BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
8 (2012) 1070–1073

nanomedicine
Nanotechnology, Biology, and Medicine

Short Communication

nanomedjournal.com

In vivo real-time confocal microscopy for target-specific delivery of hyaluronic acid-quantum dot conjugates

Ki Su Kim, BS[a], Seonghoon Kim, BS[b], Songeun Beack, BS[a], Jeong-A Yang, BS[a], Seok Hyun Yun, PhD[b,c], Sei Kwang Hahn, PhD[a,c,*]

[a]*Department of Materials Science and Engineering, Pohang University of Science and Technology Pohang, Republic of Korea*
[b]*Graduate School of Nanoscience and Technology, Korea Advanced Institute of Science and Technology, Daejon, Republic of Korea*
[c]*Wellman Center for Photomedicine, Harvard Medical School and Massachusetts General Hospital, Cambridge, Massachusetts, USA*

Received 21 December 2011; accepted 28 May 2012

Abstract

Hyaluronic acid (HA), which is a biocompatible, biodegradable, and linear polysaccharide in the body, has been widely used for various biomedical applications. In this work, real-time bioimaging for target-specific delivery of HA derivatives was carried out using quantum dots (QDs). In vitro confocal microscopy of HA-QD conjugates confirmed the intracellular delivery of HA derivatives to B16F1 cells with HA receptors by HA-receptor-mediated endocytosis. Furthermore in vivo real-time confocal microscopy of HA-QD conjugates successfully visualized the target specific delivery and accumulation of HA-QD conjugates from the fluorescence-labeled blood vessels to the liver tissues. The authors could confirm the feasibility of HA derivatives as a target-specific intracellular drug-delivery carrier for the treatment of liver diseases and the in vivo real-time confocal microscopy as a new bioimaging tool for various drug-delivery applications.

From the Clinical Editor: This study demonstrates the possibility of labeling hyaluronic acid with quantum dots for visualization and for targeted intracellular drug delivery in liver disease models.

Key words: Hyaluronic acid; Quantum dots; Confocal microscopy; Bioimaging; Drug delivery

A variety of bioimaging tools have been developed to visualize the delivery and distribution of biomolecules in the body. In particular, optical bioimaging systems have been extensively investigated using quantum dots (QDs), iron oxide nanoparticles (IONPs), and gold nanoparticles (AuNPs). However, optical fluorescence bioimaging tools are known to have several disadvantages, such as limitations in resolution, penetration depth of fluorescence, real-time accessibility, and so on.[1,2] Alternatively, in vivo real-time confocal microscopy can be a powerful bioimaging technique to circumvent those problems.[3] It has been widely exploited to visualize biological processes, such as cell trafficking, cell-cell and cell-microenvironment interactions with a high resolution.[3] In addition in vivo confocal microscopy has been investigated for the detection and monitoring of various diseases such as cancers, ophthalmic diseases, and musculoskeletal diseases.[4] In this study in vitro confocal microscopy of hyaluronic acid (HA) derivatives labeled with QDs was carried out with a competitive binding test to assess the feasibility of HA derivatives for target specific intracellular drug delivery. HA is a naturally occurring linear polysaccharide in the body and has been widely investigated as a new emerging drug delivery carrier.[5] Furthermore in vivo real-time confocal microscopy for the target-specific delivery of HA derivatives to the liver was performed for various drug-delivery applications.

Methods

Adipic acid dihydrazide-modified HA (HA-ADH, MW = 100 kDa) was synthesized as described elsewhere.[5] Then, HA-ADH was conjugated to QDs activated with 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS).[6] For in vitro bioimaging, B16F1 cells and HEK293 cells were seeded on the poly-D-lysine coated culture slides at a density of 3×10^4 cells/well and incubated in the medium

The topic of this invited contribution has been presented at the 4th IEEE International Conference on Nano/Molecular Medicine and Engineering (IEEE-NANOMED 2010), 5-9 December, 2010, Hong Kong SAR, China.

No conflict of interest was reported by the authors of this paper.

This research was supported by the Convergence Research Center Program, Mid-career Researcher Program and the WCU Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology (2009-0081871, 2009-0084578 and R31- 2008-000-10071-0).

*Corresponding author:
E-mail address: skhanb@postech.ac.kr (S.K. Hahn).

1549-9634/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.nano.2012.05.014

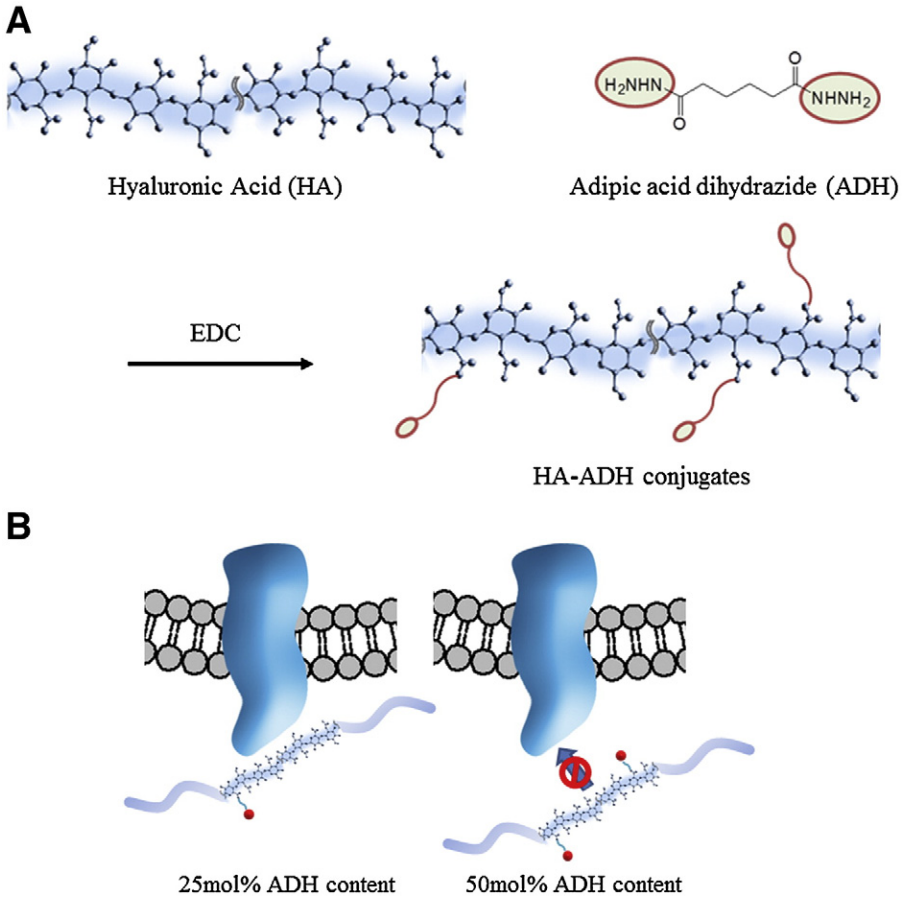

Figure 1. Schematic representations for **(A)** the synthesis of HA-ADH and **(B)** the binding of HA derivatives with HA modification degrees of 25 mol% (*left*) and 50 mol% (*right*) to the HA receptors.

for 3 days. QDs and HA-QD conjugates in 100 μL of Dulbecco's Modified Eagle Medium (DMEM) were added to the wells of culture slides. The final concentration of QDs was 5 nM. The cells were incubated for 2 hours, washed with PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI), fixed with 4% paraformaldehyde in PBS, washed again with PBS two times, and observed with a confocal scanning microscope (FluoView FV1000, ×400). As a competitive binding test, HA-QD conjugates were incubated with B16F1 cells in the absence and presence of free HA (ca. 7.5 μM) for 1 hour. Then in vivo real-time confocal microscopy was carried out after retro-orbital injection of HA-QD conjugates (150 μL, ca. 0.01 nmol) to Balb/C mice at an age of 4 weeks. QDs were also injected to the mice as a control. After cutting the abdomens, the fluorescence of injected QDs and HA-QD conjugates in the liver were observed with the home-built in vivo confocal microscope.[3] Dextran-FITC conjugates were also retro-orbitally injected to the same mice to visualize the blood vessels in the liver tissues. We have complied with the POSTECH institutional ethical use protocols for animals.

Results and discussion

HA-ADH conjugates were synthesized as schematically shown in Figure 1, *A*. The carboxyl group of HA was activated with EDC and conjugated to the amine group of ADH. Then HA-ADH conjugates were labeled with QDs via amide bond formation after activation of carboxyl groups of QDs with EDC and sufo-NHS. It has been previously reported that HA binds cluster determinant 44 (CD44) through three carboxyl groups of HA.[7] Accordingly, as schematically illustrated in Figure 1, *B*, HA-ADH conjugates with an HA modification less than 25 mol% can bind HA receptors through the unmodified three carboxyl groups of HA, whereas HA-ADH conjugates with 50 mol% HA modification cannot bind HA receptors theoretically.[7,8] The effect of HA modification on HA receptor-mediated endocytosis was previously investigated in detail and reported elsewhere.[8] To assess the feasibility of HA derivatives as a drug carrier for the treatment of liver diseases, HA-QD conjugates with a degree of HA modification less than 25 mol% was synthesized and applied to the bioimaging via in vitro and in vivo confocal microscopy.

Figure 2, *A* shows the confocal microscopic images of B16F1 cells encapsulating HA-QD conjugates by HA receptor-mediated endocytosis. B16F1 cells have highly expressed HA receptors, such as CD44 and lymphatic vessel endothelial hyaluronan receptor (LYVE)-1.[8] The cellular uptake of HA-QD conjugates was significantly low in the presence of free HA molecules and in the case of HEK293 cells without HA receptors (Figure 2, *B*) reflecting the target-specific HA receptor-mediated endocytosis.[8] Furthermore, the confocal microscopic images at the focal depths

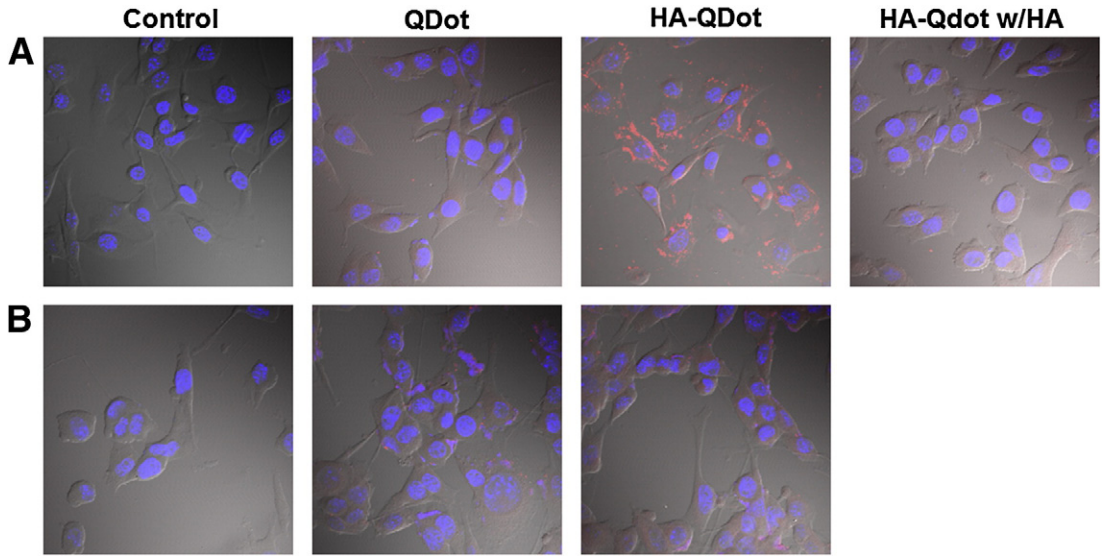

Figure 2. Confocal microscopic images of **(A)** B16F1 cells and **(B)** HEK293 cells incubated for 2 h with QDs and HA-QD conjugates with 22 mol % HA modification in the absence and presence of excess HA.

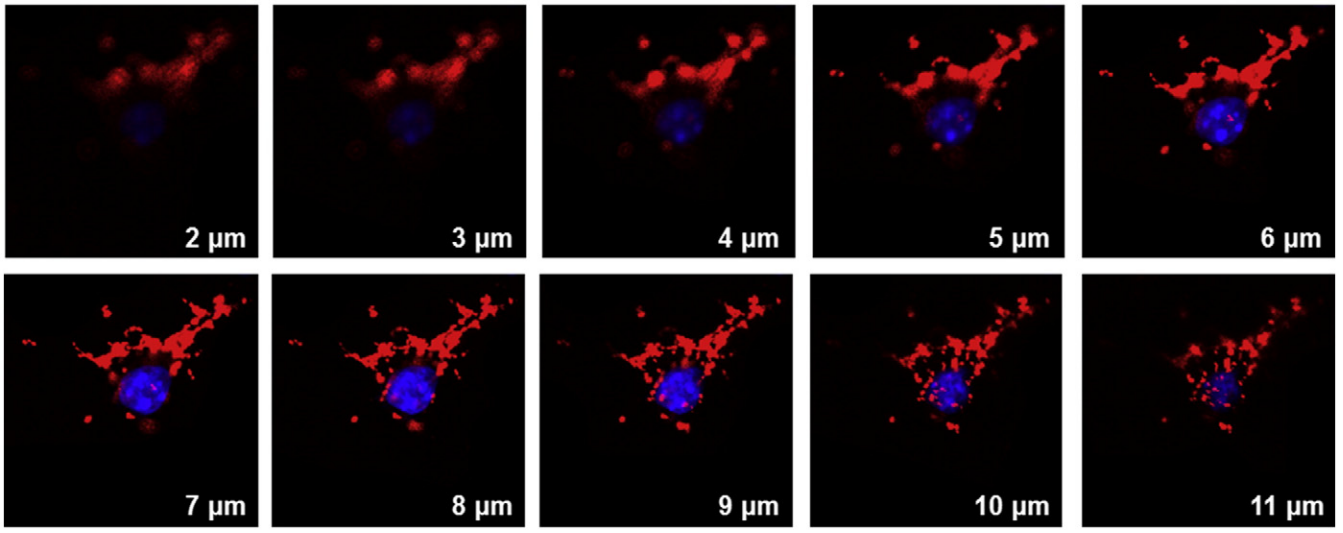

Figure 3. Confocal microscopic images of B16F1 cells incubated for 2 h with HA-QD conjugates with 22 mol% HA modification at various focal depths from 2 μm to 11 μm.

from 2 μm to 11 μm confirmed the intracellular delivery of HA-QD conjugates clearly (Figure 3).

On the basis of in vitro bioimaging, we carried out in vivo confocal microscopic analysis of HA-QD conjugates in Balb/c mice. We previously reported real-time bioimaging of HA derivatives using QDs, which confirmed the target-specific delivery of slightly modified HA-QD conjugates to the liver.[5,6] However, it was not possible to observe precisely the distribution and accumulation of HA-QD conjugates from the blood vessels to the liver tissues. Figure 4, *A* shows a photograph of the in vivo confocal microscope used to visualize the fluorescence of HA-QD conjugates in the liver tissue. Although the control of QDs mostly passed through the liver (Figures 4, *B* and *D*), HA-QD conjugates appeared to be accumulated in the liver 5 minutes after intravenous injection (Figures 4, *C* and *E*). The red fluorescence of HA-QD conjugates was concentrated in the liver tissues, but not as much in blood vessels fluorescence-labeled by the injection of dextran-FITC conjugates (Figure 4, *E*). HA-QD conjugates were thought to be uptaken to the liver cells by HA receptor-mediated endocytosis. CD44[7] and HA receptor for endocytosis (HARE)[9] have been identified as HA receptors for various biological functions in the liver. The results could be confirmed more clearly in the video provided as Supplementary Material available online at http://www.nanomedjournal.com. Recently, Ohya et al reported that HA coated micelles were target-specifically uptaken to liver sinusoidal endothelial cells by HA receptor-mediated endocytosis, whereas other particles were uptaken to Kupffer cells in the liver tissue.[10] Taken together,

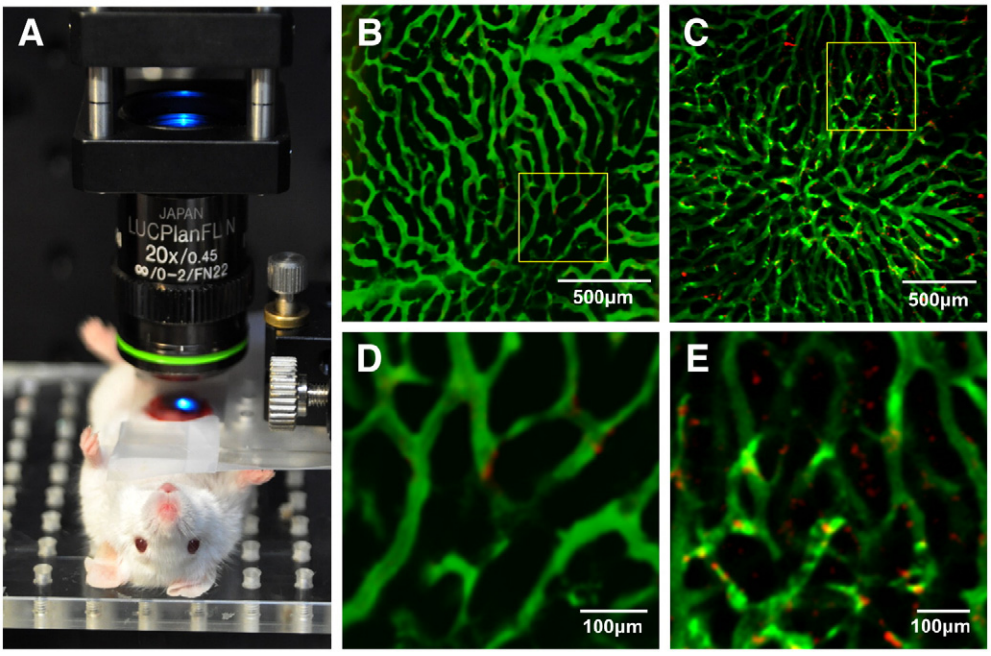

Figure 4. **(A)** A photograph of the in vivo confocal microscope. In vivo confocal microscopic images of **(B)** QDs (*red*) and **(C)** HA-QD conjugates (*red*) in the liver 5 min post injection. The blood vessels were stained green by the injection of dextran-FITC. Magnified images of the region of a yellow square **(D)** in Figure 4, *B* and **(E)** in Figure 4, *C*.

slightly modified HA derivatives might be developed as novel drug-delivery carriers for the treatment of liver diseases. In addition the in vivo real-time confocal microscopy could be successfully exploited as a new bioimaging tool for various drug-delivery applications.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2012.05.014.

References

1. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002;**296**:1869-73.
2. Jain RK, Munn LL, Fukumura D. Dissecting tumour pathophysiology using intravital microscopy. *Nat Rev Cancer* 2002;**2**:266-76.
3. Kim P, Chung E, Yamashita H, Hung KE, Mizoguchi A, Kucherlapati R, et al. In vivo wide-area cellular imaging by side-view endomicroscopy. *Nat Methods* 2010;**7**:303-5.
4. Scarcelli G, Yun SH. Confocal Brillouin microscopy for three-dimensional mechanical imaging. *Nat Photonics* 2008;**2**:39-43.
5. Oh EJ, Park KT, Kim KS, Kim JS, Yang JA, Kong JH, et al. Target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives. *J Control Release* 2010;**141**:2-12.
6. Kim KS, Hur W, Park SJ, Hong SW, Choi JE, Goh EJ, et al. Bio-imaging for targeted delivery of hyaluronic acid derivatives to the liver in cirrhotic mice using quantum dots. *ACS Nano* 2010;**4**: 3005-14.
7. Banerji S, Wright AJ, Nobel M, Mahoney DJ, Cambell ID, Day AD, et al. Structures of the CD44-hyaluronan complex provide insight into a fundamental carbohydrate-protein interaction. *Nat Struct Mol Biol* 2007;**14**:234-9.
8. Jiang G, Park K, Kim J, Kim KS, Hahn SK. Target specific intracellular delivery of siRNA/PEI-HA complex by receptor-mediated endocytosis. *Mol Pharm* 2009;**6**:727-37.
9. Weigel JA, Weigel PH. Characterization of the recombinant rat 175-kDa hyaluronan receptor for endocytosis (HARE). *J Biol Chem* 2003;**278**:42802-11.
10. Ohya Y, Takeda S, Shibata Y, Ouchi T, Kano A, Iwata T, et al. Evaluation of polyanion-coated biodegradable polymeric micelles as drug delivery vehicles. *J Control Release* 2011;**155**:104-10.