

A highly sensitive and selective viral protein detection method based on RNA oligonucleotide nanoparticle

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Abstract: Globally, approximately 170 million people (representing approximately 3% of the population worldwide), are infected with hepatitis C virus (HCV) and at risk of serious liver disease, including chronic hepatitis. We propose a new quantum dots (QDs)-supported RNA oligonucleotide approach for the specific and sensitive detection of viral protein using a biochip. This method was developed by immobilizing a HCV nonstructural protein 5B (NS5B) on the surface of a glass chip via the formation of a covalent bond between an amine protein group and a ProLinker™ glass chip. The QDs-supported RNA oligonucleotide was conjugated via an amide formation reaction from coupling of a 5'-end-amine-modified RNA oligonucleotide on the surface of QDs displaying carboxyl groups via standard EDC coupling. The QDs-conjugated RNA oligonucleotide was interacted to immobilized viral protein NS5B on the biochip. The detection is based on the variation of signal of QDs-supported RNA oligonucleotide bound on an immobilized biochip. It was demonstrated that the value of the signal has a linear relationship with concentrations of the HCV NS5B viral protein in the 1 $\mu\text{g mL}^{-1}$ to 1 ng mL^{-1} range with a detection limit of 1 ng mL^{-1} . The major advantages of this RNA-oligonucleotide nanoparticle assay are its good specificity, ease of performance, and ability to perform one-spot monitoring. The proposed method could be used as a general method of HCV detection and is expected to be applicable to other types of diseases as well.

Keywords: hepatitis C virus, viral protein, RNA oligonucleotide, quantum dots, biochip

Introduction

Hepatitis C virus (HCV) is a major human health problem worldwide which can result in acute and chronic hepatitis, cirrhosis, and/or development of hepatocellular carcinoma.^{1,2} HCV is a single-stranded positive sense RNA virus, the 9.6 kb genome of which is organized to contain a single, large translational open-reading frame that encodes a large polyprotein precursor (3010–3030 amino acids).^{3,4} The genome is replicated via a negative-stranded intermediate by the nonstructural protein 5B (NS5B), that is viral RNA-dependent RNA polymerase, a crucial and unique component of viral replication machinery.^{5–15} Because of its essential role in viral replication, HCV NS5B viral protein is regarded as a prime target for antiviral therapy.

There has been considerable interest in the development of simple and reliable methods for detection of HCV for applications in diagnostic medicine. Presently, the most widely used method for diagnosing HCV is the detection of anti-HCV antibodies using a screening enzyme-linked immunosorbent assay (ELISA), based on recombinant proteins from the genome of HCV.^{16,17} Although it is highly specific, this assay has limitations. For example, it cannot detect viruses during the early stage

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of infection, at a time when antibodies against HCV antigens are not yet produced. The ELISA method sometimes generates false-positive or false-negative results.¹⁸ In addition, antibody use is temperature-sensitive, has specific reaction conditions, and requires a secondary antibody conjugated with enzyme and fluorescent dyes.

Quantum dots (QDs), which are colloidal nanoparticles of semiconductor materials with a CdSe/ZnS core shell, have attracted considerable attention in the fields of nanotechnology and biotechnology, especially in fluorescence-based biological imaging applications.^{19–23} Semiconductor QDs possess remarkable optical characteristics compared with conventional organic fluorophores in terms of being bright, tunable, and having narrow fluorescence emission, as well as broad absorption spectra.^{24–26}

Recently, there have been many studies on the development of fluorescence-based assays for HCV RNA detection.^{27,28} Furthermore, a variety of assay methods have been developed for the qualitative and quantitative detection of HCV. Among the developed methods, biosensors based on RNA-oligonucleotide nanoparticles have attracted significant attention and are a promising method for specific detection of oligonucleotide because of their high sensitivity, low cost, rapid response, portability (compatibility for miniaturization), and low labor requirement. Several papers have been published on RNA biosensors for HCV detection,^{29,30} however, to date, little has been reported on HCV viral protein detection using QDs-supported RNA oligonucleotide. Driven by the need to detect the presence of the HCV disease, herein it was demonstrated for the first time that RNA oligonucleotide with functional signal sequence could be used for screening and quantifying HCV viral protein with selectivity and sensitivity. It is shown here that QDs-conjugated RNA oligonucleotide could be used as a probe for the detection of HCV viral protein using biochip.

Materials and methods

Materials

EDC (*N*-(3-dimethylaminopropyl)-*N*'ethylcarbodiimide hydrochloride), bovine serum albumin (BSA) and kanamycin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Quantum dots (QDs) was purchased from Invitrogen Corporation (Carlsbad, CA). The ProlinkerTM-terminated glass slide was purchased from Proteogen (Seoul, Republic of Korea). All other chemicals were of the highest grade.

Conjugation of quantum dots and RNA oligonucleotide

An amine group with a terminal modification of NS5B RNA oligonucleotide (NS5Bnor) and NS5B RNA oligonucleotide mutant (NS5Bmut) were synthesized by BIONEER Co. Ltd. (Seoul, Republic of Korea) and carboxyl-terminated QDs525 was purchased from Invitrogen (Calsbad, CA). The amino group of NS5B RNA oligonucleotide (NS5Bnor: H2N-5'-GGCCACAUGUGAGGGGCUC-3') and unspecific oligonucleotide (NS5Bmut: H2N-5'-CCCCACAUCUCACCCCCUC-3') used as a mutant of NS5B RNA oligonucleotide were first covalently conjugated onto the surface of the carboxyl-terminated QDs (10 pM, 1.25 μ L). That is, 10 pM of QDs were conjugated with 400 pM of oligonucleotide with EDC 40 nM, 1 μ L to activate amide bond formation to produce QDs-conjugated oligonucleotide (QDs-NS5B oligonucleotide) at a QDs:RNA oligonucleotide molar ratio of 1:40 for one hour at room temperature. Thereafter, QDs-oligonucleotide conjugate was collected using a centrifugal filtration at 15,000 rpm for 30 minutes, followed by several washing steps with a Tris buffer (50 mM Tris-HCl pH 7.4, 5 mM KCl, 100 mM NaCl, 1 mM MgCl₂, and 0.1% NaN₃). After centrifugal filtration and washing, the pellet of QDs-conjugated RNA oligonucleotide was dispersed by brief sonication (22 kHz, amplitude 12 μ m, and sonication time 120 seconds) using a sonic dismembrator (Model F60 Sonic Dismembrator; Fisher Scientific, Fair Lawn, NJ).

Subcloning, expression, and purification of viral protein

The gene was amplified by a PCR with the primer set, sense: 5'-CGCGAATTCATGTCCTACACATGGACAGG-3'; antisense: 5'-TTTCTCGAGTCGGTTGGGGAGCAG-GTA-3', containing restriction enzyme sites of *EcoRI/XhoI*. PCR was run with the following conditions on a thermal cycler: denaturation at 94° for one minute, annealing at 62° for 30 seconds, and extension at 72° for 2.5 minutes. The sequence was repeated 35 times followed by a seven-minute final extension step at 72°. The PCR product was digested with *EcoRI/XhoI*, and then ligated into a *EcoRI/XhoI* digested expression vector pET 28a+ (Novagen, Madison, WI), and transformed into *Escherichia coli* DH5 α (Stratagene, La Jolla, CA). The correct colony transformed with an insert gene, and was then transformed into *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA) and plated on Luria-Bertani (LB) agar containing 50 μ g mL⁻¹ kanamycin. The transformant was grown in a 250 mL flask containing 50 mL LB medium

supplemented with $50 \mu\text{g mL}^{-1}$ of kanamycin at 37° until the cell concentration reached an $\text{OD}_{600 \text{ nm}}$ of 0.6, and isopropyl-thio- β -D-galactopyranoside (IPTG) at a final concentration of 0.1 mM, followed by overnight growth at 25°C with shaking at 180 rpm. Cells were harvested by centrifugation at 4000 rpm for 30 minutes at 4°C and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by sonic dismembrator. The cell debris was removed by centrifugation at 15,000 rpm for 30 minutes. The supernatant was collected and the recombinant protein was purified using a Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen, Hilden, Germany). The supernatant was equilibrated with buffer A (10 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 8.0). The bound protein was eluted with buffer B (10 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0) at 4° . Purity of the protein was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the eluted fractions, using 10% polyacrylamide running gels.³¹ The purity of the enzyme was estimated by SDS-PAGE. The protein concentration was determined as described by Bradford³² with a BSA as standard. Enzyme samples were supplemented with 50% glycerol and stored at -20°C until use.

Electron microscopy

The free QDs and QD-conjugated RNA oligonucleotide were dried on a carbon Formvar-coated 200-mesh nickel grid, and were analyzed by transmission electron microscopy (TEM) (JEM-1400, JEOL Tokyo, Japan). The analysis of TEM was done at 80 kV.

Fluorescence microscopy

The recombinant HCV viral protein was immobilized directly onto the functional ProLinker-terminated surface. For the targeting of the specific RNA oligonucleotide, the QDs-conjugated RNA oligonucleotide was facilitated by spotting on immobilized HCV viral protein glass chip. After incubation for one hour at 25°C , the glass chip was then washed three times with the phosphate buffer (pH 7.2) for one minute. The glass chip was analyzed by a confocal laser scanning microscope LSM 510 META (Carl Zeiss, Jena, Germany). The signal intensity was determined by software for the LSM510 (LSM Image Browser). A histogram of the intensity was obtained from the region of the spotted chip. The value of the signal intensity was obtained by calculating and expressing it as the mean intensity.

Results and discussion

Schematic outline of the biochip

The biochip, a solid-phase assay method to investigate protein-RNA interaction, is becoming an attractive tool in biotechnology today for diagnostic and therapeutic purposes as well as for basic research. We designed a highly sensitive biochip using a ProteoChip coated with ProLinker, a novel calixcrown derivative with a bifunctional coupling property that permits efficient immobilization of captured viral protein on solid matrices and makes a simple analysis of protein-RNA interactions possible.³³ Specific detection of the viral protein was demonstrated by QDs-conjugated RNA oligonucleotide on the biochip. The full procedure for detection on biochip is as follows. First, the viral protein was spotted on a glass chip with carboxyl as the functional

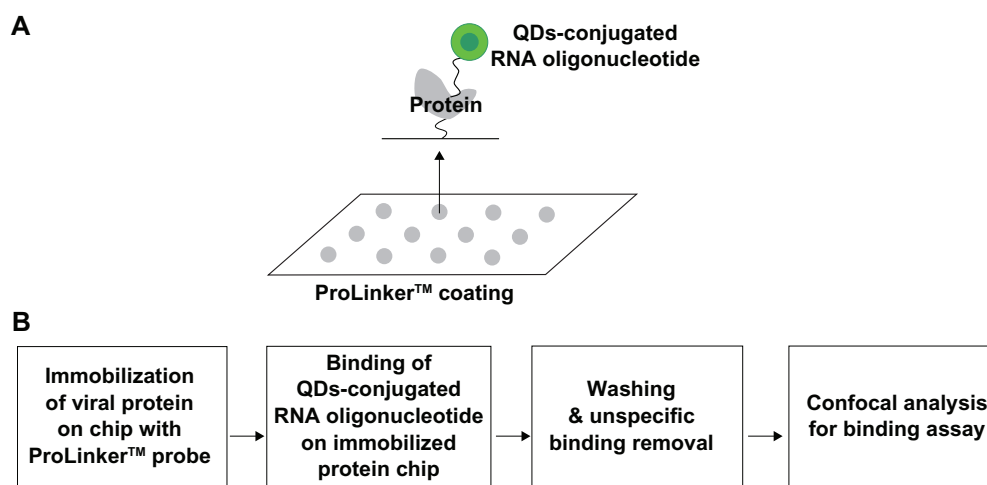


Figure 1 Representative scheme for the detection of viral protein on chip. **A)** Scheme for viral protein assay on chip. **B)** Procedure for viral protein assay.

group. Second, the QDs-conjugated RNA oligonucleotide was bound on an immobilized chip. Third, the bound chip was washed to remove nonspecific binding. Fourth, it was analyzed to identify the specific detection of viral protein on the biochip. The targeting process for effective monitoring of the viral protein on the biochip is illustrated in Figure 1. Feasibility of targeting and imaging was achieved by using QDs525 conjugates containing RNA oligonucleotide for specific NS5B viral protein.

QDs-conjugated RNA oligonucleotide

The QDs-supported RNA oligonucleotide was conjugated via amide formation from coupling of the 5'-end-amine-modified RNA oligonucleotide at the surface of QDs displaying carboxyl groups via standard EDC coupling. Formation of the QDs-conjugated RNA oligonucleotide was confirmed on 2% agarose gel at 100 V in Tris-acetate-EDTA buffer. Agarose gel electrophoresis showed a distinct mobile band pattern between the free QDs and the QDs-conjugated RNA oligonucleotide, confirming the formation of QDs-conjugated RNA oligonucleotide. The mobility shifts are compared in Figure 2. On agarose gel, both the QDs-conjugated NS5Bnor RNA oligonucleotide and the NS5Bmut oligonucleotide showed less mobility shift

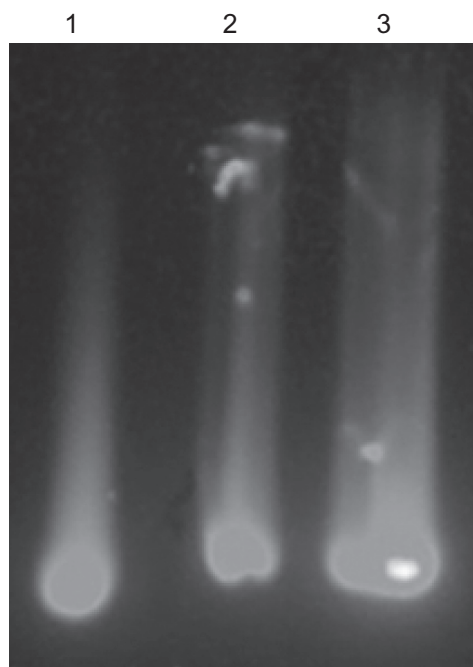


Figure 2 The conjugation pattern on 2% agarose gel electrophoresis was examined at UV excitation wavelength (345 nm), free QDs (lane 1), QDs-NS5Bnor RNA oligonucleotide (lane 2), and QDs-NS5Bmut RNA oligonucleotide (lane 3). Carboxyl-terminated QDs are conjugated with RNA oligonucleotide by an amine-carboxyl reaction.

Abbreviations: NS5B, nonstructural protein 5B; QDs, quantum dots; UV, ultraviolet.

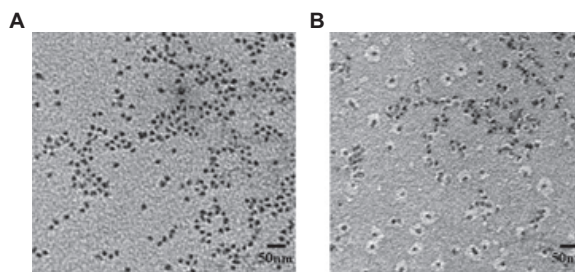


Figure 3 Characterization of QDs by TEM analysis. **A)** Free QDs. **B)** QDs-conjugated RNA oligonucleotide.

Abbreviations: QDs, quantum dots; TEM, transmission electron microscopy.

than free QDs, thereby demonstrating conjugation by amide formation between the QDs and RNA oligonucleotide.

By TEM, both the QDs and the QDs-conjugated RNA oligonucleotide appeared spherical and fairly monodispersed (Figure 3). In Figure 3A, only the free QDs nanoparticles are visible. In addition, the nanoparticles appeared to be evenly distributed on the surface. Both the QDs and the RNA oligonucleotide were conjugated in the EDC coupling reaction. The QDs (dark spot) was surrounded by a white disk of conjugated RNA oligonucleotide (Figure 3B).

Detection of viral protein on biochip

The recombinant HCV NS5B viral protein in the *E. coli* expression system was expressed and purified by Ni-NTA affinity chromatography. In the purification step, the protein was eluted in 1 mL fractions with 250 mM imidazole buffer. To verify the purity and homogeneity of the eluted NS5B, aliquots of eluted fractions were analyzed by Coomassie blue staining on SDS-PAGE. Eluates with a purity of 95% were pooled, dialyzed and stored with 50% glycerol in aliquots at -80°C . The HCV NS5B viral protein was purified by a single chromatography step on a Ni^{2+} affinity column. The C-terminally his-tagged HCV NS5B was visualized with a molecular mass of approximately 66 kDa on SDS-PAGE (Figure 4).

We demonstrated the specific interaction between QDs-conjugated RNA oligonucleotide and immobilized HCV NS5B viral protein on a chip. As illustrated in Figure 5A, the QDs-NS5Bnor RNA oligonucleotide-treated conjugates showed high fluorescent signals on the chip. In Figure 5B, no fluorescence signal was detected with a NS5Bmut RNA oligonucleotide, because of its lack of affinity. The signal of QDs-conjugated NS5Bmut RNA oligonucleotide for protein-binding affinity was very similar to the background signal. Figure 5C shows that QDs-conjugated RNA oligonucleotide is selective for HCV NS5B viral protein at varying protein concentrations. The binding affinity by confocal assay with varying concentrations of the NS5B viral protein was exam-

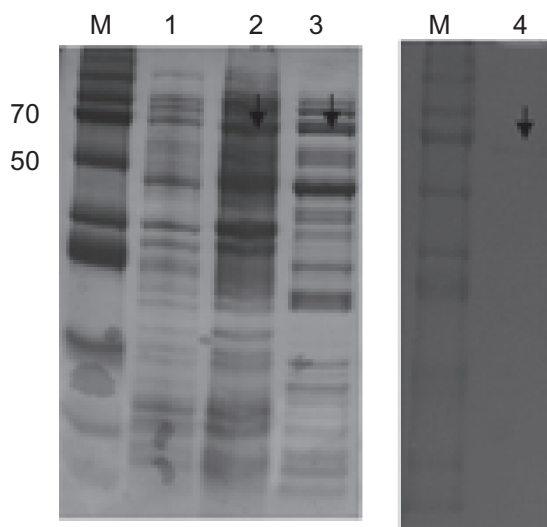


Figure 4 Purification of HCV-NS5B viral protein. The SDS-PAGE 10% gel showing NS5B protein with his-tag. M, protein marker; lane 1, before induction form of NS5B; lane 2, total form of NS5B; lane 3, soluble form of NS5B; lane 4, his-Tag form of NS5B.

Abbreviations: HCV, hepatitis C virus; NS5B, non-structural protein 5B; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

ined, and it was observed that the signal intensity of the QDs-conjugated RNA oligonucleotide could be detected even at the lowest concentration (1 ng mL^{-1}). The signal intensity was found to increase gradually by up to 1 ng mL^{-1} of protein

concentration. The insert figure in Figure 5D shows that the detection limit of this method was at the 1 ng mL^{-1} level.

These results suggest the NS5B RNA oligonucleotide could specifically target the HCV NS5B viral protein on an immobilized chip. These results show that the QDs-RNA oligonucleotide could be effective for the quantification of HCV NS5B viral protein. To confirm a competition reaction of specific binding, BSA was used as a negative control protein. No fluorescence signal was detected with BSA (data not shown). Using the QDs-RNA oligonucleotide, we developed a HCV biosensor prototype biochip that could detect and quantify the HCV NS5B viral protein. Here, the specific RNA oligonucleotide was examined for binding to the HCV NS5B viral protein by using a QDs-conjugated imaging system. HCV-specific targeting and imaging using a QDs-conjugated RNA oligonucleotide agent was demonstrated. Preferential binding of QDs-conjugated RNA oligonucleotide for detection of HCV NS5B viral protein was determined by analyzing fluorescence intensity measured by confocal microscopy. The immobilized protein chip showed no binding to NS5Bmut RNA oligonucleotide, thus demonstrating selectivity for HCV NS5B viral protein. Imaging by semiconductor nanocrystal QDs allows for monitoring with intense light emission and accuracy.

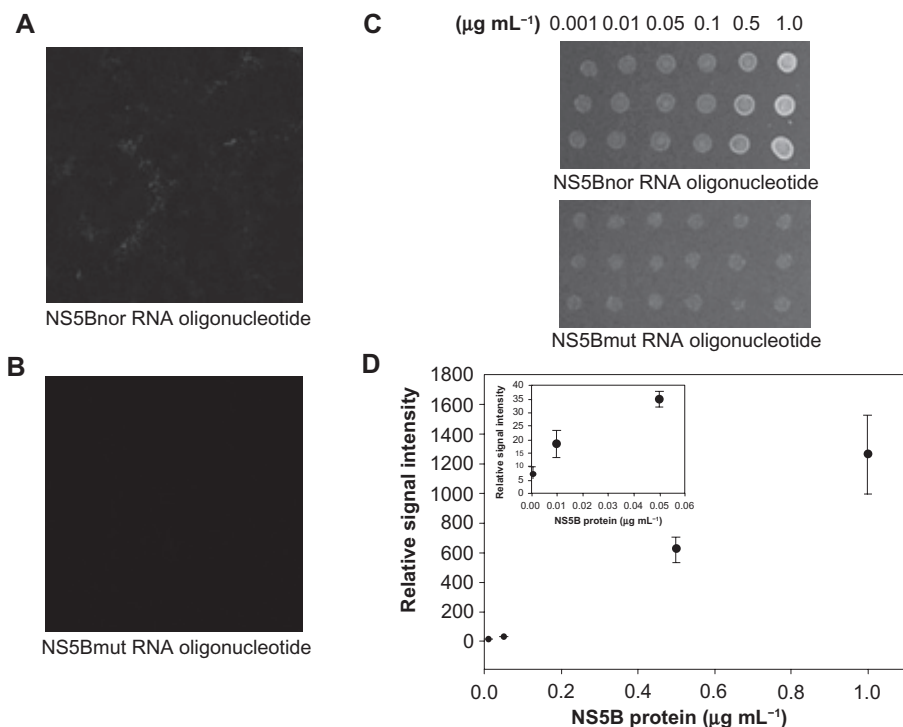


Figure 5 A, B) Selective specificity of HCV-NS5B viral protein by QDs-conjugated RNA oligonucleotide. The NS5Bnor and NS5Bmut RNA oligonucleotides were bound on an immobilized viral protein chip, respectively. **C, D)** Effect of HCV-NS5B viral protein concentrations on binding assay.

Abbreviations: HCV, hepatitis C virus; NS5B, non-structural protein 5B; NS5Bnor, normal NS5B; NS5Bmut, mutant NS5B; QDS, quantum dots.

During the last decade, there has been an increasing interest in using nanoparticles in biologic applications. The availability of colloidal semiconductor QDs with highly controlled optical properties in the nanometer size range has created widespread interest in the use of biotechnologic systems as a diagnostic platform and for biologic imaging. In this study, we report a QDs-conjugated RNA oligonucleotide probe for screening of HCV with high sensitivity and selectivity.

A RNA oligonucleotide sensor system that could quantify HCV NS5B viral protein using conjugated QDs-RNA oligonucleotide could provide an efficient strategy and a promising new platform for monitoring applications.

In summary, we have shown that QDs-RNA oligonucleotide can specifically recognize the HCV viral protein, and have demonstrated that this conjugated QDs-RNA oligonucleotide can interact on a designed biochip specifically and sensitively. This device could generate a QDs-conjugated biosensor prototype biochip for HCV diagnosis. The present visual HCV detection technique may avoid the limitations with its reported methods, namely, its high sensitivity, good specificity, simplicity, speed, and cost-effectiveness. This technique has potential applications in many fields, especially in multiple virus detection chip coupled with nanoparticle will find applications in clinic.

Conclusions

The results of this study indicate that the QDs-conjugated RNA oligonucleotide can recognize the HCV viral protein with a detection limit of 1 ng mL⁻¹ specifically and sensitively on a designed biochip. This device could generate a nanoparticle-conjugated prototype biochip for HCV diagnosis.

Acknowledgments

We thank Professor Dr Myung Heejoon and Professor Dr Seong-Wook Lee for their very kind donation of plasmid pCite. This work was supported by SNU-KAERI Degree and Research Center for Radiation Convergence Sciences from the Korea Research Council of Foundation (KRCF) Science and Technology, South Korea.

Disclosures

The authors report no conflicts of interest in this work.

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