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# A hyaluronic acid–pentamidine bioconjugate as a macrophage mediated drug targeting delivery system for the treatment of leishmaniasis†

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Leishmaniasis is still a serious public health problem worldwide, especially in tropical areas where this infectious disease is endemic. The most severe form of the disease (*i.e.* visceral) can claim victims if left untreated and the few accessible drugs have several drawbacks including major side effects and parenteral administration. In this context, the investigation of new delivery modalities which might reduce the toxicity and increase the bioavailability of the drugs currently on the market represents a valid strategy to counter these problems. Herein we present the development of a macrophage mediated drug targeting delivery system by conjugating the anti-leishmanial drug pentamidine (Pent) with the biocompatible polymer hyaluronic acid (HA), the latter employed at the same time as a delivery platform and targeting scaffold. Biological assays against *Leishmania major* amastigote-infected macrophages and primary bone marrow derived macrophages (BMDM) confirmed the validity of our strategy as the resulting bioconjugate HA–Pent increased both the potency and the selectivity index of the drug.

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## 1 Introduction

Leishmaniasis is a vector-borne tropical disease caused by different species of obligate intra-macrophage protozoa of the genus *Leishmania* transmitted to mammalian hosts during a blood meal of infected female phlebotomine sandflies. This neglected disease is endemic in 88 countries and affects approximately 12 million people worldwide, with 350 million people considered at risk.<sup>1</sup>

The clinical spectrum of Leishmaniasis ranges from a mild and usually self-resolving cutaneous form to a disfiguring mucocutaneous disease and even to a visceral form, which affects several internal organs and is lethal in the absence of chemotherapeutic treatment.<sup>2</sup>

At present, the development of an effective vaccine is far from being successful and drugs are the only available tool for treatment and control of all Leishmaniasis forms.<sup>3</sup> Pentavalent antimony compounds (*i.e.* sodium stibogluconate and meglumine antimoniate) represent the first-line of intervention. Second-line drugs, such as the polyene amphotericin B (AmB), the aromatic diamidine pentamidine (Pent) and the aminoglycoside paromomycin are important in combination therapy or in cases of antimony treatment failures.<sup>4</sup> However, all these drugs have several drawbacks including irreversible toxic effects, high costs, length of treatment, need of adequate medical care, emergence of drug resistance and parenteral administration. As an example, the main factors limiting the widespread use of AmB are the high cost of the drug and the long hospitalization period which makes it difficult to manage the disease within endemic areas strongly linked with poverty and lack of health services. Therefore, several efforts have been made over the past few years to improve the therapeutic index and to reduce the toxicity of this drug by developing lipid-based formulations and other nano- or microstructured delivery systems.<sup>5</sup> In this regard, the World Health Organization has recommended the use of liposomal AmB against Leishmaniasis based on its high levels of efficacy and safety.<sup>6</sup>

In the present work, Pent has been selected as a model drug in virtue of its biological and chemical features. In particular, it possesses a wide range of therapeutic properties including antimicrobial,<sup>7</sup> anti-inflammatory<sup>8</sup> and anti-cancer activities.<sup>9</sup> Pent has been used for more than 50 years in the therapy and prophylaxis of African trypanosomiasis and *Pneumocystis carinii*

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pneumonia in AIDS patients, as well as antimony resistant Leishmaniasis,<sup>10</sup> but its clinical use is limited by its toxicity, administration by injection and development of resistance.

On the basis of these considerations we focused our interest on the synthesis of a new Pent-bioconjugate using hyaluronic acid (HA) as delivery platform (Fig. 1), with the aim of increasing the activity of the drug and reducing its toxicity. To the best of our knowledge, the preparation of bioconjugates of Pent is to date an unexplored strategy to overcome these issues.

Chemically, Pent is a bifunctional and low-molecular weight compound that can be employed to obtain both mono-linked HA–Pent derivatives and HA–Pent cross-linked frameworks *via* amide bond formation. The two terminal amidine groups are the sole reactive moieties of the drug, allowing mild synthetic conditions without significant by-products. In addition, one of the two amidine groups could be further exploited for the conjugation with other compounds that might potentiate the activity of the drug and consequently reduce its dosage.

HA is a well-known biocompatible, biodegradable, bioresorbable, non-toxic and non-immunogenic polymer,<sup>11</sup> whose chemical versatility can be fruitfully exploited to build polymeric scaffolds able to entrap drugs with different chemophysical characteristics by means of enzyme-cleavable linkages or cross-linked networks. Additionally, HA emerges in many respects as the ideal substrate for the development of drug targeting delivery systems with sustained release properties for the treatment of macrophage-associated diseases such as Leishmaniasis.<sup>12</sup> In fact, macrophages play central roles in mediating a wide range of infectious and inflammatory diseases.<sup>13</sup> In particular, as *Leishmania* parasites are obligatory intracellular pathogens, macrophages are their primary resident cell: they phagocytose and permit parasite proliferation and they are also the major effector cells to eliminate infection.<sup>14</sup> Interestingly, HA is an attractive targeting ligand specifically recognized and internalized by macrophages that are known to express HA receptors for endocytosis (HARE/Stab2).<sup>15</sup> Targeted drug delivery to the macrophages appears to be

a useful proposition to improve therapeutic efficacy of an enclosed drug,<sup>16</sup> helping in localized delivery of the drug at the infected site.

On these bases, our unprecedented HA–Pent bioconjugate (Fig. 1) is proposed as drug targeting delivery system for the treatment of Leishmaniasis, exploiting the specific biological recognition of HA by the macrophage. The biological activity of the bioconjugate has been assessed by *in vitro* assays on *Leishmania major* amastigote-infected macrophages and primary macrophages.

## 2 Experimental

### 2.1 Materials and general methods

HA sodium salt from *Streptococcus equi* (high molecular weight), Dowex H<sup>+</sup> 50W × 8–100 sulfonic resin, pentamidine isethionate salt, 4-methylmorpholine, (NMM), 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and organic solvents were purchased from Sigma-Aldrich/Fluka. Dialysis membranes (20 kDa *M<sub>w</sub>* cutoff) were purchased from Spectrum Labs. Materials and methods for the biological assays have been described before by the authors.<sup>17</sup> AlamarBlue assays for investigation of anti-leishmanial activities against intracellular *Leishmania major* amastigotes and cytotoxicity against primary bone marrow derived macrophages (BMDM) were conducted as previously reported.<sup>17</sup>

### 2.2 Synthesis

**2.2.1 Preparation of pentamidine free base (Pent).** In a beaker, 5 g of pentamidine isethionate was dissolved in 100 mL of distilled water under vigorous stirring. Sodium hydroxide was gradually added until the pH of the solution was adjusted to ~11. The solution was then refrigerated overnight, filtered off and washed repeatedly with deionized water to obtain pentamidine free base (Pent) as a white powder (2.62 g; yield 92%). The product was eventually dried in a vacuum chamber before use.

**2.2.2 Preparation of HA-COOH.** HA sodium salt 100 mg was dissolved in deionized water (5 mg mL<sup>-1</sup>, pH = 6.85) and mixed with Dowex H<sup>+</sup> 50W × 8–100 sulfonic resin (100 mg). The resin was washed thoroughly with deionized water before use. The mixture was shaken at room temperature for 2 h (Entry 1) or 1 h (Entry 2 and 3) to ensure the ion exchange and then filtered off using a filter paper. The solution (pH = 2.85) was freeze-dried to afford 76 mg of HA in protonated form (HA-COOH).

#### 2.2.3 Preparation of HA–Pent bioconjugates

**Entry 1 (Table 1).** HA-COOH (76 mg, 0.2 mmol carboxylic acid) was dissolved in 3 mL of deionized water in a 50 mL round-bottomed flask followed by the dropwise addition of 2 mL of acetonitrile while stirring 33 µL (0.3 mmol) of NMM was added to the solution, causing the viscosity to increase temporarily. The solution (pH = 7) was then cooled to 4 °C, and 18 mg (0.1 mmol) of CDMT was added and stirred for 1 h at room temperature. The solution was mixed with 34 mg (0.1 mmol) of Pent (which corresponds to a 1 : 1 ratio between HA carboxyl groups and Pent amidine groups) and stirred for 68 h at room

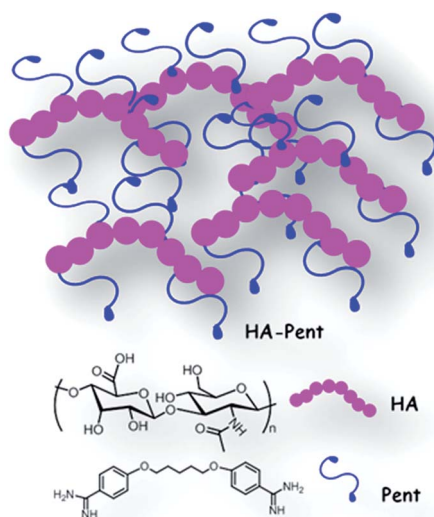


Fig. 1 Schematic illustration of HA–Pent bioconjugate.



temperature. The acetonitrile was removed under reduced pressure and the resulting mixture was filtered off to eliminate the unreacted Pent. Extensive cycles of dialysis (20 kDa  $M_w$  cutoff) against a mixture of ethanol and ultrapure water (3 : 1  $\rightarrow$  2 : 1  $\rightarrow$  1 : 1) and finally against ultrapure water were performed to purify the desired product which was recovered by freeze-drying and lyophilization as a cottony white solid and characterized by  $^1\text{H}$  NMR spectroscopy. The degree of substitution ( $\text{DS}_{100}$ ; defined as the average number of Pent groups per 100 disaccharide repeating unit) was calculated by the ratio between the integral of one aromatic proton of Pent unit (doublets at 6.99 and 7.60 ppm) and the integral of one proton of HA acetyl group (1.90 ppm) and turned out to be 12.2.

**Entry 2 (Table 1).** The time of ion exchange with resin was reduced to 1 h. The ratio of HA-COOH/Pent was increased to 1 : 1.5 using 0.15 mmol (51 mg) of Pent and the reaction time was maintained to 68 h.  $\text{DS}_{100} = 54.0$ .

**Entry 3 (Table 1).** The time of ion exchange with resin was reduced to 1 h. The ratio of HA-COOH/Pent was maintained to 1 : 1.5 using 0.15 mmol (51 mg) of Pent and the reaction time was reduced to 20 h.  $\text{DS}_{100} = 39.9$ .

### 2.3 Characterization

Nuclear magnetic resonance (NMR) measurements were performed using a Varian 500 MHz spectrometer. HA-COOH and synthesized derivatives (HA-Pent1, HA-Pent2, HA-Pent3) were dissolved in  $\text{D}_2\text{O}$  to concentrations of approximately 10  $\text{mg mL}^{-1}$  and the  $^1\text{H}$  NMR spectra were recorded with 128–256 scans. The reference signal from HDO was set to  $\delta$  4.22 ppm. The  $^{13}\text{C}$  NMR spectrum was performed dissolving the HA-Pent3 in  $\text{D}_2\text{O}/\text{DMSO-d}_6$  (95/5) and the reference signal of  $\text{DMSO-d}_6$  was set to  $\delta$  39.5 ppm. The Fourier Transform Infrared (FT-IR, Perkin Elmer Spectrum 100) spectra were collected, in Attenuated Total Reflectance (ATR) configuration, from 4000 to 450  $\text{cm}^{-1}$ .

HA-Pent3 IR: ( $\text{cm}^{-1}$ ) 3200–3500, 1712, 1643, 1611, 1492, 1403, 1270, 1033.  $^1\text{H}$  NMR, selected signals, ( $\text{D}_2\text{O}$ ; ppm):  $\delta$  1.14–1.21 (m, 2H), 1.51–1.56 (m, 2H), 1.72–1.80 (m, 2H), 1.90 (s, 3H,  $\text{N}-\text{C}(\text{O})\text{CH}_3$ ), 4.06 (t, 4H,  $J = 6.4$  Hz), 4.30–4.36 (m, 1H, H-1 proton of HA), 4.41–4.44 (m, 1H, H-1 proton of HA), 6.99 (d, 4H,  $J = 8.8$  Hz), 7.60 (d, 4H,  $J = 8.8$  Hz), 8.32 (bs, 1H,  $\text{N}-\text{H}$ ).  $^{13}\text{C}$  NMR  $\text{D}_2\text{O}/\text{DMSO-d}_6$  (95/5 ppm):  $\delta$  174.8, 173.9, 165.6, 163.1, 129.8, 119.4, 115.2, 103.1, 100.3, 82.7, 79.9, 76.3, 75.3, 73.5, 72.3, 68.3, 60.4, 50.1, 27.6, 22.4, 21.5 (ESI Fig. S2†).

Ultraviolet-visible spectrophotometry (UV-Vis) was used to quantify the amount of conjugated Pent, by dissolving a weighted amount of lyophilized bioconjugate (1 mg) in 2.5 mL of water. On the basis of optical absorbance data and molar extinction coefficient ( $\epsilon = 28\,900\text{ M}^{-1}\text{ cm}^{-1}$ ),<sup>18</sup> determined by calibration curve at a wavelength of 261 nm, a drug content of 16% was estimated using the following equation: drug content = (drug weight in the conjugate/weight of the conjugate)  $\times$  100.

No significant absorption in the UV-Vis region has been observed for HA-COOH.

Dual detector SEC analysis was performed using a GPC System equipped with a Water 1525 binary HPLC pump and

a Water 2410 refractive index detector (RI) (Waters corporation) coupled in series to a miniDAWN Treos (Wyatt Technology) light scattering detector equipped with a WyattQELS DLS Module. This procedure, with in-line refractive index and light scattering detectors, allows the determination of absolute  $M_w$  values.<sup>19</sup> The analyses were performed using three Progel-TSK columns (Tosoh Bioscience LLC) connected in series (G3000 PWXL, G5000 PWXL and G6000 PWXL, with a separation range up to 50 MDa). The mobile phase was a saline water solution, 0.1  $\text{mol L}^{-1}$  of  $\text{NaNO}_3$  with 0.02% of  $\text{NaN}_3$  (higher ionic strength shows similar results), at a flow rate of 1.0  $\text{mL min}^{-1}$ . To calculate the average molecular masses, a refractive index increment ( $\text{dn/dc}$ ) of 0.165  $\text{mg mL}^{-1}$  was used. The samples of HA and of its derivatives were prepared with a final concentration of 2  $\text{mg mL}^{-1}$  in the eluant solvent. Acquired data were analyzed by means of ASTRA 6.0.1 software (Wyatt Technology).

### 2.4 Amastigote drug screening assay and determination of cytotoxicity against macrophages

HA-Pent3 as well HA-COOH and pentamidine isethionate were tested against intracellular amastigote of *Leishmania major* as recently described using the amastigote drug screening assay.<sup>17</sup> In brief a 1801 kbp fragment of the firefly luciferase (LUC)-coding region was cut from pGL4.13 (Promega, Mannheim, Germany) by *NcoI/XbaI* and subsequently cloned into *Leishmania* expression vector pLEXSY-hyg2 (Jena Bioscience, Jena, Germany) with a marker gene for selection with hygromycin B (HYG) and cut with *NcoI/NheI*. After linearization with *SwaI*, the coding sequences for LUC and HYG were integrated into the 18S rRNA locus of nuclear DNA of *L. major*. The virulence of LUC-transgenic *L. major* was maintained by passage in BALB/c mice. Promastigotes were grown in blood agar cultures at 27 °C, 5%  $\text{CO}_2$ , 95% humidity and 50  $\mu\text{g mL}^{-1}$  hygromycin B. Bone marrow derived macrophages (BMDM) generated as previously described using L929 supernatants<sup>17</sup> were seeded ( $2 \times 10^5$  cells per mL, final culture volume: 200  $\mu\text{L}$  per well) into a white 96-well plate (Greiner Bio-One, Frickenhausen, Germany), and incubated for 4 h at 37 °C to promote adhesion. LUC-transgenic promastigotes of *L. major* were harvested and re-suspended in RPMI-1640 medium, and finally added to each well. The infection rate of macrophages with LUC-transgenic *L. major* promastigote was adjusted to 1 : 15 ( $3 \times 10^6$  promastigotes per mL). These cocultures were incubated for 24 h at 37 °C, 5%  $\text{CO}_2$ , and 95% humidity to ensure infection and differentiation to amastigotes. After washing twice with RPMI-1640 medium, infected macrophages were incubated in the absence or presence of increasing concentrations of test samples (0.8  $\mu\text{M}$  to 100  $\mu\text{M}$ ) for further 24 h at 37 °C in duplicate wells (final culture volume: 200  $\mu\text{L}$  per well). Then, 25  $\mu\text{L}$  Britelite™ (PerkinElmer, Waltham, MA, USA) a lysis buffer containing luciferin was added in each well and luminescence was measured with a Victor™ X Light 2030 luminometer (PerkinElmer, Fremont, USA). The intensity of light emission after cell lysis is proportional to the number of intracellular amastigotes in macrophages. The luminescence was reduced after treatment with a leishmanicidal compound compared to the



controls without compound (growth control). HA-Pent3 was dissolved in water before testing. HA-COOH and pentamidine isethionate were used as reference compounds and were also dissolved in water. The half-maximal inhibitory concentrations ( $IC_{50}$  values) were calculated by linear interpolation (decreasing concentrations of the samples: 100  $\mu$ M, 20  $\mu$ M, 4  $\mu$ M, 0.8  $\mu$ M) as previously reported<sup>20</sup> and are presented as mean values of two independent experiments against the parasite and primary macrophages.

The activity of HA-Pent3 was compared with the free drug activity, by normalizing for the drug content. Cytotoxicities of HA-Pent3, Pent and HA-COOH were tested against uninfected primary BMDM as described above.<sup>17</sup>

## 3 Results and discussion

### 3.1 Chemistry

The conjugation of Pent with HA has been achieved by the "triazine-activated amidation" method, a highly versatile synthetic strategy that allows the regiospecific introduction of a wide range of functionalities without any unwanted side reactions,<sup>21</sup> under relatively mild conditions (room temperature; neutral pH in aqueous media).<sup>22</sup> The strategy entails the use of CDMT as an alternative reagent for coupling amines to HA carboxyl groups. Briefly, HA sodium salt was first converted into HA protonated form by using the appropriate ion-exchange resin. Then, NMM and CDMT were added to a solution of HA to activate the free carboxylic groups. The activation proceeds according to the proposed synthetic route depicted in Scheme 1 and involves the formation of an intermediate triazine-activated ester.<sup>23</sup> Finally, pentamidine [as free base (Pent)] was added to the mixture at 1 : 1 and 1 : 1.5 molar ratios (Entry 1 and Entry 2 and 3, respectively) with respect to HA carboxyl groups to effect the amide bond formation. Extensive dialysis of the solution and lyophilization afforded the desired amide-modified hyaluronan. Each amidation reaction was performed in a mixture of water and acetonitrile (3 : 2) which enables the solubilisation of both HA and other organic reagents. The data reported in

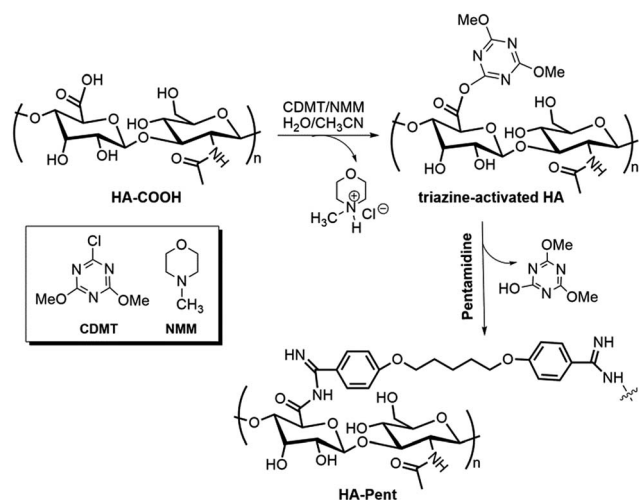
Table 1 indicate that the molar ratio HA carboxyl groups/Pent (*i.e.* Entry 1 *vs.* Entry 2) and the reaction time (*i.e.* Entry 3 *vs.* Entry 2) are key factors to increase the  $DS_{100}$ . The most notable  $DS_{100}$  was achieved by using 1.5 equivalents of Pent with a reaction time of 68 h; under these conditions, we obtained HA-Pent2 bioconjugate with 54.0 of  $DS_{100}$  (Entry 2, Table 1). Although HA-Pent2 shows the highest degree of drug loading, its low solubility in water (mainly in solution with high ionic strength) prevented the use of it for our biological application. We supposed that the coupling reaction between HA-COOH and our bifunctional drug proceeded towards the formation of a multi-branched (and therefore poorly water-soluble) complex. Thus, to limit the formation of multi-branched structures the reaction time was reduced to 20 h maintaining the 1 : 1.5 HA carboxyl groups/Pent molar ratio (Table 1, Entry 3). Under these experimental conditions we obtained a bioconjugate (*i.e.* HA-Pent3) with a good drug loading (although lower than HA-Pent2) and a proper solubility in water. On these bases, HA-Pent3 was advanced to biological screening.

The structure of HA-Pent3 was supported by spectral and analytical data; in particular the FT-IR spectrum shows the broad, strong band of O-H stretching at 3200–3500  $cm^{-1}$ , several bands associated with C=O and C=NH stretching (1712, 1643 and 1611  $cm^{-1}$ ) and the strong band of C-O stretching at 1033  $cm^{-1}$  (ESI, Fig. S1†).

The detection of the doublets of Pent aromatic protons at 6.99 ppm and 7.60 ppm in the  $^1H$  NMR spectrum (Fig. 2) indicated the successful achievement of the coupling reaction and their integral values are congruent with the coupling efficiency. Therefore, the  $DS_{100}$  was determined by comparing integrated signals of aromatic protons of Pent with the corresponding methyl signal of the *N*-acetylglucosamine unit of HA (singlet at 1.90 ppm).

### 3.2 Determination of $M_w$ by SEC/MALLS

HA is susceptible to degradation through different mechanisms due to the labile nature of its glycoside bonds.<sup>24</sup> In our case these fragmentations may occur principally during the ion-



Scheme 1 Synthesis of HA-Pent bioconjugates.

Table 1 Reaction conditions of HA-Pent synthesis, degree of substitution, theoretical molecular weight of polysaccharide and drug loading

Sample	Reaction conditions	$DS_{100}^b$	$M_{wt}^c$ (g mol $^{-1}$ )	Drug loading $^d$
HA-Pent1	Molar ratio $^a$ 1 : 1, 68 h, (Entry 1)	12.2	32 750	9.9
HA-Pent2	Molar ratio $^a$ 1 : 1.5, 68 h, (Entry 2)	54.0	43 300	33.2
HA-Pent3	Molar ratio $^a$ 1 : 1.5, 20 h, (Entry 3)	39.9	39 800	26.7

$^a$  HA carboxylic group/Pent molar ratio.  $^b$  Number of Pent groups per 100 disaccharide repeating unit.  $^c$  Theoretical molecular weight at the corresponding  $DS_{100}$ .  $^d$  Loading of Pent in the bioconjugates at the corresponding  $DS_{100}$  (% w/w) calculated considering the formation of a mono-linked derivative.





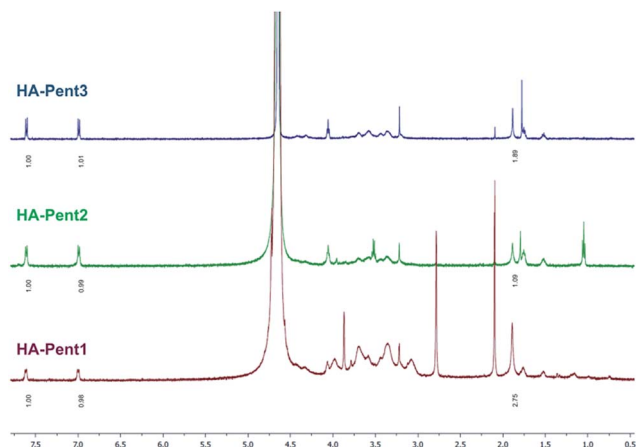


Fig. 2  $^1\text{H}$  NMR spectra of HA-Pent derivatives recorded in  $\text{D}_2\text{O}$ .

exchange with the resin and secondly during the various synthetic steps. Therefore, the average  $M_w$  of HA-COOH and its derivatives were measured by SEC-MALLS. The refractive index trace of starting HA-COOH, obtained from HA at high  $M_w$  commercially available, shows a distribution with an average  $M_w$  of about 30.0 kDa and a dispersity of 1.03 (Table 2). Instead, HA-Pent1, HA-Pent2 and HA-Pent3 peak elution volumes reveal a volume decrease (see Fig. 3) indicating an increase of both hydrodynamic volume and molecular mass. HA-Pent1, obtained by the standard coupling procedure (2 h of treatment with ionic-exchange resin and one equivalent of Pent), showed an increased polydispersity (from 1.03 to 1.48) together with an increased average  $M_w$  with respect to HA-COOH. Moreover, its GPC profile showed the presence of different oligomers with low  $M_w$  (Fig. 3). The optimization of the reaction conditions (1 h of treatment of HA with ionic-exchange resin and an increased amount of basic reagent Pent as for HA-Pent2 and HA-Pent3) avoided the oligomers formation (Fig. 3). A lower dispersity (*i.e.* 1.27; HA-Pent3) was obtained by reducing the reaction time from 68 h to 20 h. The molecular mass values obtained from SEC/MALLS are reported in Table 2 for all the samples. These data are in accordance with the qualitative evaluation deduced from the refractive index traces. However, for all HA-Pent derivatives the increase of the molar mass is much higher than expected. Probably, this is due to the bifunctional amidine moiety of Pent that, in addition to the intramolecular reactions (forming internal loop), can react also with others HA-COOH's chains (intermolecular reactions) leading to of multi-branched

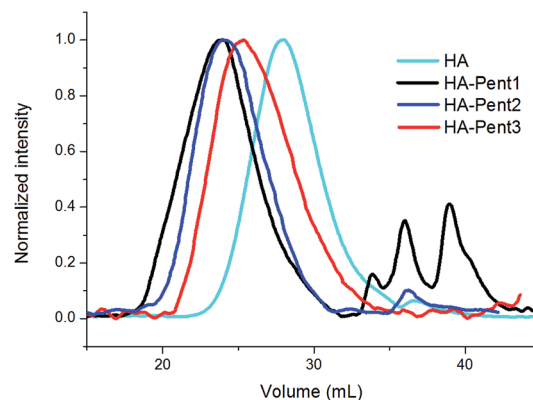


Fig. 3 GPC normalized trace of HA and HA-Pent bioconjugates.

structures. Because of this, in the case of HA-Pent2, sample with the highest drug loading, the macromolecular species turned out to be only partially water-soluble and exhibited a lower  $M_w$  value with respect to HA-Pent1 derivative (case with only about 10% of Pent loading, Table 1). This outcome suggests a lower ability of HA-Pent2 to form network structures with respect to HA-Pent1. However, this deduction is misleading because the increase of Pent leads also to an increase of the bioconjugate branching degree with the consequent formation of an insoluble multi-branched and/or cross-linked polymer fraction. Conversely, the different  $M_w$  values observed for HA-Pent2 and HA-Pent3 (both having the same HA/Pent molar ratio) are due to the different reaction times, higher for HA-Pent2 (68 h) than HA-Pent3 (20 h).

The differences between theoretical  $M_{wt}$  and  $M_n$  values of bioconjugates suggest that the coupling reaction proceeded towards the formation of linear/branched and/or partly cross-linked bioconjugates in the case of HA-Pent3 (39 800 *vs.* 73 100) and towards the formation of multi-branched or cross-linked derivatives in the cases of HA-Pent1 and HA-Pent2. Thus, the cross-linking in the coupling reaction depends mainly on  $\text{DS}_{100}$  and reaction time parameters.

### 3.3 Biological evaluation

To validate our drug targeting strategy, HA-Pent3 was employed for the biological tests. In the assay against the intracellular amastigote form of the parasite, HA-Pent3 turned out to be more potent than the reference compound (HA-Pent3,  $\text{IC}_{50}$  =

Table 2  $M_w$  by SEC/MALLS and dispersity

Sample	$M_{wt}^a$ (g mol $^{-1}$ )	$M_n^b$	$M_w^c$	$D^d$
HA-COOH	—	29 750	30 560	1.03
HA-Pent1 (Entry 1)	32 750	190 800	283 400	1.48
HA-Pent2 <sup>e</sup> (Entry 2)	43 300	149 100	196 900	1.32
HA-Pent3 (Entry 3)	39 800	73 100	89 000	1.27

<sup>a</sup> Theoretical molecular weight at the corresponding  $\text{DS}_{100}$ . <sup>b</sup> Number average molecular weight. <sup>c</sup> Weight average molecular weight. <sup>d</sup> Dispersity. <sup>e</sup> Analysis performed on the soluble fraction.

Table 3 Antileishmanial activity (after 24 h of incubation) and cytotoxicity (after 48 h of incubation) of HA-Pent3 in comparison with HA-COOH and pentamidine isethionate (reference compound)

Compound	$\text{IC}_{50}^a$ [ $\mu\text{M}$ ]	$\text{IC}_{50}^b$ [ $\mu\text{M}$ ]	SI
Pentamidine isethionate	4.5	45.2	10.1
HA-COOH	>100	>100	—
HA-Pent3	2.7	61.8	23.3

<sup>a</sup> Amastigotes (*L. major*). <sup>b</sup> BMDM.



2.7  $\mu\text{M}$  vs. pentamidine isethionate,  $\text{IC}_{50} = 4.5 \mu\text{M}$ ; Table 3), indicating an efficient internalization of the bioconjugate in the infected macrophages. At the same time HA-Pent3 showed less cytotoxicity than the standard in the assay against primary macrophages (HA-Pent3,  $\text{IC}_{50} = 61.8 \mu\text{M}$  vs. pentamidine isethionate,  $\text{IC}_{50} = 45.2 \mu\text{M}$ ; Table 3). HA did not show any anti-leishmanial or cytotoxic effect on primary macrophages. Overall, by conjugating Pent with HA, the selectivity index (SI) of the drug was raised over two-fold (Table 3).

## 4 Conclusions

In this work, we reported a straightforward synthetic method to obtain a drug targeting delivery system by using Pent and HA as drug and targeting delivery platform, respectively. The resulting HA-Pent bioconjugate turned out to be effective in the biological assay against *L. major* amastigote-infected macrophages by increasing nearly two-fold the potency of the drug with no significant cytotoxicity effects towards primary macrophages, showing a potential for the treatment of Leishmaniasis. These results suggest that the drug can be successfully internalized in the macrophages by means of HA receptors and released within the intracellular environment proposing HA as an appealing tool for the development of drug targeting delivery systems for the treatment of macrophage-associated diseases. On the other hand, since Pent is biologically and chemically versatile, our HA-Pent bioconjugate may be exploited for the development of complex/combined systems, *i.e.* by conjugation with other drugs that may produce synergistic therapeutic effects and/or further reduce the toxicity.

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## Notes and references

- 1 J. Alvar, I. D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin and M. den Boer, *PLoS One*, 2012, **7**, e35671.
- 2 C. Posch, J. Walochnik, A. Gschnait, H. Feichtinger and K. Rappersberger, *Hautarzt*, 2012, **63**, 947.
- 3 J. N. Sangshetti, F. A. Kalam Khan, A. A. Kulkarni, R. Arote and R. H. Patil, *RSC Adv.*, 2015, **5**, 32376.
- 4 K. Seifert, *Open Med. Chem. J.*, 2011, **5**, 31.
- 5 (a) Z. L. Yang, X. R. Li, K. W. Yang and Y. Liu, *J. Biomed. Mater. Res., Part A*, 2008, **85**, 539; (b) K. Shao, R. Huang, J. Li, L. Han, L. Ye, J. Lou and C. Jiang, *J. Controlled Release*, 2010, **147**, 118; (c) R. F. Carvalho, I. F. Ribeiro, A. L. Miranda-Vilela, J. Souza-Filho, O. P. Martins, C. S. D. Oliveira, A. C. Tedesco, Z. G. Lacava, Z. N. Bão and R. N. Simpaio, *Exp. Parasitol.*, 2013, **135**, 217; (d) A. E. Silva, G. Barratt, M. Cheifon and E. S. T. Egito, *Int. J. Pharm.*, 2013, **454**, 641; (e) R. Kumar, G. C. Sahoo, K. Pandey, V. N. R. Das and P. Das, *Drug Delivery*, 2015, **22**, 383; (f) P. K. Gupta, A. K. Jaiswal, V. Kumar, A. Verma, P. Dwivedi, A. Dube and P. R. Mishra, *Mol. Pharm.*, 2014, **11**, 951; (g) T. G. Ribeiro, J. R. Franca, L. L. Fuscaldi, M. L. Santos, M. C. Duarte, P. S. Lage, V. T. Martins, L. E. Costa, S. O. A. Fernandes, V. N. Cardoso, R. O. Castilho, M. Soto, C. A. P. Tavares, A. A. G. Faraco, E. A. F. Coelho and M. A. Chávez-Fumagalli, *Int. J. Nanomed.*, 2014, **9**, 5341.
- 6 World Health Organization, *W. H. O. Tech. Rep. Ser.*, 2010, **949**, 22.
- 7 (a) M. F. Minnick, L. D. Hicks, J. M. Battisti and R. Raghavan, *Int. J. Antimicrob. Agents*, 2010, **36**, 380; (b) P. Harris, C. Engler and R. Norton, *Int. J. Antimicrob. Agents*, 2011, **38**, 547; (c) X. Wang, Z. Fiorini, C. Smith, Y. Zhang, J. Li, L. R. Watkins and H. Yin, *PLoS One*, 2012, **7**, e47703.
- 8 G. Esposito, E. Capoccia, G. Sarnelli, C. Scuderi, C. Cirillo, R. Cuomo and L. Steardo, *J. Neuroinflammation*, 2012, **9**, 277.
- 9 (a) M. K. Pathak, D. Dhawan, D. J. Lindner, E. C. Borden, C. Farver and T. Yi, *Mol. Cancer Ther.*, 2002, **1**, 1255; (b) J. Smith, B. J. Stewart, S. Glaysher, K. Peregrin, L. A. Knight, D. J. Weber and I. A. Cree, *Anticancer Drugs*, 2010, **21**, 181.
- 10 A. Mukherjee, P. K. Padmanabham, M. H. Sahani, M. P. Barrett and R. Madhubala, *Mol. Biochem. Parasitol.*, 2006, **145**, 1.
- 11 (a) B. Chen, R. J. Miller and P. K. Dhal, *J. Biomed. Nanotechnol.*, 2014, **10**, 4; (b) A. Mero and M. Campisi, *Polymers*, 2014, **6**, 346.
- 12 A.-F. Tăbăran and C. Catoi, *Biotechnol., Mol. Biol. Nanomed.*, 2014, **2**, 17.
- 13 M. Kamat, K. Boubbou, D. C. Zhu, T. Lansdell, X. Lu, W. Li and X. Huang, *Bioconjugate Chem.*, 2010, **21**, 2128.
- 14 D. Liu and J. E. Uzonon, *Front. Cell. Infect. Microbiol.*, 2012, **2**, 83.
- 15 (a) T. Fernandes Stefanello, A. Szarpak-Jankowska, F. Appaix, B. Louage, L. Hamard, B. G. de Geest, B. van der Sanden, C. V. Nakamura and R. Auzély-Velty, *Acta Biomater.*, 2014, **10**, 4750; (b) S. Y. Park, M. Y. Jung, H. J. Kim, S. J. Lee, S. Y. Kim, B. H. Lee, T. H. Kwon, R. W. Park and I. S. Kim, *Cell Death Differ.*, 2008, **15**, 192.
- 16 N. K. Jain, V. Mishra and N. K. Mehra, *Expert Opin. Drug Delivery*, 2013, **10**, 353.
- 17 G. Bringmann, K. Thomale, S. Bischof, C. Schneider, M. Schultheis, T. Schwarz, H. Moll and U. Schurig, *Antimicrob. Agents Chemother.*, 2013, **57**, 3003.
- 18 N. N. Degtyareva, B. D. Wallace, A. R. Bryant, K. M. Loo and J. T. Petty, *Biophys. J.*, 2007, **92**, 959.
- 19 M. H. Ong, K. Jumel, P. F. Tokarczuk, J. M. V. Blanshard and S. E. Harding, *Carbohydr. Res.*, 1994, **260**, 99.
- 20 W. Huber and J. C. Koella, *Acta Trop.*, 1993, **55**, 257.
- 21 Z. J. Kamiński, *Tetrahedron Lett.*, 1985, **26**, 2901.
- 22 K. Bergman, C. Elvingsson, J. Hilborn, G. Svensk and T. Bowden, *Biomacromolecules*, 2007, **8**, 2190.
- 23 H. L. Rayle and L. Fellmeth, *Org. Process Res. Dev.*, 1999, **3**, 172.
- 24 G. Huerta-Angeles, D. Šmejkalová, D. Chladkova, T. Ehlova, R. Buffa and V. Velebny, *Carbohydr. Polym.*, 2011, **84**, 1293.

