Signal Amplification

DOI: 10.1002/anie.200803893

Mass Spectrometry Signal Amplification Method for Attomolar Detection of Antigens Using Small-Molecule-Tagged Gold Microparticles**

Jung Rok Lee, Juhee Lee, Sang Kyung Kim, Kwang Pyo Kim, Hyung Soon Park, and Woon-Seok Yeo**

Biologically functionalized surfaces are applied widely in biochemical/biological research. Many biochemical and biological events can be detected accurately and reliably on biosurfaces, including a variety of biochips and bio-(nano)materials.[1] Recently, enormous emphasis has been placed on the development of new bioanalytical methods to detect ultralow-abundancy analytes and to handle crude samples, such as blood or urine, without pretreatment. Such demands necessitate the amplification of obtained signals,[2] for example, the polymerase chain reaction (PCR) in the case of oligonucleotide targets and label-free detection formats for observing intact analytes.[3] Nam et al. reported an ultrasensitive protein detection method in which antibodies on microparticles and nanoparticles captured target proteins selectively.[1f,4] The large excess of oligonucleotides on the nanoparticle was applied to the detection of target proteins, and additional PCR provided increased sensitivity to concentrations of several attomoles. Zhao et al. described femtomolar detection of DNA utilizing dye-doped silica nanoparticles, which entrapped several fluorophores and therefore enhanced the fluorescent signals resulting from DNA-binding events on the DNA chip.[5]

The use of mass spectrometry (MS) as a readout system has come into the spotlight recently because it provides several advantages over current detection tools. Most importantly, MS affords the molecular weight—the intrinsic property of analytes—and hence enables the use of labels for target identification to be avoided. However, MS has limitations for ultrasensitive detection because its sensitivity depends directly on the amount of analyte. For this reason, there has been a strong request for the development of amplification methods for MS signals.

Herein, we report a new signal amplification strategy that allows attomolar detection of target proteins in solution, without additional amplification or target-labeling steps, by combining self-assembled monolayers (SAMs) on gold with laser desorption/ionization time-of-flight (LDI-TOF) MS with a matrix-free format. We utilize a small molecule as a reporter of the target protein in this signal amplification strategy (Figure 1). The target protein is captured specifically by ligands or antibodies that are present on the gold microparticles and the biochip. A gold microparticle carries small molecules, which exist in large excess over capture proteins on the gold particle. After the specific capture of target proteins, the small molecules on a gold particle are subsequently analyzed by LDI-TOF MS. In this way, the biological event can be transduced to a highly amplified mass signal by observing a large number of small molecules instead of target proteins. We term this small molecule an amplification tag (Am-tag), because the molecule is tagged on a gold particle and plays a role in amplifying the biological event.

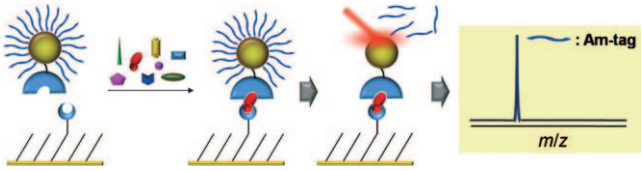


Figure 1. Signal amplification strategy for the detection of analytes by using Am-tag and LDI-TOF MS.

Our approach utilizes SAMs on gold particles of size 2 μm that possess capture proteins or antibodies among triethylene glycol moieties. The triethylene glycol groups play a dual role in our strategy. First, they ensure that the modified surfaces are inert to nonspecific protein adsorption, and therefore

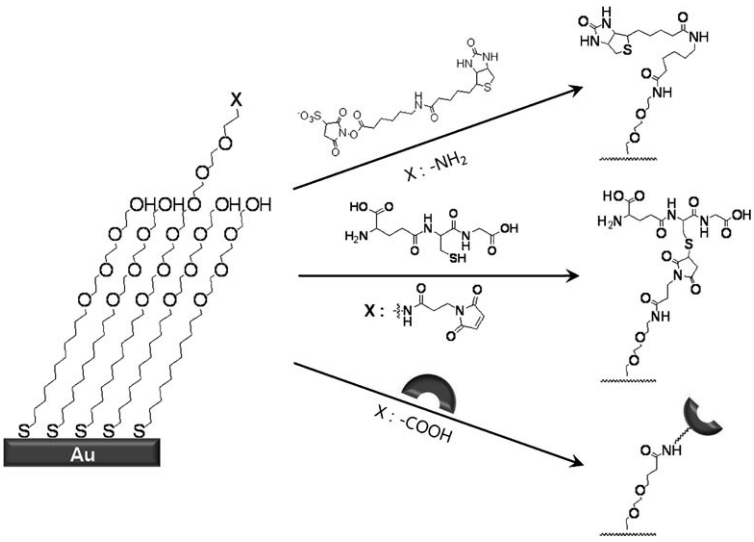
[*] Dr. H. S. Park
Probiond Co., Ltd., Gwanjin VBS Center
Seoul 143-834 (Korea)
Fax: (+82) 2-455-0089
E-mail: phsoonah@probiond.com
J. Lee,[+] Prof. Dr. W.-S. Yeo
Department of Bioscience and Biotechnology, Konkuk University
Seoul 143-701 (Korea)
Fax: (+82)-2-2030-7890
E-mail: wsyeo@konkuk.ac.kr
J. R. Lee,[+] Prof. Dr. K. P. Kim
Institute of Biomedical Science and Technology
Department of Molecular Biotechnology, Konkuk University
Seoul 143-701 (Korea)
Dr. S. K. Kim
Nano-Bio Research Center, Korea Institute of Science and Technology
Seoul 136-791 (Korea)
[+] These authors contributed equally to this work.

[**] The MS measurements were carried out at the Institute of Bioscience and Technology at Konkuk University. This work was supported by a Korea Research Foundation grant funded by the Korean Government (MOEHRD; KRF-2007-331-C00175) and by the Intelligent Microsystem Center, which carries out one of the 21st century's Frontier R&D Projects sponsored by the Korea Ministry of Knowledge Economy.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200803893.

reduce false-positive signals substantially.[6] Second, as depicted in Figure 1, they incorporate a function of Am-tag to amplify biological events, such as protein–ligand and protein–protein interactions, because they are present in large excess over capture proteins on a gold particle. In addition, the small Am-tag molecule can induce a much stronger mass signal than target proteins or biological macromolecules, which are often difficult to analyze by MS because of their size, even if they exist at high concentrations.

The use of microparticles over nanoparticles is advantageous in our strategy for the amplification of biological signals, as the colloidal stability and feasibility of surface modifications are sometimes important issues in the use of nanoparticles.[7] Furthermore, a single microparticle has a much higher loading capacity for Am-tags, which induces greater amplified signals than a nanoparticle alone. Scanning electron microscopy revealed that the size of the gold particles was uniform with an average diameter of 2 μm (Figure S1 in the Supporting Information).



Scheme 1. Structure of SAMs on gold and chemical modifications used to prepare functionalized surfaces. Biotin and glutathione were immobilized on the monolayers presenting amine and maleimide functional groups, respectively, to capture NeutrAvidin or GST. Proteins were immobilized on acid-presenting gold particles by using carbodiimide as a coupling reagent.

Scheme 1 shows the structures and chemical modifications of the monolayers used. The monolayers were prepared from a mixed solution of triethylene glycol-terminated alkanethiols and functional-group-terminated alkanethiols. Biotin and glutathione were immobilized through well-known conjugation reactions on the monolayers presenting amine and maleimide functional groups, respectively. The amine-presenting monolayers were treated with *N*-hydroxysulfosuccinimide (sulfo-NHS)-modified biotin (50 mM in pH 7.4 phosphate-buffered saline (PBS)) and the maleimide-presenting monolayers[8] were treated with glutathione (50 mM in pH 7.5 Tris buffer). The matrix-assisted LDI (MALDI)-TOF MS analyses of these monolayers with 2,5-dihydroxybenzoic acid (DHB) as a matrix confirmed that the reactions proceeded with high yield (Figure S2 in the Supporting Information).[9] For the incorporation of proteins onto gold particles, the amide coupling reaction with carbodiimide was used. Carboxylic acid groups on gold particles were activated with NHS (5 mg mL^{-1} in CH_2Cl_2) by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 20 mg mL^{-1} in CH_2Cl_2). NeutrAvidin or glutathione *S*-transferase (GST; 50 μM in pH 7.4 PBS) was then incubated with the resulting gold particles to obtain the proteins immobilized on gold particles.

To test the fidelity of our strategy for amplifying biological signals, we first examined protein–ligand interactions: NeutrAvidin binding to the biotin ligand and GST binding to the glutathione ligand. We prepared ligand-presenting monolayers with various surface densities ranging from 10^{-7} to 10%. First, we directly analyzed specifically bound proteins by MALDI-TOF MS with a sinapinic acid matrix (5 mg mL^{-1} in 50% aqueous acetonitrile). Biotin-presenting monolayers were treated with NeutrAvidin (50 μM in pH 7.4 PBS) and subsequently analyzed by MS. The monolayers with biotin density above 1% clearly gave a mass peak at *m*/*z* 14.6 kDa corresponding to NeutrAvidin, whereas no distinctive peaks were observed for the monolayers with biotin density below 0.01% (Figure 2a). In the case of GST (20 μM in pH 7.5 Tris buffer, 1 mM dithiothreitol) interaction, specifically bound GST was observed on the monolayers with glutathione

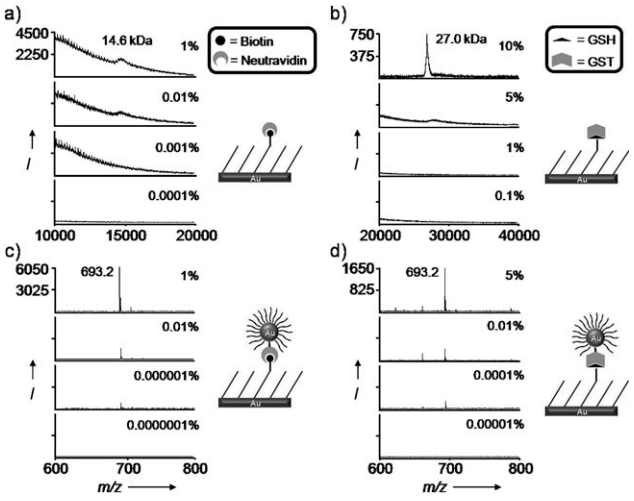


Figure 2. Amplifications of biological signals: protein–ligand interactions. a) Monolayers presenting various densities of biotin were treated with NeutrAvidin and analyzed by MS. A major peak corresponding to NeutrAvidin at *m*/*z* 14.6 kDa was observed for monolayers with a biotin density above 1%. b) Specifically bound GST was observed on monolayers with a glutathione density above 10%, as shown by a mass peak at *m*/*z* 27.0 kDa. c,d) Am-tag-containing gold particles were used to amplify biological events. An Am-tag peak at *m*/*z* 693.2 was observed on monolayers with a biotin density as low as 10^{-6}% for NeutrAvidin detection, and on monolayers with a glutathione density as low as 10^{-4}% for GST detection. Note that the spectra in (a) and (b) were obtained by using sinapinic acid as a matrix, whereas those in (c) and (d) were obtained without the use of a matrix.

density above 10%, as revealed by a mass peak at m/z 27.0 kDa corresponding to GST (Figure 2b).

We repeated this experiment with Am-tag-containing gold particles on which NeutrAvidin or GST was immobilized. We observed the Am-tag peak at m/z 693.2, which corresponded to triethylene glycol-terminated disulfide on monolayers with a biotin density as low as 10^{-6}% for NeutrAvidin detection and on monolayers with a glutathione density as low as 10^{-4}% for GST detection (Figure 2c and d, respectively).[10] These results indicate that biological signals of protein–ligand interactions were transduced to highly amplified mass signals by a factor of about 10^5. As a control, a biotin-presenting monolayer with a density of 0.01% was treated with Am-tag/myoglobin-modified gold particles, and an amine-presenting monolayer with a density of 0.01% was treated with Am-tag/NeutrAvidin-modified gold particles. The mass analyses showed no major peaks, thus indicating that the Am-tag signals of these experiments stemmed from specific protein–ligand interactions (Figure S3 in the Supporting Information).

Intriguingly, we found that mass analysis of Am-tag-modified gold particles gave a mass peak more clearly without the use of a matrix. Hence, we carried out all signal amplification experiments with the matrix-free format.[11] To verify that the Am-tag peak at m/z 693.2 shown in Figure 2 came from gold microparticles with the matrix-free format and not from the ligand-presenting monolayers, two control experiments were performed (Figure S4 in the Supporting Information). First, we prepared a monolayer modified with triethylene glycol-terminated alkanethiol, which was analyzed by MS without a matrix under the same experimental conditions as the gold particle analysis. The mass spectrum of the monolayer showed no peak at m/z 693.2. Next, an identical monolayer was treated with gold particles that were modified by tetraethylene glycol-terminated alkanethiol. Mass analysis of the monolayer without a matrix gave a major peak of only m/z 780.6, which corresponded to a tetraethylene glycol-terminated disulfide constituent of the gold microparticle surface. These results implied that the Am-tag signals represented amplified biological signals. Furthermore, signal amplification by Am-tag gold particles avoids the use of a matrix and thereby allows for simpler protocols.

Finally, our strategy was applied to the detection of analytes in solution with ultrahigh sensitivity. We demonstrated this application with specific interactions between antigens and antibodies. As targets, adiponectin (Acrp30, apM1, a possible therapeutic target for type 2 diabetes and obesity)[12] and alpha-fetoprotein (AFP, a biomarker for several cancers, such as testicular cancer and ovarian cancer)[13] were tested. To detect target antigens, two monoclonal antibodies (mAbs) were used for adiponectin, and a monoclonal antibody and a polyclonal antibody (pAb) were used for AFP. Antibodies were immobilized on acid-presenting monolayers and gold particles through amide coupling reactions with NHS and EDC as described above. The mAb-presenting monolayers were exposed to antigens at concentrations ranging from 1 pM to 1 aM, followed by Am-tag gold particles that were conjugated with mAbs for adiponectin and pAbs for AFP. Figure 3 shows the representative mass spectra

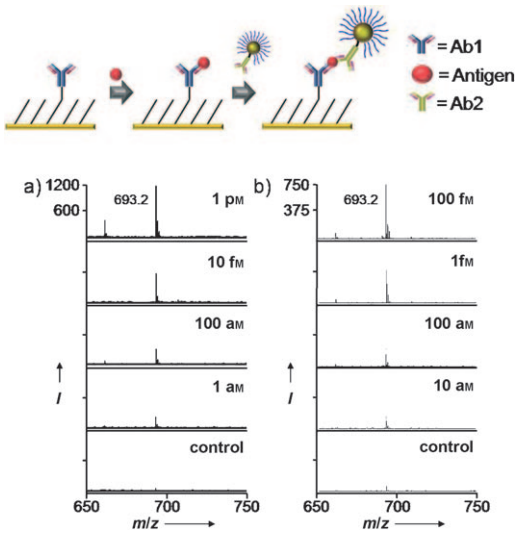


Figure 3. Amplifications of biological signals: protein–protein interactions and detection of antigens in solution by using gold particles decorated with Am-tag/Abs. a) mAb-presenting monolayers were exposed to adiponectin at concentrations ranging from 1 pM to 1 aM, followed by Am-tag gold particles that present another mAb. LDI-TOF MS analysis showed that Am-tag peaks can be observed at concentrations as low as 1 aM. b) By using the same approach with mAb on monolayers and pAb on gold particles, AFP can be detected at concentrations as low as 10 aM.

of LDI-TOF analyses without the use of a matrix. Note that Am-tag peaks at m/z 693.2 were observed at concentrations as low as 1 aM for adiponectin and 10 aM for AFP. As a control experiment, the mAb-presenting monolayers were treated with Am-tag/mAb gold particles without antigen treatment. As expected, the mass analysis gave only trace Am-tag peaks.[14] These results strongly indicate that our strategy demonstrated ultrahigh sensitivity to the detection of antigens in the range of 1–10 aM, which, to the best of our knowledge, is one of the best sensitivities to date.[15]

The use of MS as a detection tool is now routine.[9c,16] MS enables label-free assays and multiplexing analysis for multiple analytes because it provides the molecular weight, the intrinsic property of analytes. In addition, by observing molecular-ion peaks, the signals of analytes can be readily discriminated from background species. In particular, MALDI-TOF MS is well suited to the analysis of SAMs on gold. Mrksich et al. described SAMs for MALDI (SAMDI) MS to observe proteins that were specifically bound to the monolayers through protein–ligand or protein–protein interactions.[17] However, the sensitivity of this method depends directly on the amount of protein bound on the monolayers, which hampers the detection of low-abundance analytes.

The work described herein provides a new analytical tool for the detection of low-abundance analytes in solution with ultrahigh sensitivity in the attomolar range, without additional amplification or target-labeling steps. In addition, all experiments are performed without the use of a matrix, thus facilitating the analysis process. This work might be extended to multiplexing formats in detecting multiple analytes in crude samples. We are expanding our strategy to other

binding pairs, such as small-molecule capture agents, DNA/RNA, and aptamers. We believe this method can be used as an alternative to existing tools, such as ELISA, and will find applications in disease therapeutics and tissue imaging by MS.

Experimental Section

Monolayers were prepared according to standard procedures.[18] Briefly, gold-coated silicon wafers were immersed in a solution of functional-group-terminated alkanethiol and a triethylene glycol-terminated alkanethiol in ethanol, in a ratio ranging from 5:95 to 0.0000001:99.9999999, for 12 h (the total concentration of thiol was 1 mM.). The monolayers were rinsed with copious amounts of ethanol and dried under a stream of nitrogen. Biotin- or glutathione-presenting monolayers were prepared by immersing amine- or maleimide-presenting monolayers in 50 mM sulfosuccinimidyl-6-(biotinamido)hexanoate or glutathione solutions, respectively, for 2 h. The ligand-presenting monolayers were washed with ethanol and stored at 4 °C.

Mass analysis was performed with an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) with a Smartbeam laser as ionization source. All of the spectra were acquired with an accelerating voltage of 19 kV, a 50 Hz repetition rate, and positive mode with an average of about 500 shots. To confirm the reaction progression, monolayers were analyzed with DHB (5 mg mL^{-1} in acetonitrile) as a matrix. Direct protein observation was performed with sinapinic acid (5 mg mL^{-1} in 50% aqueous acetonitrile) as a matrix.

Received: August 7, 2008
Published online: October 29, 2008

Keywords: gold · mass spectrometry · monolayers · proteins · signal amplification

[1] a) R. Blonder, E. Katz, I. Willner, V. Wray, A. F. Bückmann, *J. Am. Chem. Soc.* **1997**, *119*, 11747; b) T. P. Russell, *Science* **2002**, *297*, 964; c) N. Nath, A. Chilkoti, *J. Am. Chem. Soc.* **2001**, *123*, 8197; d) R. J. Thibault, P. J. Hotchkiss, M. Gray, V. M. Rotello, *J. Am. Chem. Soc.* **2003**, *125*, 11249; e) L. Quinti, R. Weissleder, C. H. Tung, *Nano Lett.* **2006**, *6*, 488; f) D. G. Georganopoulou, L. Chang, J.-M. Nam, C. S. Thaxton, E. J. Mufson, W. L. Klein, C. A. Mirkin, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 2273; g) J. H. Ng, L. L. Ilag, *Biotechnol. Annu. Rev.* **2003**, *9*, 1; h) T. Kodadek, *Trends Biochem. Sci.* **2002**, *27*, 295.

[2] a) T. Sano, C. L. Smith, C. R. Cantor, *Science* **1992**, *258*, 120; b) B. Schweitzer et al., *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10113.

[3] a) W. D. Wilson, *Science* **2002**, *295*, 2103; b) N. Nath, A. Chilkoti, *J. Fluoresc.* **2004**, *14*, 377; c) S. R. Nicewarner-Peña et al., *Science* **2001**, *294*, 137; d) G. Zheng, F. Patolsky, Y. Cui, W. U. Wang, C. M. Lieber, *Nat. Biotechnol.* **2005**, *23*, 129; e) Y. L. Bunimovich, Y. S. Shin, W.-S. Yeo, M. Amori, G. Kwong, J. R. Heath, *J. Am. Chem. Soc.* **2006**, *128*, 16323.

[4] J.-M. Nam, C. S. Thaxton, C. A. Mirkin, *Science* **2003**, *301*, 1884.

[5] X. Zhao, R. Tapec-Dytioco, W. Tan, *J. Am. Chem. Soc.* **2003**, *125*, 11474.

[6] a) K. L. Prime, G. M. Whitesides, *Science* **1991**, *252*, 1164; b) M. Mrksich, G. M. Whitesides, *ACS Symp. Ser.* **1997**, *680*, 361.

[7] C. S. Weisbecker, M. V. Merritt, G. M. Whitesides, *Langmuir* **1996**, *12*, 3763.

[8] B. T. Houseman, E. S. Gawalt, M. Mrksich, *Langmuir* **2003**, *19*, 1522.

[9] Sodium adducts of disulfides are the major species that are observed in MALDI spectra of SAMs of alkanethiolates; see: a) J. Su, M. Mrksich, *Angew. Chem.* **2002**, *114*, 4909; *Angew. Chem. Int. Ed.* **2002**, *41*, 4715; b) J. L. Trevor, K. R. Lykke, M. J. Pellin, L. Hanley, *Langmuir* **1998**, *14*, 1664; c) M. Mrksich, *ACS Nano* **2008**, *2*, 7.

[10] The peak at *m*/*z* 661.1 in Figures 2d and 3 corresponds to the molecular ion $[M-S+Na]^+$, which is often observed in the MALDI-TOF analysis of SAMs on gold and results from the abstraction of one sulfur atom.

[11] The mechanism of desorption and ionization of Am-tag on gold microparticles without a matrix is not yet clear. We are currently performing experiments with several kinds of gold microparticles to find an explanation for this phenomenon, based on the assumption that microscopic characteristics of the gold particle surface may account for energy transfer. For more discussion, see: J. A. McLean, K. A. Stumpo, D. H. Russell, *J. Am. Chem. Soc.* **2005**, *127*, 5304.

[12] C. Hug, H. F. Lodish, *Curr. Opin. Pharmacol.* **2005**, *5*, 129.

[13] G. J. Mizejewski, *Expert Rev. Anticancer Ther.* **2002**, *2*, 709.

[14] Absolute intensities three times larger than that of the control were assigned as specific binding.

[15] D. A. Healy, C. J. Hayes, P. Leonard, L. McKenna, R. O'Kennedy, *Trends Biotechnol.* **2007**, *25*, 125.

[16] a) J. Y. Engwegen, M. C. Gast, J. H. Schellens, J. H. Beijnen, *Trends Pharmacol. Sci.* **2006**, *27*, 251; b) D.-H. Min, W. J. Tang, M. Mrksich, *Nat. Biotechnol.* **2004**, *22*, 717; c) Y.-P. Kim, E. Oh, Y.-H. Oh, D. W. Moon, T. G. Lee, H.-S. Kim, *Angew. Chem.* **2007**, *119*, 6940; *Angew. Chem. Int. Ed.* **2007**, *46*, 6816; d) J. Wei, J. M. Buriak, G. Siuzdak, *Nature* **1999**, *399*, 243; e) T. R. Northen, O. Yanes, M. T. Northen, D. Marrinucci et al., *Nature* **2007**, *449*, 1033; f) J. Sunner, E. Dratz, Y. C. Chen, *Anal. Chem.* **1995**, *67*, 4335; g) W. Y. Chen, Y. C. Chen, *Anal. Bioanal. Chem.* **2006**, *386*, 699; h) D. Nedelkov, R. W. Nelson, *Trends Biotechnol.* **2003**, *21*, 301.

[17] a) W.-S. Yeo, D.-H. Min, R. W. Hsieh, G. L. Greene, M. Mrksich, *Angew. Chem.* **2005**, *117*, 5616; *Angew. Chem. Int. Ed.* **2005**, *44*, 5480; b) V. L. Marin, T. H. Bayburt, S. G. Silgar, M. Mrksich, *Angew. Chem.* **2007**, *119*, 8952; *Angew. Chem. Int. Ed.* **2007**, *46*, 8796; c) S. M. Patrie, M. Mrksich, *Anal. Chem.* **2007**, *79*, 5878.

[18] C. D. Bain, E. B. Troughton, Y. T. Tao, J. Evall, G. M. Whitesides, R. G. Nuzzo, *J. Am. Chem. Soc.* **1989**, *111*, 321.

