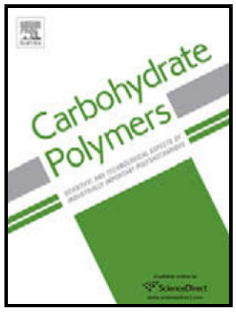

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PII: S0144-8617(17)31217-1
DOI: https://doi.org/10.1016/j.carbpol.2017.10.061
Reference: CARP 12910

To appear in:

Received date: 11-8-2017
Revised date: 5-10-2017
Accepted date: 17-10-2017

Please cite this article as: Wang, Zhaojie., Qiu, Yuanhao., Hou, Chunyan., Wang, Dongdong., Sun, Feifei., Li, Xiaojun., Wang, Feng., Yi, Hong., Mu, Haibo., & Duan, Jinyou., Synthesis of hyaluronan-amikacin conjugate and its bactericidal activity against intracellular bacteria in vitro and in vivo.*Carbohydrate Polymers* https://doi.org/10.1016/j.carbpol.2017.10.061

Manuscript: CARBPOL-D-17-02734

Title

Synthesis of hyaluronan-amikacin conjugate and its bactericidal activity against intracellular bacteria *in vitro* and *in vivo*

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Highlights

- HA-AM was obtained through reaction of HA with amikacin via “click” chemistry.
- HA-AM was more effective against intracellular bacteria than amikacin did.
- HA-AM had undetectable cytotoxicity.

Abstract

Aminoglycosides are often subtherapeutic to intracellular infections due to their high hydrophilicity. Here we used hyaluronic acid (HA) as a carbohydrate carrier of the aminoglycoside antibiotic, amikacin (AM) to deal with intracellular bacterial infections. The hyaluronan-amikacin conjugate (HA-AM) was synthesized by 'click' reaction between HA-propargyl amide (HAPA) and AM-azide. This conjugate with little cytotoxicity retained antibiotic effects on planktonic bacteria and showed better intracellular bactericidal activity than the antibiotic did. In addition, this conjugate was more efficient in reducing bacteria burden in an *in vivo* acute infection model than amikacin did. These results suggested that hyaluronic acid conjugation could reduce the dosage of antibiotic in treatment of intracellular bacterial infection, and further helping to alleviate the emergence of drug resistance in bacteria due to a long-term, high-dosage treatment.

Keywords: Hyaluronic acid; conjugate; Amikacin; Click chemistry; Intracellular bacteria.

1. Introduction

The intracellular bacteria related infections are always hard to be cured because these intracellular bacteria (e.g. *L. monocytogenes, P. aeruginosa, S. aureus* and *M. tuberculosis*) can exploit a huge range of niches within their hosts, protect them from some immune effectors such as antibodies, further gain access to and proliferate within the host cell cytosol, making the infection latent or recurrent (Ray, Marteyn, Sansonetti, & Tang, 2009; Joller et al., 2010; Leber et al., 2008).

For now, the treatment of infections caused by intracellular bacteria still presents a number of challenges, because their intercellular lifestyle protect them not only from the attack of the immune system but also from the action of the antibiotics (Imbuluzqueta, Gamazo, Ariza, & Blanco-Prieto, 2010). There are more than two thirds of prescribed antibiotics are ineffective against intracellular pathogens (Butler & Cooper, 2011). One of the major reasons is the limited intracellular active concentrations of antibiotics are often subtherapeutic, resulting in low effectiveness against intracellular pathogens and the emergence of antibiotic resistance (Baharoglu, Krin, & Mazel, 2013). To improve the therapeutic effect, high doses of antibiotics are often given, generating many side effects and toxicity. Aminoglycoside antibiotics, for instance, are poorly effective in treating intracellular pathogens *in vitro*, due to their high hydrophilicity and poor penetration into the eukaryotic cell membrane (Maurin & Raoult, 2001). But high-does or longtime aminoglycosides treatment are known to cause ototoxicity (Francis et al., 2013) and nephrotoxicity (Mingeot-Leclercq & Tulkens, 1999).

Hyaluronic acid (HA), is a nonsulfated glycosaminoglycan which consisted of alternating residues of β-4 linked D-glucuronic acid and β-3 linked N-acetyl-D-glucosamine (W. Zhang et al., 2014; Orellana et al., 2016). As a component of extracellular matrix, HA is readily chemically modified and adapted for medical uses (Dřímalová et al., 2005; Guo, Xie et al., 2014; Naderi-Meshkin et al., 2014), which shows various applications in tissue engineering or drug delivery (Borke, Winnik, Tenhu, & Hietala, 2015; Dosio, Arpicco, Stella, & Fattal, 2016; Thibeault et

al., 2011). Several chemical methods that use usually involving the activation of the carboxylate groups with reagents, such as N-(3-dimethylaminopropyl)-N'-ethy-lcarbodiimide (EDC) and 1-hydroxypyrrolidine-2,5-dione (NHS) or 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT), have been devised to facilitate the amidation of the HA carboxylate groups (Borke, Winnik, Tenhu & Hietala, 2015; Bulpitt & Aeschlimann, 1999; Huerta-Angeles et al., 2011; Kuo, Swann & Prestwich, 1990; Nakajima & Ikada, 1995; Schanté et al., 2011). 1-[Bis (dimethylamino) methylene]-1H-1,2,3-triazolo [4,5-b] pyridinium-3-oxid-hexafluorophosphate (HATU) is one of condensing reagents for solid-phase peptide synthesis (SPPS) that has been used in the reaction of carboxyl and amino peptide bonds (Saito & Wada, 2014).

Nowadays, it is possible to encapsulate, incorporate or even conjugate biologically active molecules into liposomes in order to deliver antibiotics intracellularly and hence to treat infections (Imbuluzqueta et al., 2010). However, liposomal antibiotics also display some drawbacks, such as instability of the vesicles, low drug entrapment and difficulty in sterilization procedures for liposomal antibiotics (Drulis-Kawa & Dorotkiewicz-Jach, 2010). Previously we demonstrated that a conjugate between HA and streptomycin through reductive amination could resolve the conundrum above (H. Y. Qiu et al., 2017). We asked whether this strategy was also suitable to amikacin, belonging to the third generation of aminoglycoside antibiotic family. The advantage of polysaccharide-antibiotic conjugate is that it possesses definite molecular structure and was ready to be prepared and sterilized. In

the present study, an HA-AM conjugates was synthesized by 'click' reaction between HAPA and AM-azide. The intracellular killing capacity of this conjugate was explored *in vitro* and *in vivo* (Fig. 1).

2. Results and discussion

2.1. Synthesis and characterization of HAPA derivatives

Several methods have been reported for the preparation of alkyne modified HA, but none of them was a chemical reaction in an easy way with HATU-mediated amidation. We set out first to confirm that, in our hands, the HATU-mediated amidation proceeded well under the conditions reported previously using the same amines ranging in the same pKa=6.5~7.0 (Borke, Winnik, Tenhu & Hietala, 2015). HATU in the presence of DMSO could be used to activate carboxyl functional groups in polysaccharides. In order to increase the solubility of polysaccharides in DMSO, hyaluronic acid sodium salt was exchanged for HA by cation exchange resin and the triethylamine was added to the mixture. Using this methodology, the amine bearing alkynyl terminal groups were attached to HA quickly and easily (Scheme 1). After reaction, the HAPA derivatives were purified, isolated.

The FT-IR analysis confirmed that the chemical reaction between the units and the amine by the presence of two distinct bands at 1645 cm^{-1} attributed to (C=O) stretching band and (–NH) deformation band, respectively. A small band at 2156 cm^{-1} revealed the presence of a terminal alkynyl group (Fig. 2A).

The degree of substitution (DS) values were resolved by ^{1}H NMR spectroscopy in mixed solution of DOH/DMSO-d_5 (*v/v*=1:1). The ^{1}H NMR spectroscopy showed

typical HA proton signals at 2.0 ppm belonging to –N–CO**CH$_3$** group, skeletal signals at 3.4~3.9 ppm and anomeric resonances at 4.4~4.6 ppm. The signal peaks of the propargyl protons appeared at 4.0~4.1 ppm (-NH**CH$_2$**C≡CH) and 2.8~3.0 ppm (–NHCH$_2$C≡**CH**), respectively (Fig. 3A). The DS of HAPA derivatives was determined by ^{1}H NMR spectroscopy as the integral ratio of the protons of methylene in amide, which was introduced relative to the three N-acetyl protons of HA.

2.2. Synthesis and characterization of azide-modified amikacin (AM-N$_3$)

One group has prepared and assayed 6'-N-metheyl and 6'-N-ethyl derivatives of amikacin. They showed that these derivatives were hardly affected by the 6'-N-acetyl transferase which has been shown to inactivate amikacin (Umezawa, H., Iinuma, K., Kondo, S., & Maeda, K., 1975). The primary alcohol in the 6'' position on these molecules was accessible to modification and was substituted for a variety of hydrogen bond donors and acceptors of different sizes. Analogues with guanidinium groups replacing the 6''-hydroxy group have been shown to display increased A-site affinity and, in some cases, superior antibacterial activity. Beyond imparting greater affinity for the A-site, some modifications could potentially disrupt recognition by aminoglycoside-modifying enzymes, the most common mechanism of aminoglycoside deactivation (Disney, M. D., & Childs‐Disney, J. L. 2007). Such derivatives could exhibit greater antibacterial potency against resistant bacteria (Fair et al., 2014).

In order to site-specifically immobilize the aminoglycosides onto a HAPA, the azide-modified aminoglycoside was synthesized. The parent aminoglycoside was

converted into three key intermediates (Fair et al., 2014). Firstly, four amines of AM were globally tert-butyloxycarbonyl (Boc)-protected using excess amount of di-tert-butyl dicarbonate (Tsitovich et al., 2010). The single primary alcohol of $(Boc)_4$ amikacin was then selectively converted into a sterically demanding sulfonate of active intermediate (Fair et al., 2012; Childs-Disney, Pushechnikov, Aminova, & Disney, 2007). Syntheses of AM derivatives were completed according to modification of previously published procedures in which a primary hydroxy group was activated by reaction with 2,4,6-triisopropylbenzenesulfonyl chloride in dry pyridine (Tsitovich et al., 2010). The resulting adduct was displaced with sodium azide (Mccoy. 2014; Tsitovich et al., 2010). The AM-N_3 were synthesized in above way. Acidic deprotection of all Boc groups using a one to one mixture of trifluoroacetic acid (TFA) in dichloromethane, followed by column chromatography purification (Fair et al., 2014; Tsitovich et al., 2010). Fig.2B showed the FTIR spectra of amicacin (a), 6"-O-TIPBS-tetra-N-Boc-amikacin (b), azide-modified amikacin (c). As shown in Fig.2B curve (c), a new adsorption peak at 2114 cm^{-1} was assigned to the stretching vibration of azide groups, which indicated that AM chains were successfully functionalized by azide groups. Spectra in Fig.2B curve (b) was normalized with respect to the benzenesulfonyl stretching peak at 2924 cm^{-1}. The peaks at 1645 cm^{-1} and 1046 cm^{-1} in Fig.2B curve (a), (b), (c) corresponded to the C-H stretching vibration of AM chains.

2.3. Synthesis and characterization of HA-AM

The azido-aminoglycosides and polysaccharide derivatives were arrayed onto

alkyne-agarose microarrays in the presence of copper (I) (Crescenzi et al., 2007). The water-soluble polysaccharide derivatives bearing side chains endowed with either azide or alkyne terminal functionality was mixed together in aqueous solution. When the latter two types of derivatives are mixed together in aqueous solution they give rise to a 1,3-dipolar cycloaddition reaction resulting in fast gelation (in the presence of catalytic amounts of Cu(I)) at room temperature. “Click chemistry” using azides and acetylenes is most effective when performed in water or the mixture of water and organic co-solvents （e.g. tret-butanol, ethanol, DMSO, THF and acetonitrile） (Gruškienė, Čiuta & Makuška, 2009; Parrish et al., 2005). AM-N_3 grafted HAPA in a mixed solution of water and DMSO, under the conditions of copper sulfate and sodium ascorbate, via a click reaction. The presence of a weak singlet assigned to the proton of the triazole ring at δ 7.9~8.0 ppm (Gruškienė, Čiuta & Makuška, 2009). Formation of HA-AM copolymers were confirmed by ^{1}H NMR spectra. The propargyl peaks at 2.8~3.0 ppm disappeared, while the signal originating from the newly formed triazole ring at 7.9~8.0 ppm was indicated (Fig. 3B). The overt signal protons of glycosidic ring groups of AM at 3.1~4.2 ppm appeared in the spectra and cross overlapped with those signals of the protons of skeletal signals of the HA backbone. The remaining typical proton signals of AM were prevailed at 5.4 ppm (-OH), 5.0 ppm (-CH_2-), 2.1 ppm (-CH_2-), 1.8 ppm (-CH_2-).

The DS of HA-AM conjugate (containing 13% amikacin) was determined by ^{1}H NMR spectroscopy as the integral ratio of the protons of the -CH- of the triazole ring, which was introduced relative to the two anomeric protons of HA (Fig. 3B signing 1

and 4).

2.4. Determination of molecular weight results by HPGPC

As shown in Fig. 4, both HA and HA-AM were eluted as a symmetrical narrow peak on HPGPC, indicating a high purity. The molecular weight was estimated to be 149.450 kDa, in reference to standard dextrans.

2.5. HA-AM conjugate eliminated intracellular bacteria effectively

Viability assay indicated that HA-AM (containing 13% amikacin) almost had no cytotoxicity on RAW 264.7 cells at tested concentration (Fig. S2). The cell survival rate (CSR) was above 80% even HA-AM up to the dosage of 300 μg/mL.

Macrophages function at the front line of immune defences against incoming pathogens, and therefore, are a common target for those bacterial pathogens that benefit from avoiding an encounter with the immune system, as well as those that are aiming to secure systemic spread (Parsek & Singh, 2003). After RAW264.7 macrophages were infected with *P. aeruginosa*, an opportunistic pathogen that could survive inside macrophages (Miao, Ernst, Dors, Mao, & Aderem, 2008), amikacina lone had a mild effect on bacterial counts after 12 h treatment compared to blank control (Fig. 5A). A combination of amikacin and hyaluronan did not improve reduction of intracellular bacteria counts dramatically. However, the HA-AM (containing 13% amikacin) conjugate (100 μg/mL) could kill more *P. aeruginosa* in macrophages, although hyaluronan had no bacterial activities at all (Fig. 5A).To see whether the HA-AM conjugate was able to clear other intracellular bacteria in macrophages, two other intracellular organisms, *S. aureus* (a common cause of skin

infections and respiratory disease) (Naimi et al., 2003) and *L. monocytogenes* (the causal organism of the serious foodborne illness listeriosis) (Freitag, Port, & Miner, 2009) were tested. As expected, the HA-AM conjugate (100 μg/mL) had stronger bactericidal activities towards *S. aureus* (Fig. 5B) and *L. monocytogenes* (Fig. 5C) intracellular than the respective mixture with equivalent amount of hyaluronan and amikacin did. Taken together, these results clearly indicated that the HA-AM conjugate had a potential to eliminate multiple intracellular bacteriain macrophages.

2.6. HA-AM conjugate promoted intracellular bacteria clearance *in vivo*

In order to evaluate bactericidal efficiency of HA-AM (containing 13% amikacin) conjugate against bacteria *in vivo*, mice were infected intraperitoneally with log-phase *L. monocytogenes* (10^5 CFU/mouse). 24 h later, mice received a daily subcutaneous injection of HA-AM (10 mg/kg), amikacin alone (AM) or the mixture with equivalent amount of HA and amikacin (HA+AM), or PBS for three days. Similar to *in vitro* findings, there was an improvement in the reduction of bacteria burden in the spleen and kidney for HA-AM conjugate, although amikacin alone or in combination with HA reduced the viable bacteria counts in some extent (Fig. 6).

2.7. Discussion

In an earlier study we demonstrated that covalent-coupling chitosan to streptomycin significantly improved intracellular bactericidal capacity of streptomycin towards multiple organisms within phagocytic or nonphagocytic cells (Mu, Niu, et al., 2016). However, chitosan-streptomycin conjugate showed an undesirable cytotoxicity to RAW264.7 cells, maybe because of the poly cationic

features of chitosan. Furthermore chitosan has been reported exhibited cytotoxicity at high concentrations (>500 μg/mL) (Huang, Khor, & Lim, 2004). Recently we synthesized a conjugate between HA and streptomycin through reductive amination from our group (Qiu et al., 2017). We asked whether this strategy was suitable for other antibiotic in the aminoglycoside family.

The HA-AM conjugate was synthesized by 'click' reaction between HA-propargyl amide and AM-azide. The HATU was used as coupling agent to carry out the amidation of HA in DMSO under mild conditions with excellent yields (59.3%). Compared to other coupling agents, such as EDC/NHS (Crescenzi, Cornelio, Di Meo, Nardecchia, & Lamanna, 2007) or CDMT/NMM (Bergman, Elvingson, Hilborn, Svensk, & Bowden, 2007) with yeild of 31% and 50%, this method described here was economical and convenient. This reaction condition meets the modification requirements for HA.

HA could be quickly transported into the cell by carriers (e.g. CD44), which exists on the cell surface of epithelial cells or certain types of hematopoietic cells (Mackay et al., 1994). CD44 receptors were over-expressed on certain cellular populations such as activated inflammatory cells (Vafaei, Esmaeili, Amini, Atyabi, Ostad & Dinarvand, 2016). Our earlier studies also discovered over-expressed CD44 receptor in infected RAW 264.7 (Yin et al., 2008; Zhang et al., 2014), thus HA-AM can be delivered into these infected cells by binding to CD44 receptor with high efficiency and be used for eradication of intracellular bacteria (Fig. 7). Compared to amikacin alone, HA-AM could reduce bacteria counts in infected RAW264.7 cells

(Fig. 5) or infected mice (Fig. 6), although HA-AM didn't show better activities against planktonic bacteria (Table S1). On the other hand, this indicated that HA conjugation did not reduce the antibiotic activity of amikacin.

3. Experimental

3.1. Materials

Hyaluronic acid sodium salt (Mw = 129 kDa) was obtained form Aladin. Amikacin disulfate (AM, 99%), Propargyl amine (PA), dichloromethane (DCM), 1-[Bis(dimethylamino)me-thylene]-1H-1,2,3-triazolo[4,5-b]-pyridinium-3-oxid-hexaf luorophosphate(HATU, 99%), di-tert-butyl dicarbonate (BOC, 99.5%), 2,4,6-triisopropylbenzenesulfonyl chloride (TIPBS-Cl, 99%), sodium ascorbate (BC), trifluoroacetic acid (TFA, 99.8%), pyridine (99%), Fluorescein5(6)-isothiocyanate (FITC) were purchased from Aladdin and used as received. Sodium azide, methanol (MeOH), triethylamine (TEA, 99.5%), N, N-dimethylformamide (DMF) were purchased by Xiya (Shangdong, China). 5-(4,6-Dichlorotriazinyl)aminofluorescein (5-DTAF) was buy from AAT Bioquest (USA). Water was obtained from a Millipore Milli-Q water purification system. Dialysis was conducted in CelluSep T_2 regenerated cellulose tubular membranes (Membrane Filtration Products, U.S.) with a molecular weight cut off (MWCO) of 5,000 Da. Samples for ^{1}HNMR spectroscopy were prepared in deuterium oxide (D_2O, 99.96% D) and dimethyl sulfoxide-d6 (DMSO-d_6, 99.80% D) obtained from Aladdin. DOWEX 50WX8 cation resin exchange (H type) was prepared. High performance gel permeation chromatography (HPGPC) was used on a Waters PL-GPC50. ^{1}H was carried out at room temperature on a BRUKER

Avance III 500 MHz (Switzerland). The Fluorescence Microscopy was used on OLYMPUS BX53-DP72 (JAPAN). Other chemicals were reagent grade and used without further purification unless otherwise noted.

Listeria monocytogenes (ATCC 19114), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (PAO1) were generous gifts received from Prof. Xia (College of Food Science and Engineering, Northwest A&F University). Female Kunming mice (weighing 28 ± 2 g) were purchased from School of medical laboratory animal science, The Fourth Military Medical University, China.

3.2. Preparation of HA form

As described (Huerta-Angeles et al., 2016), 1g hyaluronic acid sodium salt was dissolved in 100 mL distilled water. After filtrated through DOWEX 50WX8 cation resin exchange (H type)，the resulting solution was concentrated by rotary evaporator and lyophilized. The molecular weights of the polymer after the cationic exchange were determined by HPGPC. The obtained HA acid form was further used for the production of the amide derivate.

3.3. Synthesis of HAPA derivatives

The HAPA was synthesized as previous described (Huerta-Angeles et al., 2016). Briefly, HA acid form (200mg, 0.04mmol) was dissolved in 12mL of dry DMSO at 60 ℃. After dissolution of the polymer, the solution was cooled to room temperature. Then, 0.611 mL of TEA (3.3 mmol) was added to the reaction solution and allowed to be stirred for 15 min. At this moment 36mg of HATU (0.09mmol) was added. The mixture was stirred for 30 min at room temperature under nitrogen atmosphere (N_2).

After that, the propargyl amine (10 uL, 0.09mmol) was added to the reaction and stirred at room temperature for 24h under N_2. The products were dialyzed (Mw cut off = 5000Da) against 0.1 M of sodium chloride for 24 h, and then dialyzed against water for five days. The samples were freeze-dried and named as HAPA. The DS was determined by ^{1}H NMR as the integral ratio of the enantiomers protons of methylene α and β in amide which was introduced relative to the three N-acetyl protons of HA. FT-IR spectra were recorded using 500 cm^{-1} to 4000 cm^{-1} collecting 32 scans range with a resolution of 4 cm^{-1}. Samples were studied as KBr pellets.

3.4. Synthesis of azide-modified amikacin (AM-N_3)

As described (Childs-Disney, Pushechnikov, Aminova, & Disney, 2007; Disney & Barrett, 2007; Fair et al., 2014; Tsitovich et al., 2010; Michael, Wang, & Tor, 1999), all amines of aminoglycoside antibiotics were globally tert-butyloxycarbonyl (Boc)-protected using di-tert-butyl dicarbonate. The primary alcohol in the 6" position was modified by sulfonate of active intermediate. Then, the 6" position was displaced by azide group. Finally, the protecting group BOC was removed in trifluoroacetate.

3.5. Synthesis of Hyaluronan acid-amikacin conjugate (HA-AM) by "click chemistry"

In a typical procedure, HAPA 0.5mg, 0.017mmol (12.5μmol of propargyl groups) and AM-N_3 (10 mg, 17.7 μmol, 1.1eq) were dissolved in 0.5 mL DMSO and 1.0 mL H_2O. The solution was bubbled with argon gas for 15min to remove oxygen. Solutions of copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$, 0.006 mmol, 0.6 eq) and sodium ascorbate (0.021 mmol, 1.3 eq) in 0.5 mL water were prepared separately and

also deoxygenated by bubbling with argon gas for 10 min. Subsequently, the copper and ascorbate solution were added to the first mixture under an inert atmosphere via a syringe. The mixture was stirred under constant N_2 at room temperature for 24h. The product was purified by dialysis against 0.1 M of sodium chloride for one day, and then going on to be dialyzed against water for three days and then lyophilized. The pure product (HA-AM) was obtained in 61% yield.

3.6. Determination of molecular weight results by high performance gel permeation chromatography (HPGPC)

As previously (Mu, Niu, et al., 2016), the molecular weight was measured using HPGPC on three columns (Waters Ultrahydrogel 250, 1,000 and 2,000; 30 cm×7.8 mm; 6 μm particles) in series. The columns were calibrated with T-series Dextrans (5.2, 10, 48.6, 668, 2,000 kDa). Sodium acetate (3 mM) was used as eluant and the flow rate was kept at 0.5 mL/min. A 50 μL aliquot was injected for each run. The calibration curve of Log(Mw) *vs.* elution time (t) is:

$$\text{Log Mw} = -0.1869t + 12.061 \qquad 3\text{-}1$$

Mw = molecular weight.

t = Retention time.

3.7. Cytotoxic activity of HA-AM

The cytotoxicity test was performed using the MTT method (Mu, Tang, et al., 2016). The RAW264.7 cells (5×10^3cells/well) were cultured in DMEM (high glucose) medium supplemented with 10% fetal bovine serum. Approximately 8000 RAW264.7 (200 μL RPMI-1640 complete medium) was placed in 96-well plates for 8 h. Then

different concentration of the HA-AM (100 μg/mL, 200 μg/mL, 300 μg/mL) were added and continued for 24 h, the medium was removed. The complete medium containing 0.5 mg/mL MTT was added for 4 h, and then removed the medium. Then 100 μL DMSO was added to dissolve the blue violet crystal formazan. After 15 min, the absorbance was measured at 570 nm using a microplate reader. PBS was used as blank control.

3.8. Anti-intracellular bacteria activities

As previously (Mu, Niu, et al., 2016), the RAW 264.7 cells were plated on 24-well plate for 6h. Then, macrophages were infected at an MOI=2 for 1 h, washed thrice with PBS, and media with 50 μg/mL gentamicin was added for another 1h. After washed thrice with PBS, cells were incubated in 1 mL fresh medium supplemented with compounds for HA-AM, HA+AM, HA and AM for 12 h. Then cells were lysed with 0.25% Triton X-100 for 30 min. Serial dilutions were plated on TSB plates and colonies were counted the next day to determine CFU.

All data were made using GraphPad Prism 5.0. Results were expressed as mean ± SD. Data were analyzed by *t*-test and $P < 0.05$ was considered statistically significant.

3.9. *In vivo* bacterial infections

Female Kunming mice (weighing 28 ± 2 g) were infected intraperitoneally with 5×10^5 CFU of log-phase mouse passaged. Next day, mice received subcutaneous injection of HA-AM, HA+AM, AM or PBS (10 mg/kg of HA-AM, n=5 mice) once every day for three times. Spleen and kidney were harvested. CFU were determined

by dilution plating as previously described (Mu, Niu, et al., 2016).

3.10. The minimum inhibitory concentration (MIC) determinations for extracellular bacteria

MIC of HA-AM against bacteria was performed using microdilution assay. Briefly, two-fold serial dilutions of procedures were prepared in 96-well microplates in TSB with the final concentration of 25% after *P. aeruginosa*, *S. aureus*, *L. monocytogenes* (10^5 CFU/mL) addition. All assays were carried out in triplicate. The antibacterial activities were examined after incubation at 37°C for 24 h (Bastian, Warszawik, Panduru, Arenz & Herrmann, 2013; Mu, Zhang, Zhang, Cui, Wang & Duan, 2012).

3.11. Immunofluorescence

This was measured by fluorescent labels (FITC and 5-DTAF). The FITC bond amino and hydroxyl groups of HA-AM. The 5-DTAF labeled hydroxyl groups of HA and HA-AM. As described (Li, G. G. M, Li et al., 2002), Fluorescent labeling of HA-AM was obtained. RAW264.7 cells were plated on coverslips 12 h before incubation with fluorescent labeling of HA-AM conjugate (containing 13% amikacin), equivalent HA alone, PBS for 4 h. Then the coverslips were fixed in 4% paraformaldehyde for 15 minutes at room temperature . The fluorescent was stained by the Fluorescence Microscopy (OLYMPUS, Japan). For blocking experiments, cells were incubated with Anti-CD44 (20 μg/mL, eBioscience, CA) or free hyaluronic acid (100 μg/mL) for 1 h prior treatment with HA-AM.

4. Conclusion

Our results highlighted that HA conjugation could improve intracellular bactericidal capacity of amikacin against different pathogens. In vivo acute infection models indicated it elicited a better therapeutic effect on intracellular infection than amikacin did. Moreover, HA-AM showed neglectable cytotoxicity on RAW264.7 cells. These might be contribute to reduce antibiotic consumption in intracellular bacterial infection treatment, and then help to reduce the emergence of antibiotic resistance due to a long-term, high-dosage treatment.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (NSFC) (Grant No. 31570799), and Fundamental Research Funds for the Central Universities, Northwest A&F University (2452017026、Z109021713)

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Figure legends:

Fig.1. Schematic of the procedure for Anti-intracellular bacteria.

Fig.2. (A).FT-IR spectra of HA (1) and HA-Propargyl amide Derivatives (2), (B).FT-IR spectra of Amicacin (a) and 6"-O-TIPBS-tetra-N-Boc-amikacin (b) and azide-modified amikacin (c).

Fig.3. (A).^{1}H NMR spectra of HAPA and HA, (B).^{1}H NMR spectra of amikacin (AM), HA and HA-MA conjugate (HA-AM).

Fig.4. HPGPC chromatograms of HA, HAPA, HA-AM conjugate and AM on waters ultrahydrogel in series.

Fig.5. The colony-forming units of residual *P. aeruginosa* (A), *L.monocytogenes* (B) and *S.aureus* (C) in infected macrophages treated with different samples.

Fig.6. HA-AM conjugate removed intracellular bacteria efficiently in Kunming mice (Kunming mice peritoneal were infected with *L. monocytogenes* and treated with HA-AM, HA+AM, AM or PBS at a dose of 10 mg/kg. Mice were harvested 120 hpi for bacterial colony counts. Data points represent each mouse, means ± SD from all mice. n = 5 mice per group.)

Fig. 7. (A) The conjugation facilitated the entry of amikacin into host cells via a CD44-mediated pathway. (B) The conjugation promoted amikacin entrance into macrophages.

Scheme.1. Synthesis of HA-AM conjugate with AM-N_3 derivatives and HAPA via "click chemistry" reactions.

Fig. 1

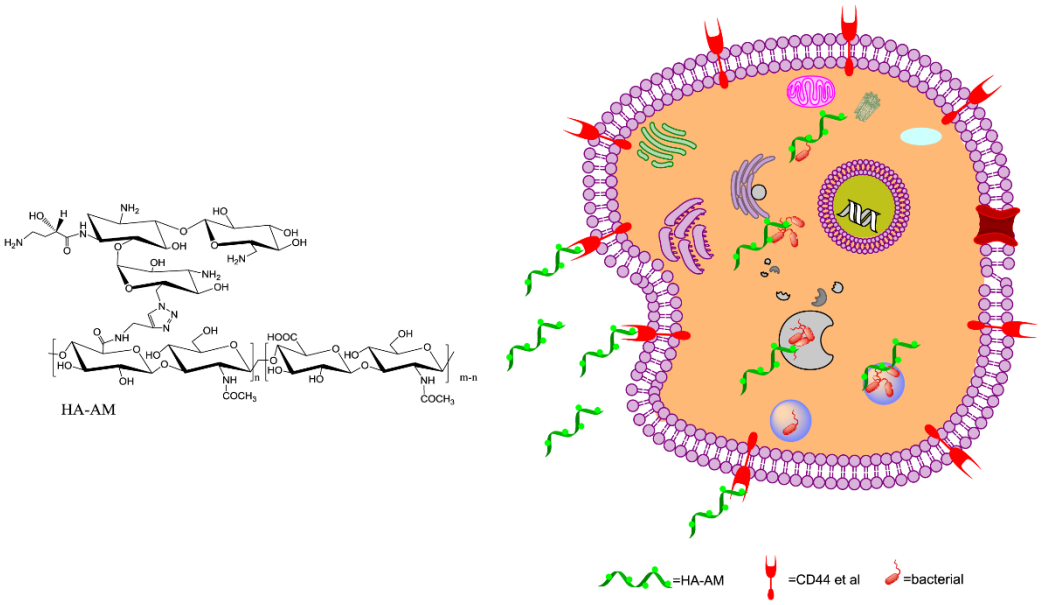


Fig. 2

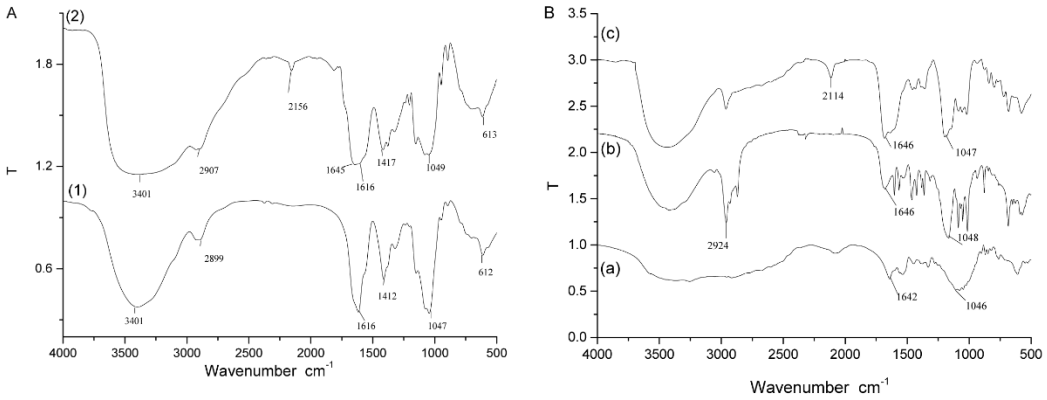


Fig. 3

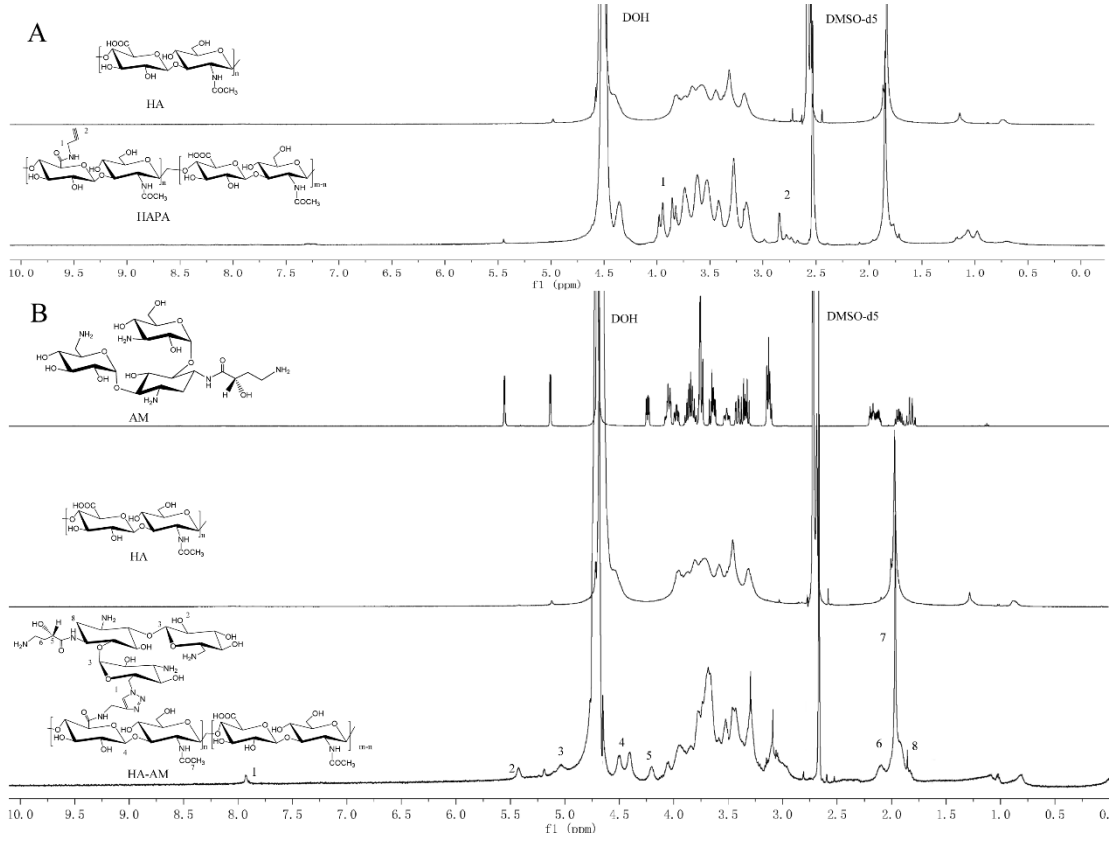


Fig. 4

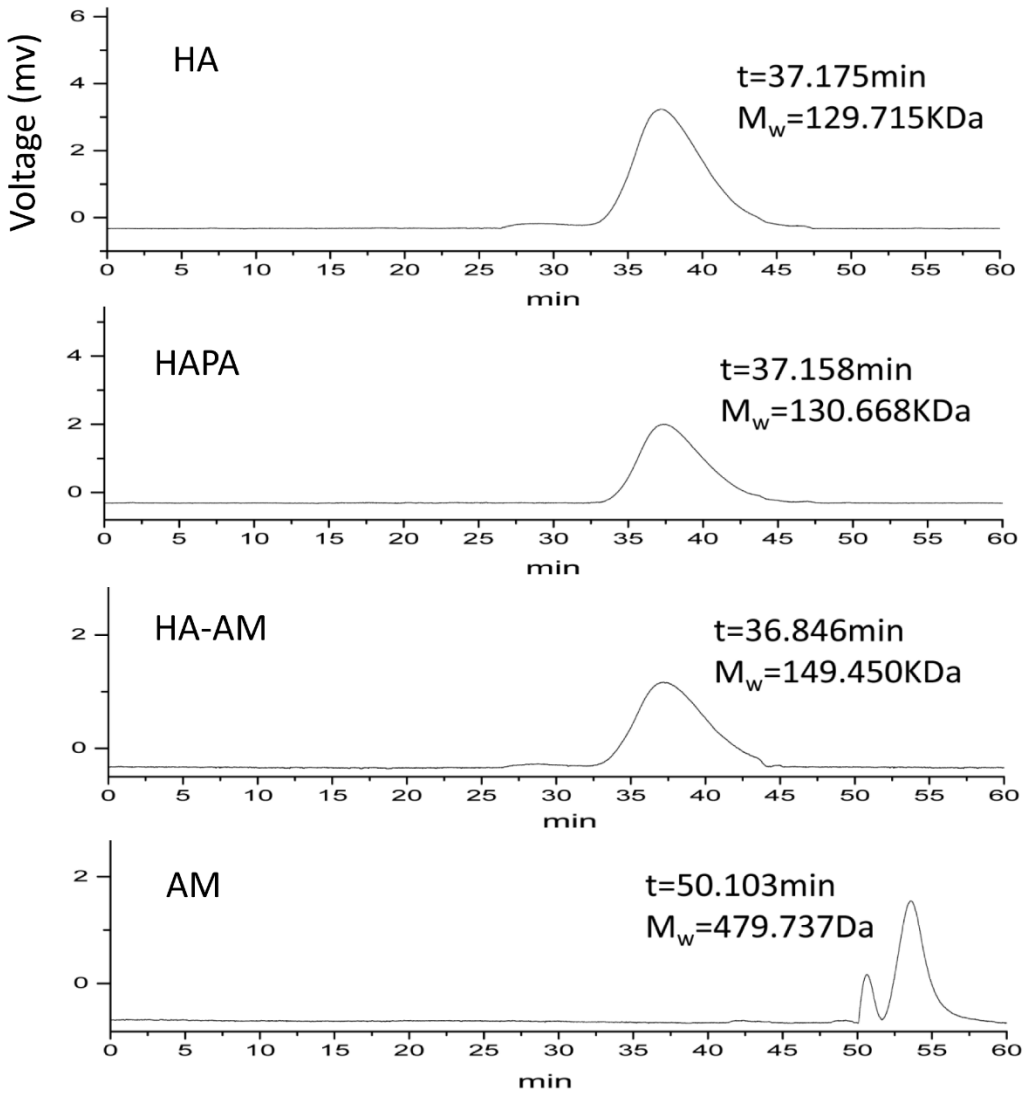


Fig. 5

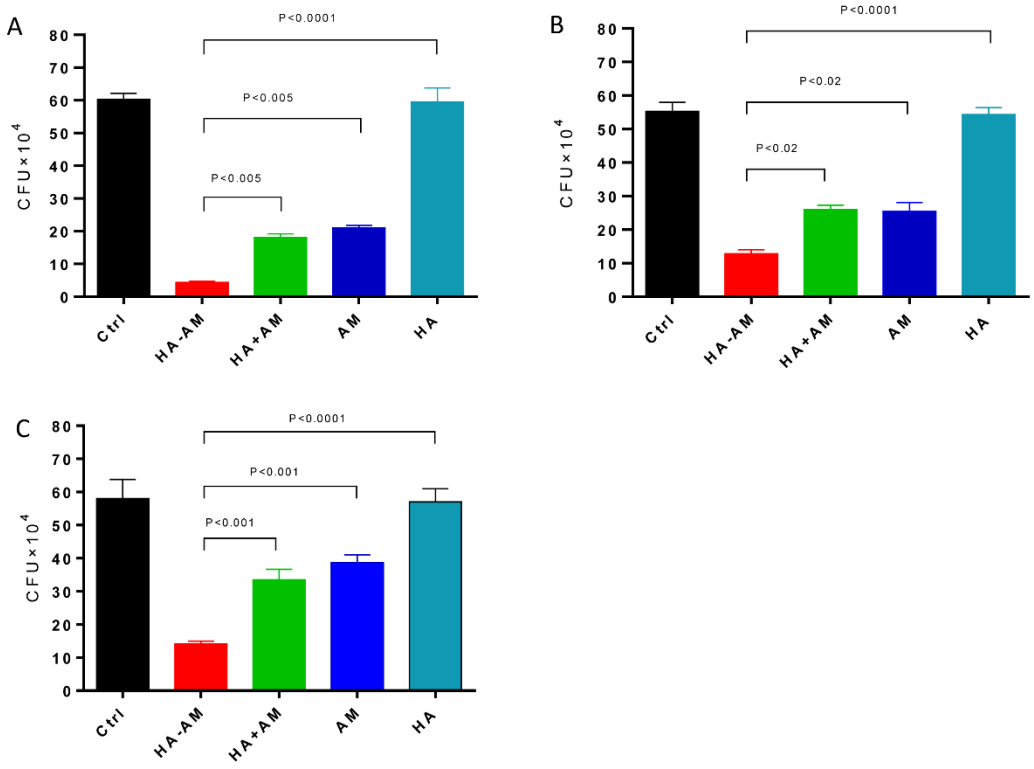


Fig. 6

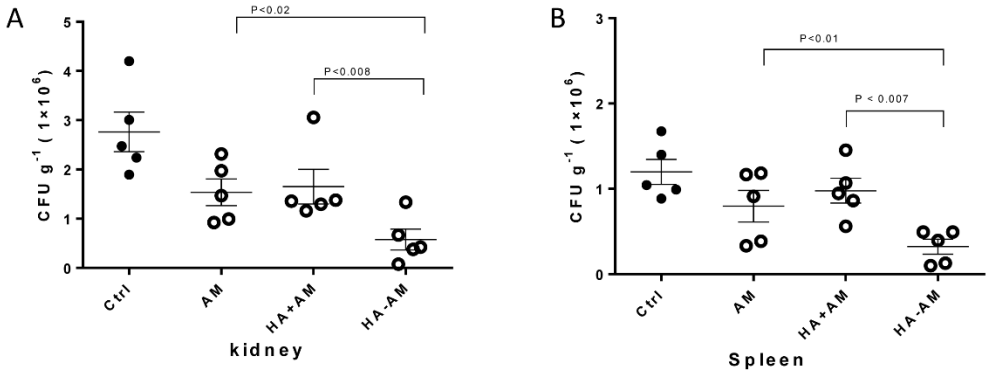
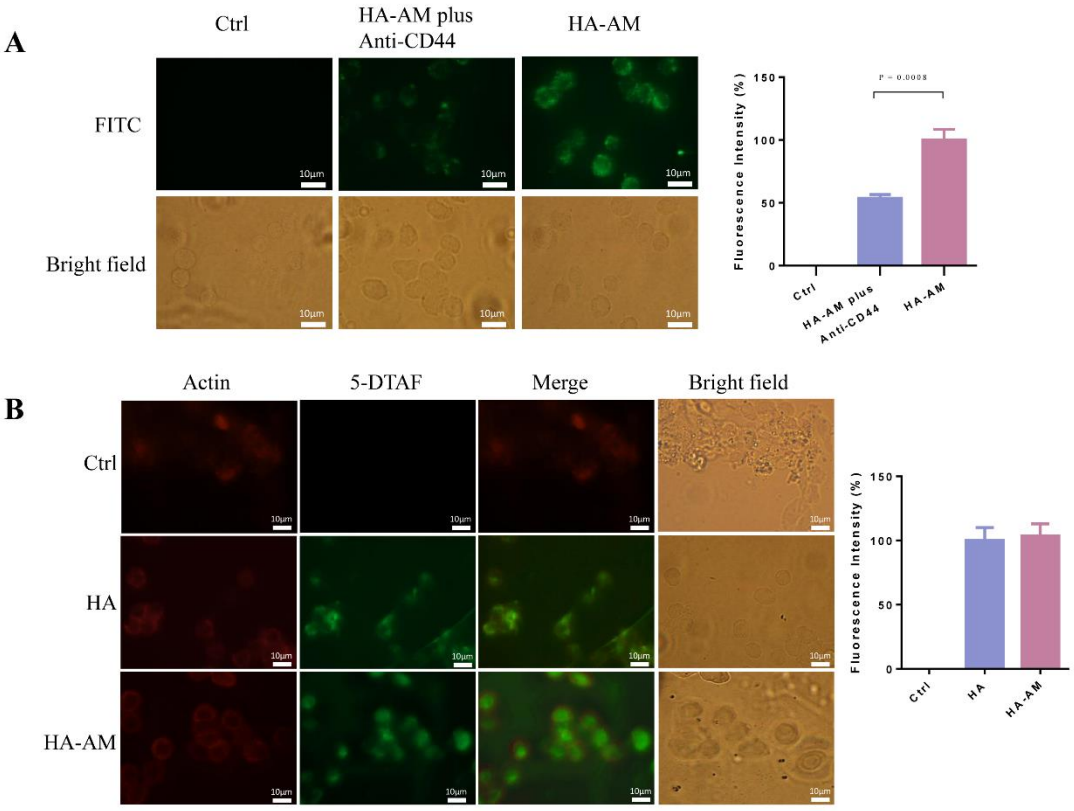


Fig. 7



Scheme. 1