European Journal of Pharmaceutics and Biopharmaceutics 79 (2011) 54–57

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Note

A new drug nanocarrier consisting of polyarginine and hyaluronic acid

Felipe A. Oyarzun-Ampuero, Francisco M. Goycoolea, Dolores Torres, Maria J. Alonso *

Department of Pharmacy and Pharmaceutical Technology, University of Santiago de Compostela, Spain

ARTICLE INFO

Article history:
Received 30 September 2010
Accepted in revised form 18 April 2011
Available online 27 April 2011

Keywords:
Nanoparticles
Hyaluronic acid
Polyarginine
Drug delivery

ABSTRACT

The purpose of this study was to produce and characterize a variety of nanostructures comprised of the polyaminoacid polyarginine (PArg) and the polysaccharide hyaluronic acid (HA) as a preliminary stage before evaluating their potential application in drug delivery. PArg was combined with high- or low-molecular-weight HA (HMWHA or LMWHA, respectively) to form nanoparticles by simply mixing polymeric aqueous solutions at room temperature. The average size of the resulting nanocarriers was between 116 and 155 nm, and their zeta potential value ranged from +31.3 to −35.9 mV, indicating that the surface composition of the particle could be conveniently modified according to the mass ratio of the polymers. Importantly, the systems prepared with HMWHA remained stable after isolation by centrifugation and in conditions that mimic the physiological medium, whereas particles that incorporated LMWHA were unstable. Transmission electron microscopy showed that the nanostructures made with HMWHA were spherical. Finally, the systems were stable for at least three months at storage conditions (4 °C).

1. Introduction

Because of their unique size and surface characteristics, nanostructures have emerged as a platform for the delivery of macromolecules and low-molecular-weight drugs for the treatment of a variety of diseases. In nanomedicine, there is a need for suitable biomaterials for optimization of the interaction/transport of drug carriers to target tissues. Concerning their characteristics, glycosaminoglycan hyaluronic acid (HA) and polyaminoacid polyarginine (PArg) are molecules used for the transport and biorecognition of nanomedicines.

HA is a natural non-toxic mucoadhesive polysaccharide that is negatively charged and biodegradable. It is widely distributed throughout the human body but primarily resides in connective tissue, eyes, intestine, and lungs. Importantly, the overexpression in a variety of tissues of the CD-44 receptor, the endogenous ligand for HA, makes this biopolymer a viable candidate for specific targeting. Several studies have tested the efficacy of nanosystems based on HA using various applications, namely gene delivery [1], cancer [2], and asthma [3] among others.

In turn, polyaminoacids are promising tools for the development of drug delivery systems, mainly due to their safety profile. These molecules are structurally similar to polypeptides and are thus degraded by human enzymes; their accumulation within the organism is minimal. Interestingly, the cationic PArg is able to translocate through cell membranes and facilitate the uptake of molecules associated with polyaminoacid [4]. This interesting feature of PArg has been exploited to harness drug delivery systems used for gene therapy [5], protein/vaccine delivery [6], and cancer [7]. Furthermore, PArg enhances the absorption of hydrophilic compounds across the nasal epithelium [8], a property that may be utilized for mucosal drug delivery.

The nanoparticles preparation method can affect its pharmacological and functional properties. Some procedures include the use of organic solvents or covalent cross-linkers that can compromise the safety of the formulation. Other preparation protocols use stringent processes, such as high temperature or sonication, which may destroy or alter drug bioactivity. In addition, nanoparticle stability in conditions that mimic biological medium or during long-term storage is a key factor that may determine the success of the formulation.

The aim of the present work was to develop and characterize a variety of PArg-containing nanostructures that were formed by the combination of the polyaminoacid with HA. We have also addressed the possible influence of the molecular weight of HA on the physicochemical and stability characteristics of the systems by using a high and a low-molecular-weight hyaluronic acid (HMWHA or LMWHA, respectively). These particles were formed using an extremely mild and simple procedure that involves mixing two aqueous phases at room temperature. The prepared nanostructures could be applied in different fields considering the established properties such as mucoadhesivity and cell-penetrating capacity of the constituent polymers. Of note, a recent study by Kim et al. reported the formation of a nanosystem composed

* Corresponding author. Campus Universitario Sur s/n, Faculty of Pharmacy, Block c, First Floor, Postcode 15782, Spain. Tel.: +34 981 563100x14885; fax: +34 981 547148.

E-mail addresses: foyarzuna1@gmail.com (F.A. Oyarzun-Ampuero), fm_goycoolea@yahoo.com (F.M. Goycoolea), dolores.torres@usc.es (D. Torres), mariaj.alonso@usc.es (M.J. Alonso).

0939-6411/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2011.04.008

of HA (19 kDa), PArg (15–70 kDa), and small interference RNA (siRNA) [9]. Such study focused on the biological behavior of siRNA-loaded nanosystems and not on the physicochemical and technological parameters of the unloaded nanoparticles.

2. Materials and methods

2.1. Chemicals

HMWHA (Mw ~ 165 kDa) was a gift from Bioiberica (Barcelona, Spain). LMWHA (Mw ~ 29 kDa) was purchased from Inquiaroma (Barcelona, Spain), and polyarginine (PArg, Mw ~ 5–15 kDa) was purchased from Sigma Aldrich (Madrid, Spain). All other reagents were of the highest analytical grade. MilliQ water was used for experimentation.

2.2. Preparation of nanoparticles

Nanoparticles were prepared by mixing HA and PArg aqueous solutions. Briefly, 4.5 mL of an aqueous solution containing HA (0.44–2.67 mg/mL) was added to 4.5 mL of a solution containing PArg (0.53 mg/mL) by stirring at room temperature. For isolation, 1 mL of the nanoparticles was transferred to Eppendorf tubes and centrifuged (16,000*g*, 30 min, 25 °C) in 20 μL of a glycerol bed. Supernatants were discarded, and the nanoparticles were resuspended in water by vigorous shaking. It is thought that the nanosystems were formed by electrostatic interactions between the positively charged amino group of the guanidine moiety on the PArg and the negatively charged carboxylate groups with the HA.

2.3. Physicochemical characterization of nanoparticles

The size and zeta potential of the colloidal systems were determined by photon correlation spectroscopy and laser Doppler anemometry using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, United Kingdom), MilliQ water was used as solvent. Each batch was analyzed in triplicate. Morphological examination of the nanoparticles was carried out by transmission electron microscopy (TEM) (CM12 Phillips, Eindhoven, Netherlands). The samples were stained with 1% (w/v) phosphotungstic acid for 10 s, immobilized on copper grids with Formvar®, and dried overnight before TEM analysis.

2.4. Stability of nanoparticles

Nanoparticle formulations were prepared and centrifuged in the presence of glycerol. The stability of the nanoparticles was evaluated according to size and precipitation in phosphate-buffered saline (PBS, pH 7.4) at 37 °C and in MilliQ water at 4 °C.

The composition of PBS was as follows: 137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 1.3 mM Na_2HPO_4.

3. Results and discussion

Table 1a and b show the mass ratio, charge [HA]/[PArg] ratio, size, polydispersity index, and zeta potential of the tested formulations prepared with either HMWHA or LMWHA, respectively. Our data show that when the [HA]/[PArg] charge ratio was lower than 0.975, nanostructures with a positive zeta potential were obtained, indicating that the surface of these systems is mainly composed of positively charged PArg. This feature is attributed to excess of PArg (relative to that of HA) in the formulation. Consequently, when the [HA]/[PArg] charge ratio increased to 0.975 and higher, inversion of the zeta potential values was observed, indicating that the nanocarrier surface was now shielded by excess of HA, which bears a negative charge. It can be appreciated that the zeta potential values (and average size) are not further modified beyond this ratio. This indicates that HA is incorporated into the nanoparticles up to saturation limit while surplus HA remains unassociated in solution and is in agreement with yield studies (data not shown).

Alteration of the surface charge of the nanocarriers as a function of the polymer ratio allows optimization of the surface composition (and, presumably, the biological behavior) to interact with targets that have affinity for PArg or HA. Additionally, knowledge of the relative contribution of each charged species to the nanosystems may allow nanoparticle customization for the incorporation of positively or negatively charged drug molecules.

The transmission electron micrographs indicated that each formulation was reasonable spherical, which is in agreement with previous works describing a spherical shape of nanoparticles made with either natural or synthetic polymers [3]. Fig. 1 shows a micrograph of a formulation with a [HMWHA]/[PArg] charge ratio of 1.3.

Importantly, characterization of HMWHA-containing systems was conducted after isolation of the nanocarriers by centrifugation, whereas characterization of LMWHA-containing systems was performed without isolation. The reason for that was because the latter nanoparticles were unstable during the centrifugation process (the lower tested conditions of centrifugation were 500*g* during 30 min) as evidenced by the disappearance of the turbidity of the system and by photon correlation spectroscopy (PCS) size measurements. The systems containing HMWHA were effectively isolated at 16,000*g* during 30 min, maintaining the same characteristics that the non-isolated formulations had. As shown in Table 1, at a charge ratio of 0.325, nanosystems were obtained that incorporated HMWHA, whereas those containing LMWHA were not

Table 1
Physicochemical properties of the nanocarriers prepared with different ratios of HMWHA-PArg or LMWHA-PArg and evaluated in MilliQ water (mean ± SD, $n = 3$).

Mass ratio	Charge ratio [HA]/[PArg]	Size (nm)	Polydispersity index	Zeta potential (mV)
HMWHA-PArg				
2–2.4	0.325	128 ± 8	0.2–0.3	+31.3 ± 1
4–2.4	0.65	136 ± 16	0.1–0.2	+25.3 ± 4
6–2.4	0.975	154 ± 7	0.1–0.2	−32.5 ± 5
8–2.4	1.3	150 ± 7	0.1–0.2	−33.4 ± 4
10–2.4	1.625	147 ± 7	0.1–0.2	−35.9 ± 5
12–2.4	1.95	155 ± 7	0.1–0.2	−33.8 ± 4
LMWHA-PArg				
2–2.4	0.325	Not formed	Not formed	Not formed
4–2.4	0.65	131 ± 26	0.1–0.2	+25 ± 1
6–2.4	0.975	172 ± 18	0.1–0.2	−19 ± 1
8–2.4	1.3	139 ± 9	0.1–0.2	−27 ± 3
10–2.4	1.625	137 ± 13	0.1–0.2	−31 ± 3
12–2.4	1.95	146 ± 13	0.1–0.2	−33 ± 3

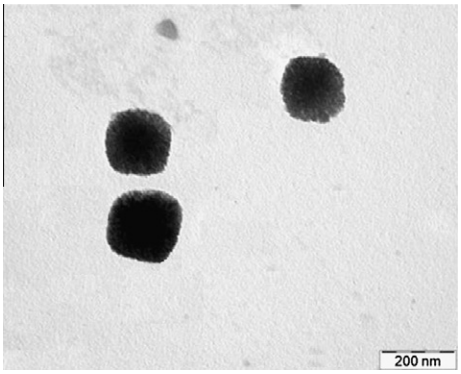

Fig. 1. Transmission electron micrograph of HMWHA-PArg nanocarriers; HA-PArg charge ratio = 1.3.

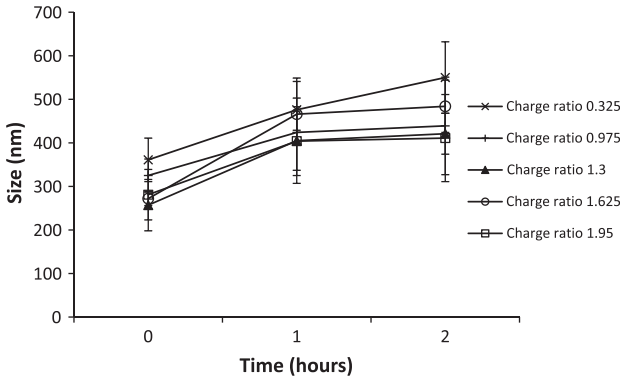

Fig. 2. Stability profiles of HMWHA-PArg nanocarriers in phosphate-buffered saline pH 7.4 at 37 °C; HA-PArg charge ratios: 0.325 (×), 0.975 (+), 1.3 (▲), 1.625 (○), and 1.95 (□) (mean ± SD, $n = 3$).

formed. In addition, the ζ values of the LMWHA-containing systems were generally lower than those containing HMWHA systems. Thus, fewer charged species were located on the surface of the low-molecular-weight polymer surface. Greater charge compensation for systems that have low-molecular-weight species may also account for the lower ζ values.

Hence, we postulate that the observed differences among nanostructures comprising HMWHA or LMWHA are probably due to the preferential organization of the polymers, particularly at the nanoparticle surface. Variance in particle assembly may also account for the instability of LMWHA nanosystems during centrifugation. The formation of local regions with a large number of consecutive associated residues (i.e., greater cooperativity) of both polyelectrolytes in the HMWHA-containing systems may explain its superior stability during centrifugation in comparison with LMWHA-containing systems, which presumably have lower cooperativity. These regions, in which charges are compensated, become more hydrophobic due to their neutral character, and hence, they are expected to lie within the inner core of the nanostructure. This distribution would provide the systems with more stability. A similar interpretation was offered in a previous work studying the behavior of hybrid nanoparticles of chitosan and alginate (of various molecular weights) cross-linked with tripolyphosphate [10]. It is important to point out that the ability to isolate formulations by simple centrifugation avoids tedious time-consuming procedures, such as those performed by Kim et al. [9], whose developed nanosystems were isolated by dialysis over 2 days.

Colloidal stability under physiological conditions is a crucial characteristic for successful biomedical application of the nanosystems. Therefore, we investigated the stability of the systems

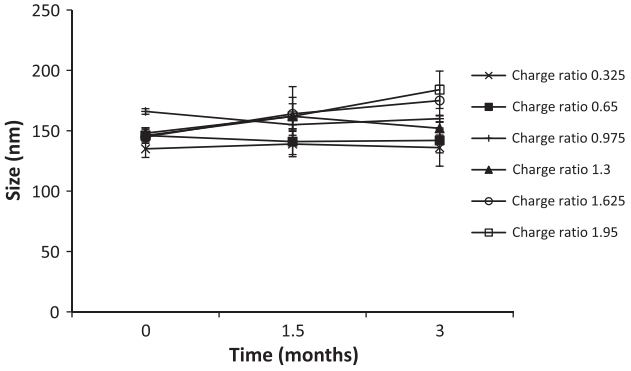

Fig. 3. Stability profiles of HMWHA-PArg nanocarriers in water during storage at 4 °C; HA-PArg charge ratios: 0.325 (×), 0.65 (■), 0.975 (+), 1.3 (▲), 1.625 (○) and 1.95 (□) (mean ± SD, $n = 3$).

composed of HMWHA and LMWHA in PBS at pH 7.4 and 37 °C. Fig. 2 shows the stability profiles of the HMWHA-containing systems. The majority of the formulations were stable for at least 2 h, indicating that the stability of the nanosystems was maintained independent of their surface composition (PArg or HMWHA). The stability profiles of each LMWHA-containing formulation and that of 0.65 charge ratio-containing HMWHA are not shown in the plot because they either immediately aggregated after addition to the medium or were larger than ~1000 nm. Differences in the stability of HMWHA- and LMWHA-containing nanostructures may also be related to the specific organization of HA and PArg on the nanoparticle surface. This would affect the colloidal stability conferred by charge repulsion and steric hindrance. In the case of the formulation of charge ratio 0.65 with HMWHA, its instability could be attributed that this formulation shows the lowest magnitude in zeta potential before the point of charge inversion, thus effectively perturbing the stability of the systems mediated by charge repulsion. Importantly, in Fig. 2 it is possible to appreciate that, from the beginning of the experiment (0 h), all the formulations showed an increased size compared with the original one evaluated in MilliQ water (Table 1). Considering that PBS presents a significant quantity of salts, this can weaken and dissociate the ionic interactions between the oppositely charged polymers leading to swelling of the systems. Additionally, the salt ions could diffuse inside the nanosystems and attracting, by osmotic forces, water inside the nanosystems also exerting a possible swelling of the formulations.

Finally, the stability of nanocarriers during long-term storage is an important step for the adequate handling of the formulations. Fig. 3 shows the stability profiles of each formulation composed of HMWHA at 4 °C for 3 months. Every formulation was stable and showed no significant change in particle size. Each system composed of LMWHA was also stable during storage (data not shown).

As future work, we are planning to introduce macromolecular hydrophilic drugs into the nanocarriers whose final targets could be solid tumor cells (where CD-44 receptors are overexpressed) or mucosal surfaces (that can avidly interact with both hyaluronic acid and polyarginine). More concretely, oral peptide delivery, in which polyarginine has demonstrated to be very promising, will be explored.

4. Conclusions

In conclusion, a nanoparticulate system composed of PArg and HA was successfully prepared using an extremely mild process. Negatively and positively charged nanoparticle formulations, with surfaces composed preferentially of HA or PArg, were obtained. Importantly, we demonstrated that the molecular weight of HA is a crucial determinant of formulation stability during mechanical

isolation and in physiological conditions. This knowledge is useful not only for systems comprising polyarginine and hyaluronic acid but also for systems composed by other polymers. Further studies testing the potential of these systems as mucoadhesive nanocarriers for targeted drug delivery will be carried out, and the in vitro–in vivo behavior of these systems will be also evaluated.

Acknowledgements

The authors acknowledge the Spanish Government for financial support (Consolider-Ingenio CSD 2006-00012 and Xunta de Galicia PGIDIT 08CSA045209PR) and CONICYT for a scholarship to F.A. Oyarzun-Ampuero.

References

[1] M. de la Fuente, N. Csaba, M. Garcia-Fuentes, M.J. Alonso, Nanoparticles as protein and gene carriers to mucosal surfaces, Nanomedicine 3 (2008) 845–857.
[2] V.M. Platt, F.C. Szoka Jr., Anticancer therapeutics: targeting macromolecules and nanocarriers to hyaluronan or CD44, a hyaluronan receptor, Mol. Pharm. 5 (2008) 474–486.
[3] F.A. Oyarzun-Ampuero, J. Brea, M.I. Loza, D. Torres, M.J. Alonso, Chitosan-hyaluronic acid nanoparticles loaded with heparin for the treatment of asthma, Int. J. Pharm. 381 (2009) 122–129.
[4] M. Lundberg, S. Wikström, M. Johansson, Cell surface adherence and endocytosis of protein transduction domains, Mol. Ther. 8 (2003) 143–150.
[5] V.P. Torchilin, TAT peptide-mediated intracelular delivery of pharmaceutical nanocarriers, Adv. Drug Deliv. Rev. 60 (2008) 548–558.
[6] K. Lingnau, K. Riedl, A. von Gabain, IC31® and IC30, novel types of vaccine adjuvant based on peptide delivery systems, Expert Rev. Vaccines 6 (2007) 741–746.
[7] Z. Miklán, E. Orbán, G. Csík, G. Schlosser, A. Magyar, F. Hudecz, New daunomycin–oligoarginine conjugates: synthesis, characterization, and effect on human leukemia and human hepatoma cells, Biopolymers 92 (2009) 489–501.
[8] M. Miyamoto, H. Natsume, S. Iwata, K. Ohtake, M. Yamaguchi, D. Kobayashi, K. Sugibayashi, M. Yamashina, Y. Morimoto, Improved nasal absorption of drugs using poly-L-arginine: effects of concentration and molecular weight of poly-L-arginine on the nasal absorption of fluorescein isothiocyanate-dextran in rats, Eur. J. Pharm. Biopharm. 52 (2001) 21–30.
[9] E.J. Kim, G. Shim, K. Kim, I.C. Kwon, Y.K. Oh, C.K. Shim, Hyaluronic acid complexed to biodegradable poly-L-arginine for targeted delivery of siRNA, J. Gene Med. 11 (2009) 791–803.
[10] F.M. Goycoolea, G. Lollo, C. Remuñán-López, F. Quaglia, M.J. Alonso, Chitosan-alginate blended nanoparticles as carriers for the transmucosal delivery of macromolecules, Biomacromolecules 10 (2009) 1736–1743.