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Biopolymeric nano/microspheres for selective and reversible adsorption of coronaviruses

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ABSTRACT

A novel biopolymeric material in the form of nano/microspheres was developed which was capable of adsorbing coronaviruses. The biopolymer was obtained by crosslinking of chitosan (CHIT) with genipin, a nontoxic compound of plant origin, in inverted emulsion and reacting the chitosan nano/microspheres obtained (CHIT-NS/MS) with glycidyltrimethyl-ammonium chloride (GTMAC). As a result the nano/microspheres of N-(2-hydroxypropyl)-3-trimethyl chitosan (HTCC-NS/MS) were obtained. HTCC-NS/MS were studied as the adsorbents of human coronavirus NL63 (HCoV-NL63), mouse hepatitis virus (MHV), and human coronavirus HCoV-OC43 particles in aqueous virus suspensions. By studying cytopathic effect (CPE) caused by these viruses and performing PCR analyses it was found HTCC-NS/MS strongly adsorb the particles of HCoV-NL63 virus, moderately adsorb mouse hepatitis virus (MHV) particles, but do not adsorb HCoV-OC43 coronavirus. The adsorption capacity of HTCC-NS/MS well correlated with the antiviral activity of soluble HTCC against a given virus. Importantly, it was shown that HCoV-NL63 particles could be desorbed from the HTCC-NS/MS surface with a salt solution of high ionic strength with retention of virus virulence. The obtained material may be applied for the removal of coronaviruses, purification and concentration of virus samples obtained from biological matrices and for purification of water from pathogenic coronaviruses.

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1. Introduction

The multistep procedures of virus separation, concentration and purification are of great importance for the preparation of the products containing a virus as the principal component, such as cell culture-derived virus stocks for vaccine manufacturing, research and gene therapy [1–4]. On the other hand, virus separation is also required during procedures involving virus removal such as preparation of biopharmaceutical products, which should be free of any viral contamination [5] and in the purification of drinking water from water-borne viruses such as hepatitis A virus, hepatitis E virus, norovirus, rotavirus, and adenovirus [6].

Typical virus separation methods encompass membrane filtration [7] including tangential flow filtration [8], ultracentrifugation [9], electrophoresis [10,11], agglutination [12], and chromatography [13]. However, they are often expensive (cost of the purification may constitute up to 80% of the total cost (14)), time- and labor-consuming and difficult to scale-up.

An alternative method of virus purification/removal is virus adsorption which takes advantage of the attractive interaction between a virion and an adsorbent. Such interaction may be of various nature. Since the surface of viruses may become charged depending on their isoelectric point (IEP) and pH of the environment, their interactions with the adsorbents may be primarily electrostatic (at pH values sufficiently different from IEP) or mostly hydrophobic (at pH ≈ IEP) [15]. The surface of virions may be both positively (at pH < IEP) and negatively (at pH > IEP) charged so they may be adsorbed by sorbents with both negatively and positively charged surface, respectively. Consequently, materials previously reported as virus adsorbents are of different nature. The examples of inorganic adsorbents are clays such as kaolinite, montmorillonite, and bentonite [16]. Their adsorption efficiency for viruses is very different but generally low. For kaolinite at the concentration of 50 g/l the log removal value (LRV) was 2.18, while montmorillonite and bentonite were much less efficient. Thermodynamic studies indicated that the adsorption of some bacteriophages to clays depended on the hydrophobicity of the surface of both clays and viruses

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[17]. The ability to adsorb viruses was shown for hybrid organic-inorganic materials, e.g., cellulose nanofibers enriched with α-Fe_2O_3 nanoparticles [18]. The hybrid inorganic nanoparticles with virus particles covalently bound to their surface have been also reported and used as virus delivery systems [19].

Most of the organic adsorbents used for virus separation studied so far have been (modified) natural polymers, both neutral and charged, but synthetic polymers such as polystyrene substituted with arginine [20] have been also reported. Polymers used as adsorbents are mainly polysaccharides. The examples of polysaccharide adsorbents include anionic sulfated polysaccharides such as heparin [13], rhamnan [21], and fucan [22], and cationic chitosan. Polymeric membranes formed from chitosan and polyvinyl alcohol nanofibres cross-linked with glutaraldehyde have the ability to adsorb porcine parvovirus (PPV) and sindbis virus (SINV) particles [23,24]. Influenza A virus particles may be adsorbed by the carbohydrate-functionalized chitosan filaments [25]. The application of proteins and peptides as adsorbents has been also reported [26,27].

Adsorbents for various viruses have been described including alfaherpesviruses (pseudorabies virus (PrV) and bovine herpesvirus 1 (BHV-1) [28]), influenza A virus [25], porcine parvovirus (PPV) [26], sindbis virus (SINV) [25], retroviruses [13], poliovirus [29], and foamy virus [8]. Importantly, the only adsorbent for coronaviruses that has been tested so far, based on clay minerals, could adsorb bovine coronavirus. The adsorption was irreversible, making the adsorbent useless for aforementioned applications [30].

In our previous study we demonstrated that a chitosan derivative, i.e., (N-(2-hydroxypropyl)-3-trimethyl chitosan (HTCC)) shows anticoronaviral properties [31]. The polymer showed high specificity towards coronaviruses, as it inhibited replication of coronaviruses that belong to two separate genera: HCoV-NL63 (alphacoronavirus) and the mouse hepatitis virus (MHV; betacoronavirus). Further study proved that fine-tuning of HTCC substitution degree enables it to inhibit replication of all human coronaviruses [32].

Study on the mechanism of action showed that the polymer interacts with the S protein of HCoV-NL63 and forms a protein-polymer complex, which results in virus neutralization. Although neutralized virions retained the ability to bind to the attachment receptor (heparan sulfate proteoglycans), their interaction with the actual fusion receptor was blocked [33]. Others also reported that HTCC may be used in the form of electrospun HTCC-PVA fibers to adsorb porcine parvovirus (PPV) and sindbis virus (SINV) [23].

The aim of our present study was to exploit high affinity of HTCC to the S protein of coronaviruses and to obtain a convenient adsorbent for isolation and/or removal of coronaviruses form biological matrices for research purposes and for removal of coronaviruses from water. For this purpose chitosan nano/microspheres (CHIT-NS/MS) crosslinked with genipin, a natural compound obtained from the fruit of *Gardenia jasminoides*, were prepared and then cationized with GTMAC to obtain nano/microspheres of HTCC (HTCC-NS/MS). Chemical composition of obtained biomaterials was investigated using elemental analysis (EA) and spectroscopic methods. The morphology and size of the polymeric objects obtained was visualized using microscopic techniques, i.e., confocal microscopy and transmission electron microscopy (TEM). The hydrodynamic radius of the nanospheres was determined using dynamic light scattering (DLS) technique. Surface charge in different media was assessed using zeta potential measurements. The ability of the biomaterial to adsorb selected coronaviruses (HCoV-NL63, MHV and HCoV-OC43) was assessed. Changes in the infectivity of viral samples, due to interaction with adsorbent, were evaluated by virus titration according to Reed and Muench method and compared with total amount of viral RNA copies using real time PCR analysis. Adsorption and desorption of coronaviral particles on/from the surface of HTCC-NS/MS was directly visualized using atomic force microscopy (AFM) technique. Careful optimization of the material allowed for recovery (desorption) of infective coronaviral particles, which further broadens its possible applications. An advantage of the studied material is that chitosan (used in many other virus-related applications involving antiviral materials [34] and antiviral drugs [35,36]) and genipin, are both non-toxic substances.

2. Materials and methods

2.1. Materials

Chitosan (CHIT, low molecular weight, 75–85% deacetylated, Sigma-Aldrich), genipin (98%, Challenge Bioproducts), sorbitan monopalmitate (Span®40, Sigma-Aldrich), sorbitan monooleate (Span®80, Sigma-Aldrich), glycidyltrimethylammonium chloride (GTMAC, ≥90%, Sigma-Aldrich), cyclohexane (p.a, POCh), glacial acetic acid (CH_3COOH, 99,85% pure p.a., CHEMPUR), acetone (POCh), methanol (POCh) and sodium chloride (NaCl, POCh) were used as received. Doubly distilled water was deionized using the Millipore Simplicity system.

2.2. Preparation of CHIT-NS/MS and functionalization of their surface

In order to obtain the polymer material in the form of nano/microspheres previously described method was used [37]. The method is based on cross-linking of chitosan in an inverted emulsion with genipin, a non-toxic cross-linking agent. In view of the need to obtain the objects of the smallest size, the synthesis was performed in an ultrasonic bath at the stage of chitosan cross-linking.

In the first step of the synthesis, 0.5 g of Span 40 and 0.26 ml of Span 80 were dissolved in 200 ml of cyclohexane. Then, 35 ml of 2% (w/w) chitosan solution dissolved in 2% (v/v) acetic acid was added. The mixture was stirred with a mechanical stirrer at 1200 rpm and a reversed emulsion was formed. Then, the emulsion was placed in an ultrasonic bath and sonicated for 15 min to further reduce the size of the droplets in the emulsion. To sonicated emulsion 1 ml of 5% (w/v) ethanol solution of genipin was added dropwise. The sonication was continued in pulsed mode for approximately 8 h at 40 °C until a blue color appeared. The dispersion was left for 12 h in the dark. After completion of the crosslinking process the supernatant was decanted and the polymer material was washed thrice with cyclohexane. The nano/microspheres crosslinked with genipin (CHIT-NS/MS) were separated by centrifugation at 10,600 rcf for 15 min and washed thrice with cyclohexane. Finally, CHIT-NS/MS were washed with methanol, concentrated by partial drying and used in the subsequent synthesis steps.

The surface of CHIT-NS/MS was cationically modified using glicydyltrimethylammonium chloride (GTMAC). Briefly, the material obtained in the first step of the synthesis was separated into four equal parts. One of them was left as reference material (CHIT-NS/MS), and three other parts were used for synthesis of cationically modified chitosan nano/microspheres (HTCC-NS/MS). In order to obtain material with three different degree of substitution, the synthesis was carried out at 57 °C for 6, 24 and 48 h yielding the chitosan hydrogel with low, medium and high degree of surface modification (L HTCC-NS/MS, M-HTCC-NS/MS, and H-HTCC-NS/MS, respectively). Obtained materials were separated by centrifugation at 10,600 rcf for 15 min and purified by washing with distilled water until conductivity of the flow through was close to the conductivity of distilled water. At no stage of the purification process the material was allowed to dry completely. The adsorbent obtained was stored in distilled water before using in biological studies.

2.3. Physicochemical characterization of CHIT-NS/MS

Elemental analysis (C, H, and N) was performed with an EuroEA 3000 Elemental analyzer and IR spectra were recorded using dried samples at room temperature on a Nicolet IR200FT-IR spectrophotometer equipped with an ATR accessory. In both cases, solvent was removed by freeze-drying. Dynamic light scattering (DLS) and zeta potential

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