

Fig. 3b

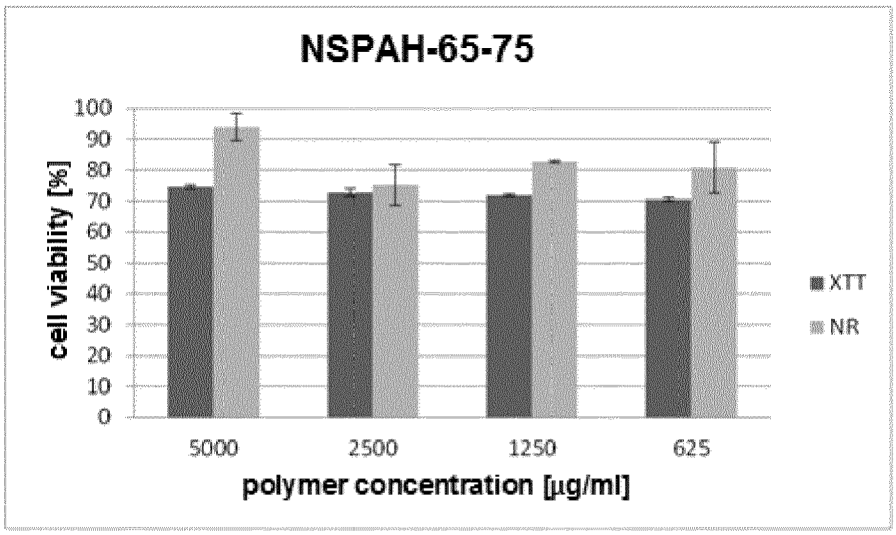
NSPAH-65-75
cell viability [%]
100
90
80
70
60
50
40
30
20
10
0
5000
2500
1250
625
polymer concentration [µg/ml]
XTT
NR

Fig. 3c

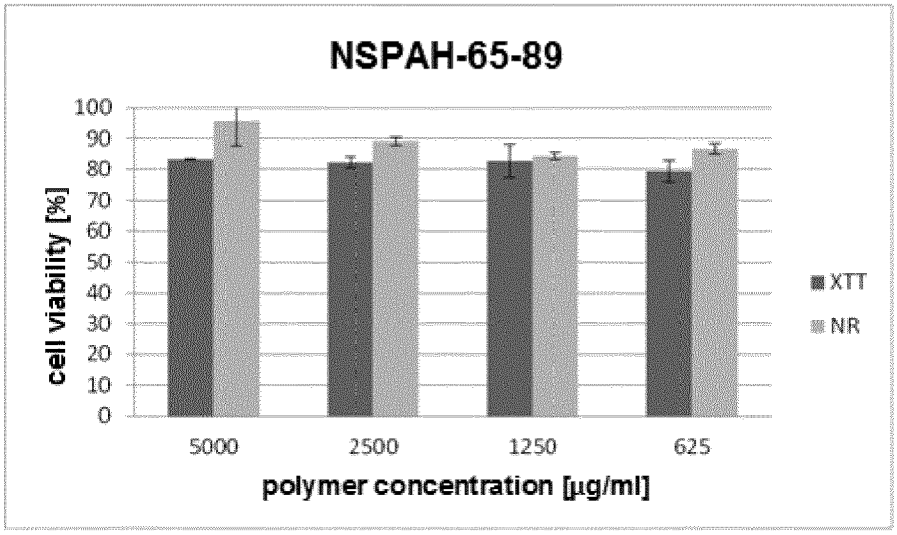
NSPAH-65-89
cell viability [%]
100
90
80
70
60
50
40
30
20
10
0
5000
2500
1250
625
polymer concentration [µg/ml]
XTT
NR

Fig. 3d

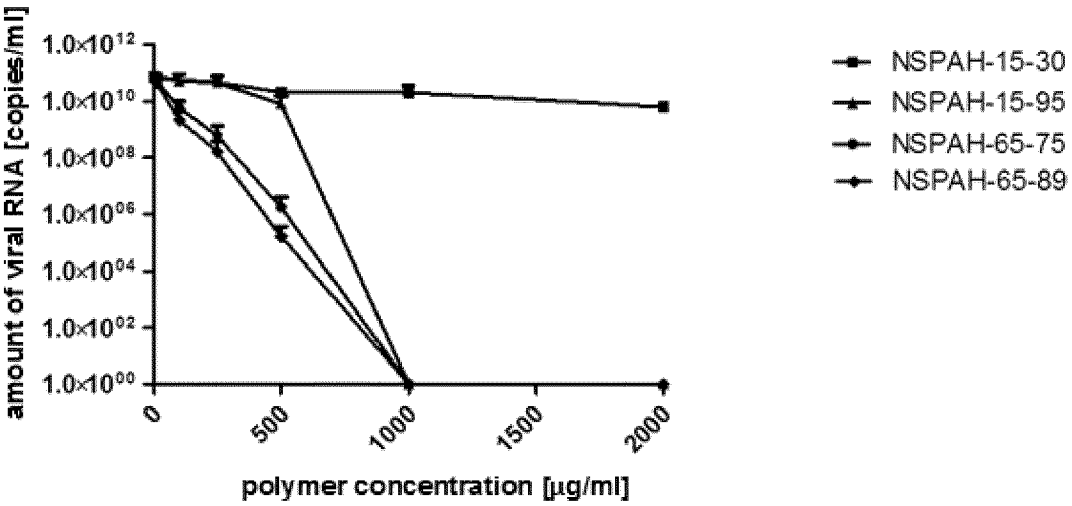
amount of viral RNA [copies/ml]
1.0×10^12
1.0×10^10
1.0×10^08
1.0×10^06
1.0×10^04
1.0×10^02
1.0×10^00
0
500
1000
1500
2000
polymer concentration [µg/ml]
NSPAH-15-30
NSPAH-15-95
NSPAH-65-75
NSPAH-65-89

Fig. 4

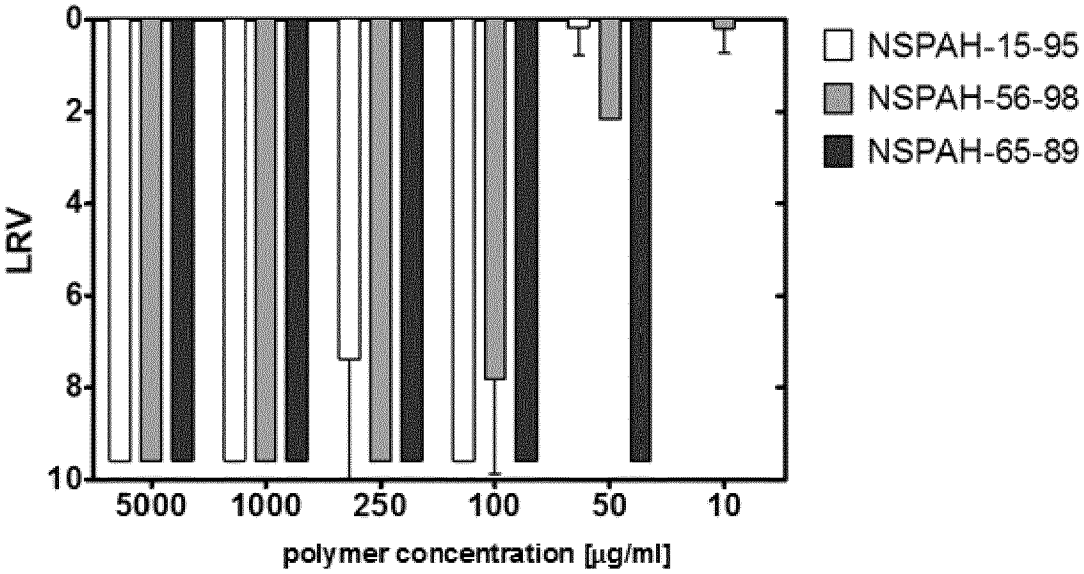
0
2
4
6
8
10
LRV
5000
1000
250
100
50
10
polymer concentration [µg/ml]
NSPAH-15-95
NSPAH-56-98
NSPAH-65-89

Fig. 5

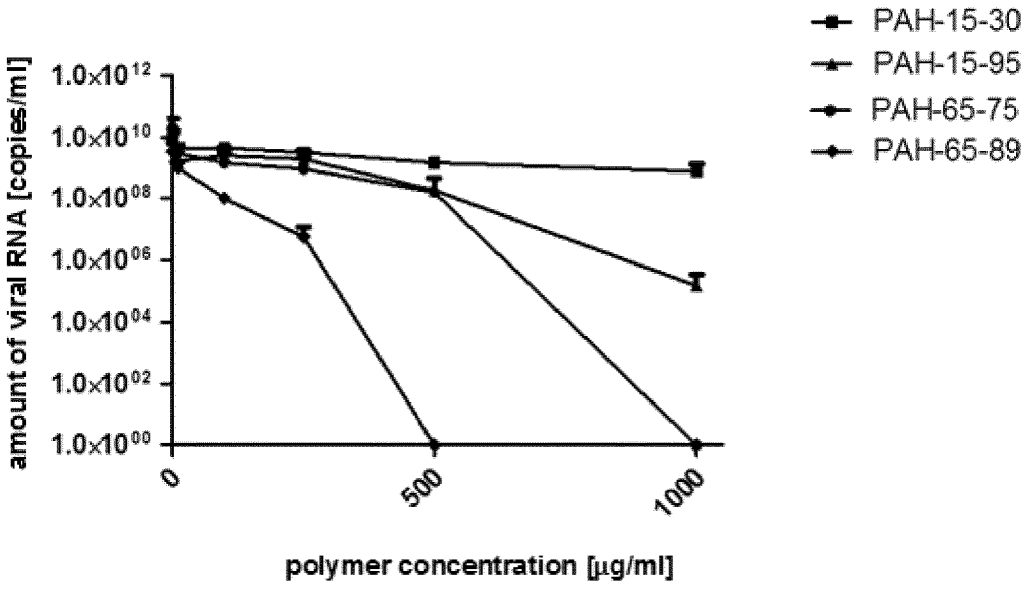
PAH-15-30
PAH-15-95
PAH-65-75
PAH-65-89
amount of viral RNA [copies/ml]
1.0x10^12
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
0
500
1000
polymer concentration [µg/ml]

Fig. 6

REFERENCES CITED IN THE DESCRIPTION

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