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Research paper

Auto-associative heparin nanoassemblies: A biomimetic platform against the heparan sulfate-dependent viruses HSV-1, HSV-2, HPV-16 and RSV

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ABSTRACT

A new, simple and green method was developed for the manufacturing of heparin nanoassemblies active against the heparan sulfate-dependent viruses HSV-1, HSV-2, HPV-16 and RSV. These nanoassemblies were obtained by the auto-association of *O*-palmitoyl-heparin and α-cyclodextrin in water. The synthesized *O*-palmitoyl-heparin derivatives mixed with α-cyclodextrin resulted in the formation of crystalline hexagonal nanoassemblies as observed by transmission electron microscopy. The nanoassembly mean hydrodynamic diameters were modulated from 340 to 659 nm depending on the type and the initial concentration of *O*-palmitoyl-heparin or α-cyclodextrin. The antiviral activity of the nanoassemblies was not affected by the concentration of the components. However, the method of the synthesis of *O*-palmitoyl-heparin affected the antiviral activity of the formulations. We showed that reduced antiviral activity is correlated with lower sulfation degree and anticoagulant activity.

1. Introduction

The first step in the infection of mucosal surfaces by viruses involves their attachment to cellular receptors exposed on the surface of epithelial cells. In many instances, virus–cell interaction is mediated by cell surface heparan sulfate proteoglycans (HSPGs) [1,2]. These negatively charged molecules are a core protein linked to glycosaminoglycan (GAG) chains of unbranched sulfated polysaccharides known as heparan sulfates (HS). HS are structurally related to heparin except that heparin has higher level of sulfation and higher content of iduronic acid [3,4].

The interaction between the viruses and HSPGs occurs between the basic amino acid residues of viral proteins and the negatively charged sulfated/carboxyl groups of the GAG chains. For this reason heparin and other GAGs can competitively interfere with virus attachment to cells. Many viruses exploit HSPGs as attachment receptors, namely the herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), the human papilloma virus (HPV) and respiratory syncytial virus (RSV) [5–7].

So far, GAGs have been explored as potential candidates in the prevention of viral infections [8,9]. Accumulated data from the literature indicate that the inhibitory effect of heparin and HS was demonstrated on HSV by acting on its earliest phase [10,6], while the binding of HPV-like particles to cells has been shown to be inhibited by heparin [11]. HS has proved to play an important role in the prevention of HPV infections [12].

Surprisingly, although numerous research works were already described in the literature on the activity of GAGs against viral mucosal infections, there is a clear gap concerning the design of efficient locally-administrated formulations. Besides the prevention of the infection, the formulation of GAGs as a drug delivery system able to target the viruses, to load antiviral drugs and to control their release over time represents an interesting strategy

Abbreviations: ATR-FTIR, Attenuated total reflectance-Fourier transform infrared; CC_{50}, cytotoxic concentrations; CIs, confidence intervals; CNRS, Centre National de la recherche Scientifique; DCM, dichloromethane; DMF, dimethylformamide; GAG, glycosaminoglycan; HPV, human papilloma virus; HS, heparan sulfates; HSPGs, heparan sulfate proteoglycans; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; IC_{50}, The concentration producing 50% reduction in plaque formation; OPH, *O*-palmitoyl-heparin; RSV, respiratory syncytial virus; SDS, sodium dodecyl sulfate; α-CD, α-cyclodextrin.

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against viral mucosal infections. However, although the large number of publications in the field of drug delivery systems, current nanotechnologies have, unfortunately, important limitations due to the complexity of the processes used to their manufacturing and thus the difficulty for the scaling-up of their production to pharmaceutical companies. Furthermore, manufacture processes require the use of toxic solvents (acetone and ethanol for flash nanoprecipitation), surfactants, polymerization initiators and extremely reactive monomers (anionic and radical emulsion polymerization). Expensive techniques must be employed to completely remove the solvents and the surfactants at the end of the preparation process. Solvent and surfactant traces may persist and constitute a drawback for the medical applications of these systems while monomers present in the polymerization medium could interact with the drug leading to its instability and the formation of toxic products.

In this context, Bouchemal's group has designed an innovative drug delivery system composed of nanoassemblies spontaneously formed in aqueous medium without using surfactants, pH modification and without heating or purification steps [13]. In this process, nanoassemblies were obtained by mixing a hydrophobically-modified polysaccharide and an α-cyclodextrin (α-CD).

The aim of the present work is to use this process to obtain new GAG-based nanoassemblies and to evaluate their ability to inhibit viral attachment to cells. We took advantage of structural similarities between heparin and cell surface HS and evaluated the antiviral activity of heparin-based formulations. The preparation of heparin nanoassemblies was achieved by the self-association in aqueous media of *O*-palmitoyl-heparin (OPH) and α-CD. This biomimetic barrier could act like a "trap" able to specifically catch up the viruses and avoid their attachment to the cells. The effect of the chemical modification of OPH on the antiviral activity was evaluated against HSV-1, HSV-2, RSV and the high-risk type of HPV (HPV-16) involved in cervical cancers. Rotavirus, a HSPG-independent virus was used as control.

2. Materials

Heparin sodium salt from porcine intestinal mucosa 500 kU, palmitoyl chloride, anhydrous pyridine, sodium chloride and sodium acetate were from Sigma (Saint-Quentin Fallavier, France). Acetone was from Carlo Erba (Val de Reuil, France). α-CD was from Cyclolab (Budapest, Hungary). Anhydrous dimethylformamide (DMF), anhydrous dichloromethane (DCM), diethylether, ethanol, and methanol were from VWR (Fontenay sous-bois, France).

2.1. Cells

African green monkey fibroblastoid kidney cells (Vero) (ATCC CCL-81), human epithelial cells Hep-2 (ATCC CCL-23), A549 (ATCC CCL-185) and African green monkey kidney epithelial (MA-104) cells (ATCC CRL-2378.1) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat inactivated fetal calf serum and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany). The 293TT cell line, derived from human embryonic kidney cells transformed with the simian virus 40 (SV40) large T antigen, was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal calf serum (FCS; Gibco-BRL), Glutamax-I 1% (Invitrogen, Carlsbad, CA) and nonessential amino acids 1% (Sigma Aldrich, Steinheim, Germany). 293TT cells allow high levels of protein to be expressed from vectors containing the SV40 origin due to over replication of the expression plasmid [14].

2.2. Viruses

Clinical isolates of HSV-1 and HSV-2 were kindly provided by Prof. M. Pistello, University of Pisa, Italy. HSV-1 and HSV-2 strains were propagated and titrated by plaque assay on Vero cells. RSV strain A2 (ATCC VR-1540) was propagated in Hep-2 and titrated by the indirect immunoperoxidase staining procedure using an RSV monoclonal antibody (Ab35958; Abcam, Cambridge, United Kingdom) as described previously [15]. Human rotavirus strain Wa (ATCC VR-2018) was activated with 5 μg/mL of porcine pancreatic trypsin type IX (Sigma, St. Louis, Mo.) for 30 min at 37 °C and propagated in MA104 cells by using MEM containing 0.5 μg of trypsin per mL as described previously [16]. Virus stocks were maintained frozen (−80 °C).

2.3. HPV PsV production

Plasmids and 293TT cells used for pseudovirus (PsV) production were kindly provided by John Schiller (National Cancer Institute, Bethesda, MD). Detailed protocols and plasmid maps for this study can be seen at http://home.ccr.cancer.gov/lco/default.asp. HPV16 PsVs were produced according to previously described methods [17]. Briefly, 293TT cells were transfected with a plasmid, named p16LLw, expressing the papillomavirus major and minor capsid proteins (L1 and L2, respectively), together with a reporter plasmid expressing the secreted alkaline phosphatase (SEAP), named pYSEAP. Capsids were allowed to mature overnight in cell lysate; the clarified supernatant was then loaded on top of a density gradient of 27% to 33% to 39 % Optiprep (Sigma-Aldrich, St. Louis, MO) at room temperature for 3 h. The material was centrifuged at 28,000 rpm for 16 h at 4 °C in an SW41.1 rotor (Beckman Coulter, Inc., Fullerton, CA) and then collected by bottom puncture of the tubes. Fractions were inspected for purity in 10% sodium dodecyl sulfate (SDS)–Tris–glycine gels, titrated on 293TT cells to test for infectivity by SEAP detection, and then pooled and frozen at −80 °C until needed. The L1 protein content of PsV stocks was determined by comparison with bovine serum albumin standards in Coomassie-stained SDS-polyacrylamide gels.

3. Methods

3.1. Preparation of O-palmitoyl heparin

Two methods for the esterification of heparin were used:

Method 1. Synthesis of OPH-1: Heparin (1 g) was suspended into 11 mL of anhydrous DCM and heated at 60 °C under magnetic stirring. Then, anhydrous pyridine (5 mL) was added followed by palmitoyl chloride (2.5 g) dissolved in 6 mL anhydrous DMF under continuous magnetic stirring at 60 °C during 2 h and 1 h at room temperature. Then, 100 mL of cold ethanol (at 4 °C) was added. The precipitate was collected and washed with 100 mL of ethanol and then with 100 mL of diethylether using a Buchner filter. The solid materials were dried under vacuum at room temperature.

Method 2. Synthesis of OPH-2: Heparin (2 g) was added to 10 mL of anhydrous DCM and palmitoyl chloride (2.5 g) under continuous magnetic stirring at room temperature during 72 h. Then, 20 mL of a solution of 10% of sodium acetate in methanol was added. The precipitate was collected and washed with 100 mL of methanol and then with 100 mL of acetone using a Buchner filter. The solid materials were dried under vacuum at room temperature. The ester was then purified by dissolution in 10 mL of water and progressive addition of NaCl until the concentration reaches 10%. After the addition of 20 mL of methanol, the precipitate formed was collected and washed with methanol and acetone and dried under vacuum at room temperature.