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Boronic acid-modified lipid nanocapsules are shown to be efficient inhibitors for hepatitis C virus (HCV) entry.
Boronic acid-modified lipid nanocapsules: novel platform for the highly efficient inhibition of hepatitis C viral entry

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Abstract

The search for viral entry inhibitors that selectively target viral envelope glycoproteins has attracted increasing interest in recent years. Amongst the handful of molecules reported to show activity as hepatitis C virus (HCV) entry inhibitors are a variety of glycan-binding proteins including the lectins, cyanovirin-N (CV-N) and griffithsin. We recently demonstrated that boronic acid-modified nanoparticles are able to reduce HCV entry and through a similar mechanism to that of lectins. A major obstacle to any further development of these nanostructures as viral entry inhibitors is their only moderate maximal inhibition potential. In the present study, we report that lipid nanocapsules (LNCs), surface-functionalized with amphiphilic boronic acid (BA) through their post-insertion into the semi-rigid shell of the LNCs, are indeed far superior as HCV entry inhibitors when compared with previously reported nanostructures. These 2nd generation particles (BA-LNCs) are shown to prevent HCV infection in the micromolar range (IC50 = 5.4 µM of BA moieties), whereas the corresponding

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BA monomer show no significant effects even at the highest analyzed concentration (20 µM). The new BA-LNCs are the most promising boronolectin-based HCV entry inhibitors reported to date and are thus seen to show great promise in the development of a pseudolectin-based therapeutic agent.

**Key words:** Lipid nanocapsules, amphiphilic boronic acid, post-insertion, viral entry inhibition.
1. INTRODUCTION

The development of designer receptors that enable the selective and potent recognition of a particular glycan or glycoconjugate epitope is an intriguing area of research. The study of such synthetic receptors promises to inform on the molecular basis of protein-ligand interactions and will, it is hoped, eventually see the elaboration of alternatives to lectins for the specific detection and isolation of glycoproteins and glycolipids. The possibility of developing such pseudo-lectins to interfere with specific carbohydrate-based binding interactions in vivo would be expected to provide powerful tools for research into countless biological phenomena. Synthetic receptors promise also to have potential as therapeutic agents given that more than half of all proteins are predicted to be glycosylated.1-4

A wealth of literature supports that certain boronic acid derivatives can bind particular glycans very well, and have been developed for the enrichment of glycopeptides or as indicators for diseases such as diabetes and cancer.3-9 Boronic acid analogs have also been proposed as low toxicity, therapeutic agents for inhibiting the entry of various highly glycosylated enveloped viruses such as HIV type-1.10-12 Phenylboronic acid-based structures have proved most attractive in this respect, several analogs having been demonstrated to form covalent complexes with cis-diol motifs, found on envelope glycoproteins and to do so reversibly and at physiological pHs.10 However, the binding affinities of free monomeric boronic acid derivatives with viral glycoproteins are found to be too weak for them to efficiently inhibit viral entry.10 This situation has been shown to be easily reversed when multiple boronic acid moieties are presented on appropriate nanometric scaffolds as this increases the probability of multiple diol units either on the same or on neighboring viral envelope glycoproteins interacting simultaneously with multiple boronic acid entities. Benzoboroxole-functionalized polymers have been shown to give particularly promising results in this respect when applied to the inhibition of HIV entry.13

We have explored this same boronolectin-based strategy for inhibiting the entry of Hepatitis C virus (HCV).2 HCV, like HIV is adorned with highly glycosylated envelope proteins and these glycoproteins have been shown to modulate the accessibility of various viral co-receptors.14-16 Amongst the handful of molecules reported to inhibit HCV viral entry is included a variety of glycan binding proteins such as cyanovirin-N (CV-N) and griffithsin.17,18 However, the projected high cost of large-scale production and purification of these protein-based antivirals together with their low stability, vulnerability to proteolytic cleavage as well as their mitogenicity undermine their eventual clinical use. We have recently demonstrated that various boronic acid-modified nanodiamond (BAD-ND) particles represent a potentially cheap and safe alternative to lectins as HCV entry inhibitors.2 However, while serving as a valuable “proof of concept” study, the total level of HCV entry inhibition with these 1st generation boronolectin particles was only moderate and could not be improved above around 62 % with maximal particle suspensions of 60 µg/mL (incorporating 3.6 µg/mL boronic acid
ligand). The poor particle dispersibility was believed to be the limiting feature of these 1st generation BA-NDs in terms of their HCV entry inhibitory potential.

We thus required improved particles that would not only feature multiple phenyl boronic acid moieties, but in addition be more stable and dispersible in aqueous media than our 1st generation BA-NDs over a wider concentration range and turned to investigation of lipid nanocapsules (LNCs) as a viable alternative. The nanometric dimensions of LNCs along with their attractive biomimetic properties has seen their use in various nanomedical settings.\(^\text{19}\) For example, these water-soluble nanocapsules appear to be useful vectors for the solubilization of hydrophobic drugs and have already been explored in drug delivery.\(^\text{20-23}\) LNCs benefit from the advantage that they are conveniently prepared from low-toxicity materials such as triglycerides and PEGylated surfactants by a solvent-free process. The process facilitates the controlled assembly of nanocapsules of less than 100 nm in diameter that show good stability and monodispersity. While the LNCs provide considerable drug encapsulation capacity and posses sustained-release capability at the site of action, we envisaged that the semi-rigid shell of these nanocapsules would also allow their modification by post-insertion of amphiphilic molecules capable of subsequent functionalization with targeting ligands such as boronic acid moieties required in this project.\(^\text{24, 25}\)

Herein we report on the fabrication of boronic-acid modified lipid nanocapsules (BA-LNCs) and their behavior as HCV entry inhibitors (Figure 1A). These novel BA-LNCs prove to be far superior as HCV entry inhibitors when compared with previously reported nanostructures. The 2nd generation particles prevent viral infection in the micromolar range (IC\(_{50}\) = 5.4 µM of BA moieties), whereas the corresponding BA monomer showed no significant activity even at the highest analyzed concentration (20 µM).

2. EXPERIMENTAL SECTION

2.1. Materials
Lipid nanocapsules were made of Labrafac\textsuperscript{TM} Lipophile WL 1349 (caprylic/capric triglyceride), Phospholipon® 90G (soybean lecithin at 97.1% of phosphatidylcholine), and Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) generously provided by Gattefosse S.A.S. (Saint-Priest, France), Phospholipid GmbH (Köln, Germany), and Laserson (Etampes, France), respectively. Deionized water was obtained from a Milli-Q plus system (Millipore, Paris, France). Other chemical reagents and solvents were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France) and used as received.
2.2. Synthesis of amphiphilic boronic acid compound (2)

Synthesis of compound (1): 2.2 g (2 mmol, 1 equiv) of Brij-58P, 1.2 g (12 mmol, 6 equiv) of succinic anhydride, and 950 µL (12 mmol, 6 equiv) of pyridine were dissolved in 40 mL of dry dichloromethane (DCM). The reaction medium was stirred for 24 h and was concentrated under vacuum. The residue was precipitated in dry diethyl ether at 4°C and then filtered. The resulting solid was collected, dissolved in DCM and again triturated twice with dry diethyl ether at 4°C. The white solid filtered was dried under vacuum in the presence of P₂O₅ overnight. 2 g of the acid derivative was collected. Yield: 83%.

1H NMR, CDCl₃, (δ) ppm: 4.27 (t, 4.5 Hz, 2H); 3.65 (m, 82H); 3.44 (t, 6.9 Hz, 2H); 2.65 (m, 4H); 1.57 (quint, 6.9 Hz, 2H); 1.25 (s, 28H); 0.88 (t, 7.0 Hz, 3H). FTIR: 717, 843, 964, 1061, 1110, 1147, 1242, 1280, 1344, 1360, 1472, 1735, 1784, 2850, 2886, 2916. Compound (2): 450 mg (0.37 mmol, 1 equiv) of compound (1) was dissolved in 20 mL of dry DCM with 115 mg (0.55 mmol, 1.5 equiv) of dicyclocarbodiimide (DCC), and 205 µL (1.50 mmol, 4 equiv) of triethylamine (TEA) was added. Then, 96 mg (0.55 mmol, 1.5 equiv) of 4-aminophenylboronic acid hydrochloride dissolved in 10 mL of dry DCM was added dropwise. The reaction medium was stirred for 24 h and filtered. The organic phase was washed with water, dried over MgSO₄ and evaporated under reduced pressure. The crude product was purified on silica gel column (DCM/MeOH 95:5). The white solid was dried under vacuum in the presence of P₂O₅ overnight. 400 mg of the boronic acid derivative was collected. Yield: 81% (ratio (1)/(2) 1:1). 1H NMR, CDCl₃, (δ) ppm: 8.56 (s, 0.5H, NH); 7.80 (d, 8.1Hz, 1H, ArH); 7.59 (d, 8.1Hz, 1H, ArH); 6.19 (s, 1H); 4.25 (m, 2H); 3.64 (m, 80H); 3.44 (t, 6.7 Hz, 2H); 2.76 (m, 5.5 Hz, 2H); 2.69 (t, 5.5 Hz, 2H); 2.47 (s, 2H); 2-1.5 (m, 6H); 1.25 (s, 28H); 0.88 (t, 6.9 Hz, 3H). FTIR: 717, 843, 964, 1061, 1110, 1147, 1242, 1280, 1344, 1359, 1404, 1468, 1531, 1595, 1638, 1670, 1708, 1737, 2851, 2885, 2916.

2.3. Preparation of boronic acid-modified LNCs

2.3.1. Formulation of LNCs

LNCs were formulated at a nominal size of 25 nm using a phase inversion method of an oil/water system. Briefly, the oil phase was mixed with the appropriate amounts of Solutol, Phospholipon 90G, NaCl and distilled water, and heated under magnetic stirring up to 85°C. The mixture was subjected to 3 temperature cycles from 70 to 90°C under magnetic stirring. Then, it was cooled to 78°C, 3.3 mL of distilled cold water (0°C) were added, and the suspension was still stirred at room temperature for another 10 min before further use. The composition of the formulation is summarized in Table 1. The percentage composition is just the mass percentage of the components for the lipid nanocapsules (a total initial mass of 1.26 g). LNCs were purified from supernatant using disposable PD-10 desalting columns (Sephadex® G-25 for gel filtration as stationary phase, Amersham Biosciences). A column
was stabilized with 25 mL of distilled water. Then 2 mL of a suspension of LNCs were deposited on the column, 0.5 mL of water were added to complete the dead volume of the column, then 4 mL of distilled water eluted and the LNCs were collected in this eluant. The suspension was then dialyzed (pore size: 6000-8000 Da) against deionized water overnight. The solution was freeze-dried and weighed in order to estimate the mass concentration.

2.3.2. Formulation of BA-LNCs

BA-functionalized LNCs were obtained by a post-insertion method operating at room temperature. An excess of the amphiphilic component vis a vis that of the LNCs was used to favor the highest density of active boronic molecules grafted at the NP surface (mass ratio of compound (2)/LNC=1/2). Briefly, preformed nanocapsules (4 mg/mL) were incubated for 24 h with an aqueous suspension of amphiphilic boronic acid component (2) (2 mg/mL) under planetary stirring (200 rpm) at room temperature in a final volume of 600 µL. After 24 h, the suspension of LNCs decorated by boronic acid molecules was removed and the nanostructures dialysed (RC membranes, 12000-14000 Da during 4 days).

2.4. Instrumentation

2.4.1. Characterization

Fourier transform infrared (FTIR) spectra in grazing-angle attenuated total reflectance mode (gATR) were recorded using a ThermoScientific FTIR instrument (Nicolet 8700) equipped with a VariGATR™ accessory (Harrick Scientific Products Inc.). Spectra were collected at an incidence angle of 62.5° from normal with a resolution of 4 cm⁻¹ by accumulating a minimum of 64 scans per sample. MALDI-TOF mass spectra were recorded with a Voyager-DE™ Pro Workstation of Applied Biosystems using 2,5-dihydroxybenzoic acid as matrix. ¹H NMR spectra were recorded with a Bruker Advance 300 MHz spectrometer using the deuterated solvent as the lock and TMS as an internal standard. Chemical shifts (δ) and coupling constants (J) are expressed in ppm and Hertz (Hz), respectively.

X-ray photoelectron spectroscopy (XPS) experiments were performed in a PHI 5000 VersaProbe - Scanning ESCA Microprobe (ULVAC-Phi, Japan/USA) instrument at a base pressure below 5×10⁻⁹ mbar. Monochromatic AlKα radiation was used and the X-ray beam, focused to a diameter of 100 µm, was scanned on a 250×250 µm surface, at an operating power of 25 W (15 kV). Photoelectron survey spectra were acquired using a hemispherical analyzer at pass energy of 117.4 eV with a 0.4 eV energy
step. Core-level spectra were acquired at pass energy of 23.5 eV with a 0.1 eV energy step. All spectra were acquired with 90° between X-ray source and analyzer and with the use of low energy electrons and low energy argon ions for charge neutralization. After subtraction of the Shirley-type background, the core-level spectra were decomposed into their components with mixed Gaussian-Lorentzian (30:70) shape lines using the CasaXPS software.

UV-vis spectroscopic measurements were carried out on a Perkin-Elmer Lambda 950 dual-beam spectrophotometer operating at a resolution of 1 nm.

2.4.2. Particle size and zeta potential measurements

LNCs were characterized in terms of size and charge. The average diameter and polydispersity index (PI) were determined by dynamic light scattering using a Zetasizer® Nano ZS (Malvern Instruments S.A., Worcestershire, UK). The zeta potential was measured using the electrophoretic mode with the Zetasizer®. All the batches were diluted at 1 mg.mL⁻¹ in distilled water (filtered over 0.22 µm) prior to the analysis and analyzed in triplicate.

2.4.3. UV-vis spectroscopy for the quantitative analysis of boronic acid molecules incorporation into LNCs

Amphiphilic boronic acid compound (2) in dichloromethane shows an absorption band with a maximum at 259 nm (Figure 2A). A calibration curve of different concentrations of (2) in dichloromethane (12.5 to 100 µg/mL) versus the intensity of the absorption at 259 nm was established (Figure 2B). The amount of incorporated boronic acid was calculated by measuring the concentration of the non-incorporated boronic acid compound (2) in dichloromethane and subtracted from the initial amphiphilic boronic acid compound (2) concentration of 2 mg/mL.

2.5. Biological assays

2.5.1. Cytotoxicity (MTS) assay

Huh-7 cells²⁶ were seeded, a day before initiating the assay, in 96 well plates at a density of 7x10³ cells/well, in Dulbecco’s modified essential medium (DMEM) containing 10% fetal calf serum. The well mixture was replaced with fresh culture medium containing the desired concentrations of modified nanocapsules and incubated for 2 h. The nanocapsules were then washed away and an MTS assay was performed as described by the company (CellTiter 96 AQueous One Solution cell
proliferation assay; Promega) after 48 h. Thus, first the cells were washed with PBS once, then cells were incubated for 30 to 45 min with 20 % of MTS solution prepared in fresh culture medium. The optical density of each well was measured using a microplate reader at a detection with absorbance of 490 nm. Each experimental condition assayed was replicated four times and wells without added nanocapsules were taken as negative control.

### 2.5.2. Virus entry inhibition assay

For experiments with HCV, a modified JFH1 virus containing mutations at the C-terminus of the core protein leading to amino acid changes F172C and P173S were used. These mutations have been shown to lead to increased viral titers and a mutation was introduced in E1 glycoprotein to reconstitute the A4 epitope (SSGLYHVTNDC) as described previously. JFH1 virus was pre-incubated with varying concentrations of nanocapsules for 1 h at room temperature. Then the virus/nanocapsule complexes were added to Huh-7 cells that had been seeded at a density of 3x10^4 cells/well in 24-well plates containing microscopy coverslip, a day prior to incubation. After 2 hours incubation the nanocapsule treated infected cells were washed twice with PBS to remove complexes completely, and then incubated for a further 48 h with fresh culture medium. After a further 48 h incubation, cells were fixed with 100 % methanol (-20°C) and the percentages of infected cells were determined by immunofluorescence using a monoclonal antibody recognizing HCV glycoprotein E1 (A4 epitope), followed by detection with a secondary antibody carrying a fluorophore (CY-3 tagged goat anti-mouse) that recognizes the primary antibody. Nuclei were labeled by using 4′-6-diamidino-2-phenylindole (DAPI), a DNA-specific fluorochrome. Infected cells were labeled by Cy-3. Five images (for both nuclei and infected cells) were collected using fluorescent microscopy (Zeiss Axiophot 2) from different areas of each coverslip. In addition, the total number of cells and infected cells from each image was counted using Image J software. Percentages of infected cells were calculated using the following formula:

\[
\text{% infected cells in each conditions} = \frac{\text{total number of infected cells}}{\text{total number of cells}} \times 100
\]

All conditions tested were repeated in duplicate. Experiments without nanocapsules were taken as negative controls. To determine whether the nanostructured boronic acid act specifically on the viral entry step, BA-LNCs were added at different time points before, during and after inoculation of Huh-7 cells with JFH1. (See also 2.5.4)
2.5.3. Competitive assay with mannose and Man-9

BA-LNCs containing 620 µM of grafted BA moieties were incubated with 520 µg/mL of mannose (2889 µM) in excess. In parallel BA-LNCs containing 382 µM of grafted BA moieties were incubated with 190 µg/mL of N-linked high mannose oligosaccharides mannonanose-di-(N-acetyl-D-glucosamine) (Man-9, 162 µM) at room temperature for 1 h. Subsequently further dilutions were carried to allow pre-incubation of the thus fabricated sugar coated-BA-LNCs with HCV at room temperature. Huh-7 cells were infected for 2 h at 37°C with these various sugar-nanocapsule/virus complexes. The complexes were then washed away and the wells replaced with fresh cell culture medium and incubated for a further 48hrs. The wells were then analyzed using immunofluorescence to score the infected cells.

2.5.4. HCV pseudotyped particles (HCVpp)

The luciferase-based HCV pseudotyped (HCVpp) retroviral particle infection assay was used to further confirm the effect of BA-LNCs on the HCV entry. These are retroviral cores carrying HCV glycoproteins in their envelope. In this context, only the early steps of the viral life cycle, i.e. virus interaction with receptors, uptake and fusion, are HCV specific, whereas all later steps are dependent on the retroviral nucleocapsid elements, and luciferase expressed from the retroviral genome is measured to determine HCVpp entry efficiency. The following plasmids encoding HCV envelope glycoproteins of different genotypes kindly provided by J. Ball (Nottingham University, UK) were used in this work: UKN2B-1.1 (genotype 2b), UKN4-11.1 (genotype 4), UKN6-5.340 (genotype 6). In addition, the plasmid encoding HCV envelope glycoproteins from genotype 2a (strain JFH-1) was kindly provided by T. Pietschmann and R. Bartenschlager (University of Heidelberg, Germany). The plasmid encoding HCV envelope glycoproteins from genotype 1a (H77) has been described previously. As a control, retroviral particles pseudotyped with vesicular stomatitis virus glycoprotein G (VSVg) were used. The level of infection was determined by measuring luciferase activity. Briefly, the particles at different concentrations were incubated with the pseudoparticles for 1 h at room temperature then the Huh-7 cells were infected by the nanocapsules/virus complex. Infection time was set for 2 h in case of HCVpp and 1 h for VSVg. Later, the complex was replaced with fresh cell culture medium and intracellular firefly luciferase signal was measured after 72 h of incubation and normalized to untreated conditions.
3. RESULTS AND DISCUSSION

3.1. Synthesis of the amphiphilic boronic acid compound (2)

The amphiphilic boronic acid (BA) compound (2) (Figure 1B) was synthesized in two steps. The nonionic detergent Brij-58P consisting of a C16 alkyl chain bearing an average of 20 ethylene glycol units (PEG) was used as starting material. Coupling the free hydroxyl groups at the chain end of Brij-58P with succinic anhydride results in the formation of a carboxylic acid modified Brij-58P (1). Interaction of 4-aminophenylboronic acid with the –COOH groups of Brij-58P at room temperature for 24 h using N,N'-dicyclohexylcarbodiimide (DCC) as a coupling agent results in the formation of the corresponding boronic acid derivative (2) in 81% yield where the amphiphilic boronic acid component (2) presents 50%. The other 50% corresponds to carboxylic acid modified Brij-58P (1) as determined from the $^1$H NMR analysis (see experimental part and supplementary information: Figure S1).

3.2. Post-insertion of the amphiphilic boronic acid ligand (2) onto lipid nanocapsules (LNCs)

The formulation of LNCs was based on a phase inversion process. These thermal manipulations allow the preparation of very small LNCs, consisting of an oily liquid triglyceride core surrounded by a hydrophilic surfactant, which exposes a medium PEG chain containing an average of 15 ethyleneglycol units (Figure 1A). In the present study, LNCs were achieved by mixing labrafac, solutol and phospholipon at optimized concentrations. The composition of the formulation is summarized in Table 1. This process offers a good control over the size of the nanocapsules (31.6 ± 0.4 nm) with narrow size distributions (PI<0.1) (Table 2). Due to the presence of Solutol HS-15 which exposes PEG chains, the zeta potential of LNCs was -10.0 ± 0.3 mV. This value is similar to the zeta potential of other pegylated vectors such as liposomes and identical to those previously reported in the literature.

BA-LNCs were obtained by a classical post-insertion method on preformed LNCs as first demonstrated by Uster et al. The preformed LNCs solution (4 mg mL$^{-1}$) was incubated with 2 mg mL$^{-1}$ of the amphiphilic boronic acid ligand (2) (Figure 1B). As the amphiphilic boronic acid compound shows limited solubility in aqueous conditions, unreacted boronic acid ligands precipitate out of solution. To ensure the purity of BA-LNCs, dialysis (RC membranes, 12000-14000 Da during 4 days) was performed in addition.

The quantity of phenylboronic acid moieties incorporated into LNCs was established using UV/vis spectroscopic data (Figure 2) as an absorption band at 259 nm is observed for the amphiphilic
boronic acid compound (2) (Figure 2A). As seen from Figure 2B, the absorption intensity scales linearly with the concentration of ligand (2) from 12.5 to 100 µg mL⁻¹. The concentration of incorporated BA is estimated as 0.83 ± 0.06 mg mL⁻¹ (0.62 ± 0.04 mM), which corresponds to 16.6% of mass concentration. The success of the post-insertion process was in addition confirmed by the presence of 3.9 at % of BIs in XPS analysis. The BIs high resolution Xps spectrum shows a band at 191.1 eV corresponding to -C-B(OH)₂ bond (Figure 2C) as expected for the BA-LNCs. The mean diameter of the BA-LNCs is 38.8 ± 0.3 nm, slightly larger than the mean diameter of blank LNCs (Table 2). The low size distribution (PDI = 0.225 ± 0.004) was also retained (Table 2). The zeta potential of BA-LNCs remained negative at -17.6 ± 0.8 mV.

3.3. Effect of lipid nanocapsules on Huh-7 cell viability

The cell viability of blank LNCs, BA-LNCs and the amphiphilic boronic acid compound (2) was analyzed on the Huh-7 cell line using the colorimetric change of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) as described by the company (CellTiter 96 AQueous One Solution cell proliferation assay; Promega). Addition of phenazine methosulfate to MTS results in the production of a formazan product that has an absorption maximum at 490-500 nm in phosphate-buffered saline. Huh-7 cells were incubated with the three samples at increasing concentrations for 2 h (to be used in the virus entry inhibition assay). Thereafter, the nanocapsules were washed away and the MTS assay was carried out after 48 h of incubation with fresh medium. The blank LNCs did not show any cytotoxicity even at the highest analyzed concentration (100 µg mL⁻¹, data not shown) for 2 h incubation (Figure 3). The BA ligand (2) is non-toxic up to a 20 µM concentration of BA moieties. For BA-LNCs, a slight decrease in cell viability was observed for concentrations higher than 8 µM of BA moieties (Figure 3). However no further decrease was observed at higher concentrations even up to the highest concentration examined in this assay.

3.4. Virus entry inhibition assay using BA-LNCs

Modified JFH1 cell cultured virus (HCVcc) was used in this assay to evaluate the potential of BA-LNCs as HCV entry inhibitor. The assay was performed as previously established using inorganic nanoparticles modified with boronic acid ligands. Serially increasing concentration of BA-LNCs and BA compound (2) were incubated together with HCVcc for 1 h before the mixture (HCVcc-BA-LNCs and HCVcc-BA) was brought into contact with a fixed number of Huh-7 target cells. After 2 h of incubation, the complexes were removed and replaced by fresh medium. After an additional 48 h
incubation, the number of infected cells at each dilution was quantified by immunofluorescence. **Figure 4A** shows typical fluorescence images of the Huh-7 target cells after having been incubated with HCVcc-BA-LNCs and HCVcc-BA. The red colored cells are those that have been infected and counting the number of infected cells allows the inhibition level to be estimated (**Figure 4B**). For example, in the case of BA-LNCs a dose-dependent reduction in HCVcc infection is observed with a 50% inhibitory concentration (IC\textsubscript{50}) for particles with a 5.8 µM concentration of BA moieties (**Figure 4B**). In contrast, the equivalent amounts of monovalent BA compound did not show any significant effect on the inhibition of HCVcc even at the highest concentration tested.

3.5. Mode of action of the inhibitory effect of BA-LNCs

To determine whether BA-LNCs specifically act on the early stage of HCV virus entry, BA-LNCs with BA moieties at a concentration of 12.4 µM were added at different time points before, during and after infection of Huh-7 cells with HCVcc (**Figure 5A**). The results presented in **Figure 5B** clearly indicate that the pre-incubation of virus with nanocapsules before infection remarkably inhibits viral infection in cell culture, whereas the addition of BA-LNCs after 2 h infection has no effect. This suggests that BA-LNCs are specifically acting on the early phase of virus entry. This conclusion is also supported by the observation that when nanocapsules were incubated with Huh-7 for 2 h before infection no inhibition was observed. In contrast to studies where lectins such as griffithsin and cyanovirin N which can also bind to glycan structures present on the surface of epithelial cells, BA-LNCs are seen to interact directly with viral particles. Indeed, we have demonstrated previously that boronic acid modified nanostructures when saturated with mannose prior to infection of Huh-7 cells with HCVcc lose their antiviral activity.\textsuperscript{2}

To validate that this was also true in the case of BA-LNCs, interference with their inhibitory potential through blocking surface boronic acid moieties through treatment with a monosaccharide such as mannose or the oligosaccharide mannonanose-di-(N-acetyl-D-glucosamine) (Man-9) known to be present on the envelope glycoproteins of HCVcc was investigated. The data are represented in **Figure 6A** and clearly shows that mannose saturated BA-LNCs have a diminished inhibitory effect compared with that of untreated particles, a masking effect of free boronic acid moieties that is even more pronounced when the particles are instead saturated with Man-9. These data support the hypothesis that the inhibitory effect of BA-LNCs is dependent on the recognition by boronic acid moieties of N-linked high-mannose glycans present on the envelope glycoproteins of HCVcc.

To establish that the effect of BA-LNCs is on the HCV entry step additional proof was obtained using the infectious HCV pseudo-virus (HCVpp) system.\textsuperscript{31} HCVpps are retroviral cores carrying HCV glycoproteins in their envelope. In this context, only the early steps of the viral life cycle, i.e. virus
interaction with receptors, uptake and fusion, are HCV specific, whereas all later steps are dependent on the retroviral nucleocapsid elements. Luciferase expressed from the retroviral genome can be measured and is used to determine HCVpp entry efficiency. Moreover, to determine the conservation of the antiviral effect of BA-LNCs on different HCV isolates, HCV envelope glycoproteins from different genotypes were used (Figure 6B). These pseudoparticles are defective for reinfection since they do not contain the genetic information to express envelope glycoproteins in their genome. Since they only contain the envelope glycoproteins of HCV, these pseudoparticles have been widely used as a surrogate model to study HCV entry. The level of virus entry is therefore determined by measuring the luciferase activity. As a control, retroviral particles pseudotyped with vesicular stomatitis virus glycoprotein G (VSVg) instead of HCV envelope glycoproteins were used. In this study, BA-LNCs at a BA concentration of 8.3 µM inhibited HCVpp infection of the different genotypes tested, which is likely due to the high level of glycosylation of HCV envelope glycoprotein (Figure 6B). In the same assay, BA-LNCs at the similar BA moieties concentration did not have any significant inhibitory effect on entry of pseudoparticles containing the envelope glycoprotein G of VSV. This might be due to the presence of only two \( N \)-linked glycans of a complex type, which might not be recognized by BA-LNC as also observed when HCVpp are pre-incubated with griffithsin lectin. This is an important finding for itself, as one of the major difficulties in HCV vaccine preparation lies on the fact that there exist variable genotypes and particularly its envelope glycoprotein. This reality encourages for the search of more-conserved features of the virus as an attractive targets for the development of antiviral therapeutics. Envelope glycoproteins are highly glycosylated at well defined locations in specific variants and the fact, that these glycans play an essential role in HCV entry motivates investigations of several cost effective and efficient therapeutic systems.

Conclusions

This study demonstrates clearly the potential of boronic acid-modified lipid nanocapsules to interfere with HCV entry. The great success of this nanocarrier is believed to be due to the stable and highly soluble nature of the BA-LNCs over an enlarged concentration range. The synthesis of the amphiphilic boronic acid ligand and its incorporation into the LNCs can be easily accomplished by post-insertion. BA-LNCs inhibited HCVcc as well as HCVpp entry regardless of the genotypes. The BA-LNCs can efficiently prevent HCV infection in the micromolar range (IC\(_{50}\) = 5.4 µM of BA moieties) whereas monomer BA compound did not show any significant inhibition even at the highest analyzed concentration (20 µM). The low infection level allowed the investigation of mechanistic concepts for HCV inhibition using BA-LCNs. When the active boronic acid moieties were blocked by oligosaccharides such as Man-9, no reduction in infection was encountered. This strongly supports the hypothesis that BA-LNCs act through cyclic di-ester-formation between the LNCs-appended BA
moieties and glycans present on the HCV envelope proteins. Moreover, the lack of effect of BA-LNCs in ‘pre’ and ‘post’-incubations of infection further suggests that the BA-LNCs exhibit the inhibition property by specifically blocking the entry of HCV into the target cell. This finding was further supported by using the HCVpp system where inhibition of different genotypes is clearly shown, which is probably due to the high degree of glycosylation of the envelope glycoprotein of all genotypes. Therefore, this study has brought up the most promising stable and efficient nanocarrier that can be further studied in vivo and applied as a potential therapeutic strategy for blocking viral entry. A potential concern of carbohydrate-binding antivirals is the degree of selectivity they may eventually show for virally derived glycoproteins over those of other cells. Although, the HCV envelope glycoproteins carry a higher proportion of high-mannose-type glycans than do mammalian glycoproteins, 17, 39 this would not necessarily ensure effective selectivity towards the desired target. Clearly, this important issue will have to be thoroughly examined, if such nanostructures are to be considered as a therapeutic strategy in the future.

ACKNOWLEDGEMENTS

M.K, A.B, R.B and S.S. gratefully acknowledge financial support from the Centre National de la Recherche Scientifique (CNRS), the Université Lille 1 and the Nord Pas de Calais region. J.D. was supported by a grant from the French National Agency for Research on AIDS and Viral Hepatitis (ANRS). Support from the European Union through a FP7-PEOPLE-IRSES (PHOTORELEASE) is acknowledged. M. K. gratefully acknowledges financial support from the Nord Pas de Calais region for a PhD fellowship. L.F. was supported by a PhD fellowship from the French Ministry of Research and T.V. was supported by a PhD fellowship from ANRS. Support from the ANRS, CEIFPRA and the CNRS is acknowledged by A.S. We thank J.K. Ball, R. Bartenschlager, F.L. Cosset and T. Wakita for providing us with essential reagents. We thank H. Drobecq for MALDI-TOF mass spectra. The fluorescence microscopy data were generated with the help of the Bio-imaging Center Lille Nord-de-France (BICeL).
REFERENCES


Table 1. Composition of the components for the lipid nanocapsules preparation for a total initial mass of 1.26 g

<table>
<thead>
<tr>
<th></th>
<th>Labrafac (%)</th>
<th>Solutol (%)</th>
<th>Phospholipon 90G (%)</th>
<th>NaCl (%)</th>
<th>Water (%)</th>
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<tr>
<td>LNC</td>
<td>20.0</td>
<td>32.4</td>
<td>3.0</td>
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<td>42.9</td>
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Table 2: Physicochemical characteristics of the different ligands investigated

<table>
<thead>
<tr>
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<th>Mean diameter / nm</th>
<th>PDI</th>
<th>Zeta potential / mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA ligand (2)</td>
<td>205.5 ± 10.9</td>
<td>0.328 ± 0.022</td>
<td>-27.7 ± 4.2</td>
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<tr>
<td>Blank LNCs</td>
<td>31.6 ± 0.4</td>
<td>0.065 ± 0.014</td>
<td>-10.0 ± 0.3</td>
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<tr>
<td>BA-LNCs</td>
<td>38.8 ± 0.3</td>
<td>0.225 ± 0.004</td>
<td>-17.6 ± 0.8</td>
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</table>
**Figure 1:** (A) Schematic representation of the formulation of boronic acid-modified lipid nanocapsules (BA-LNCs); (B) Synthetic route to amphiphilic BA compound (2).
Figure 2: (A) UV–Vis spectra of amphiphilic boronic acid compound (2) in dichloromethane; (B) Calibration curve of amphiphilic boronic acid compound (2) in dichloromethane: \((A_{259} - A_{310}) = f\) [compound (2)]; (C) High resolution B1s XPS spectrum of BA-LNCs.
Figure 3: *In vitro* cytotoxicity of different ligands (ratio BA/LNCs 1:6.5): MTS assay data for BA ligand (2) (black), BA-LNCs (green) and blank LNCs (blue) at different concentrations on the Huh 7 cell culture after 2 h incubation. Thereafter, the medium with particles was replaced with fresh cell culture medium and MTS assay was performed after 48 h.
Figure 4: HCV infection tests on the Huh 7 cell culture. (A) Fluorescent image (10×) of the Huh 7 target cells after incubation with modified JFH1 virus for 48 h, incubation in the presence of the blank LNCs (80 µg/mL), BA compound (concentration of boronic acid moieties = 15 µM) and BA-LNCs (concentration of boronic acid moieties = 12.4 µM), along with absence of nanocapsules (red color and blue color correspond to infected cells and nucleus, respectively); (B) Effect of BA ligand (2) and BA-LNCs at different concentrations on the infection activity.
Figure 5: HCV infection (a) and pre-(b), co-(c) and post-incubation (d) of BA-LNCs on the Huh 7 cell culture. (A) Schematic representation of different incubation concepts investigated; (B) Effect of BA-LNCs (concentration of boronic acid moieties = 12.4 µM) on the infection activity.
Figure 6: Competitive assays and selectivity. (A) Effect of mannose blocking BA functions on BA-LNCs on the viral entry inhibition activity: negative control (MQ water), BA-LNCs at concentration of 12.4 µM of boronic acid moieties and BA-LNCs (at similar concentration of boronic acid moieties) covered with mannose (57.78 µM) and Man-9 (5.4 µM); (B) Selectivity of BA-LNCs at concentration of 8.3 µM of boronic acid moieties on the entry of different HCVpp genotyped over pseudotyped (control) virus particle with envelope protein of VSVg.