ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Pancreatitis-Associated Extracellular Vesicle Identification through an Allosteric Probe-Initiated Cascade Amplification System

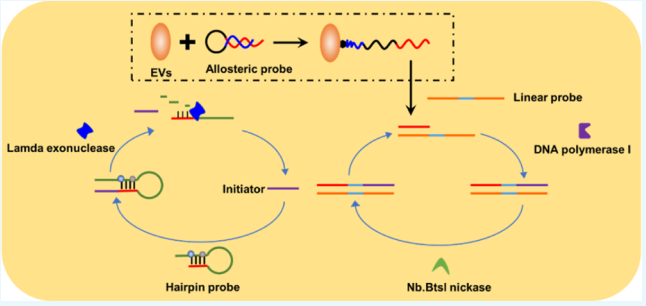
Yuan Zhou,[§] Feng Yu,[§] Heng Cheng, and Linhong Ning*

Cite This: *ACS Omega* 2021, 6, 17776−17781

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: As an emerging class of noninvasive biomarkers, accurate quantification of circulating extracellular vesicles (EVs) is essential to both the basic biological research and early diagnosis of pancreatitis. We report here an allosteric probe-initiated cascade amplification system for a highly sensitive detection of serum-circulating EVs. In this method, the special recognition of EVs by the allosteric probes triggers allosterism of the probe and thus induces the release of a signal amplification initiator. Through the following multiple rounds of cascade amplifications, a large number of fluorescence moiety are released, generating an enhanced fluorescence signal. This method exhibits a large dynamic range of 5 orders of magnitude. In addition, this strategy could also be performed under isothermal conditions in a wash-free way, indicating its potential applications in early diagnosis and prognosis of pancreatitis.



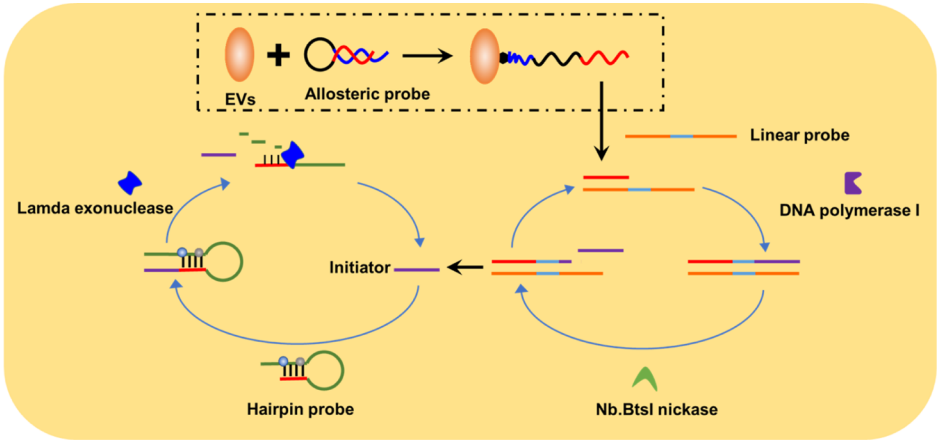
INTRODUCTION

Extracellular vesicles (EVs) are a kind of small-membrane vesicles secreted from the parent cell with the diameter ranging from 40 to 1000 nm.[1−3] EVs constitute a totally novel but significant signal communication paradigm between parent cells and target cells through the carried diverse cargos including RNA species (including mRNA, miRNA, lncRNA, and other RNA species), DNAs (mtDNA, ssDNA, and dsDNA), and proteins.[4,5] The transport of complicated cargos in EVs to the recipient cells would elicit pleiotropic response in the recipient cells and thus maintain the normal and pathophysiological conditions of cells.[6] Therefore, EVs play a pivotal role in diverse biological processes, including reproduction, embryonic development, tissue repair, bone calcification, and the nervous system. Among all the related diseases, acute pancreatitis attracted abundant attention due to the gradually increased incidence and significant morbidity and mortality.[7,8] Herein, a comprehensive understanding of the relations between the amounts of EVs and related pancreatitis will provide references for the early diagnosis and therapeutic of pancreatitis.

Because the EVs have the characteristics of complicated diameter range, origination from diverse cells, and low abundance of certain subtypes, accurate quantification of EVs from clinical or experimental samples combined with the comparable analysis performance with standardized methods remains challenging.[9−12] Conventional EV detection methods include ELISA, western blot, microarrays, and nanoparticle tracking analysis (NTA). However, these methods are criticized for some of their intrinsic shortcomings, such as the complicated data analysis process in microarrays, low repeatability between batches, high labor, and cost requirements in western blot, and especially unattainable machine in NTA. In recent years, a variety of alternative methods have been reported including colorimetric, luminescent, electrochemical, and Raman scattering spectroscopic measurements, but still with the shortcomings of complicated compound synthesis, short luminescence lifetime, cumbrous electrode preparations, and high interference.[13−15] Fluorescent assay integrated with various signal amplification approaches have been developed for quantitative EV analysis. For example, Zhao et al proposed a CRISPR-Cas12a-based exosome detection method with a carefully designed capture probe for signal conversion.[16] Benefiting from the trans-cleavage activity of the Cas12a enzyme, the method exhibited a sensitive detection performance. In addition, they also developed an allosteric probe-based wash-free method and successfully applied it for sensitive EV detection. The dual cycle-assisted CRISPR-Cas12a in the method provided a significantly enhanced sensitivity for EV detection.[17] Even though these methods made certain progresses to the conventional EV detection strategies, the complicated primer design, multiple enzyme requirements, and multiple steps made these methods inappropriate for further clinical practices.

Received: December 30, 2020
Accepted: February 19, 2021
Published: July 6, 2021

Scheme 1. Principle of the Proposed Allosteric Probe-Initiated Cascade Amplification System for EV Identification



Herein, we exhibited here a novel allosteric probe-initiated cascade amplification system for accurate and highly sensitive pancreatitis-related EV detection. In this method, an allosteric probe is designed with an integrated aptamer of EV membranal proteins and signal amplification initiators. The special recognition of EVs by the allosteric probes triggered allosterism of the probe and thus induced the release of a signal amplification initiator. The successful hybridization of the signal amplification initiator with the linear probe may successively induce the following two-cycle strand displacement amplification (SDA) processes and eventually generate fluorescence signals due to divorce of fluorescence moiety and the corresponding quenching moiety in the process of fluorescence probe cleavage by the lamda exonuclease. After a series of experimental verification, the proposed method exhibited a favorable sensitivity and selectivity for EV quantification and showed a correlation with pancreatitis. Furthermore, the method could be applied for EV identification in a wash-free manner, which would be potentially applied for point-of-care cancer diagnosis.

RESULT AND DISCUSSION

Principle of the Proposed Strategy. The principle of the allosteric probe-initiated cascade amplification system for highly sensitive and wash-free circulating EV identification assay is illustrated in Scheme 1. The whole sensing system is composed of two components: an allosteric probe for EV membrane protein recognition and initiation of the subsequent signal amplification and a two-cycle SDA process for signal enrichments and readout. Inspired by a former report by Zhao et al., the allosteric probe is designed with two parts: (i) a CD63 aptamer for special recognition of membrane proteins and (ii) an amplification initiator to induce the following signal amplification; before the recognition of target EVs, the allosteric probe is locked and does not initiate the signal amplification process. Therefore, the allosteric probe is designed with a hairpin structure through two complementary sections in its two terminals. After the recognition of CD63 protein in target EVs, the hairpin structure of the allosteric probe changed and thus a signal amplification initiator was released.

In the signal amplification process, we have designed a linear probe and a hairpin structure probe. In detail, the linear structure probe is composed of three components: a signal amplification initiator binding section, Nb.BtsI nicking enzyme recognition section, and the second circle initiator. Through the integrated three components, the linear probe could continuously generate the second circle initiator as the polymerization template for SDA. In the SDA process, the hairpin structure probe is designed with two functional parts: a fluorophore generator and the amplification primer. As illustrated in Scheme 1, the upper strand of the totally complementary section in the probe is labeled with a fluorescence moiety and quenching moiety about 9 and 14 bases away from the loop section and the down strand was designed as a complement with a signal amplification initiator in allosteric probes. In order to avoid the possible digestion and interferences, the terminal of the hairpin structure probe is labeled with 3′-NH_2 and 5′-phosphoryl (PO_4)-end.

When target EVs existed, the hairpin structure of allosteric probe changed after recognizing the target EVs through the CD63 aptamer section. As a result, the signal amplification initiator in the probe was exposed and could then partly hybridize with the linear probe. With the signal amplification initiator as the primer and linear probe as the template, the polymerization reaction was initiated to generate double-strand DNA (dsDNA) under the assistance of DNA polymerase I. Afterward, a nicking site was generated in the newly hybridized strand with the assistance of Nb.BtsI enzyme, and thereby, the second circle initiator was released from the dsDNA with the assistance of DNA polymerase I (cycle I).

When hairpin structure probes were added, the second circle initiator could then hybridize with the complementary section through the interaction with the protruding 5′-PO_4-end, and the lambda exonuclease substrate was formed. On this basis, the resultant probe could be digested by lambda exonuclease, leading to the simultaneous release of fluorescence moiety molecules and inducing the simultaneously release of the second circle initiator. Therefore, the released second circle initiator could hybridize with hairpin structure probes and thus form the second cycle. Through the dual cycles of the amplification process, the second circle initiator was generated and a greatly enhanced fluorescence was obtained in response to the circulating EVs.

Validation of Circulating EV Assay. Before all the experiments, we first extracted EVs from a cell culture supernatant through differential centrifugation and characterized the morphology of the extracted EVs through transmission electron microscopy (TEM) and size distribution through NTA. From the result given in Figure 1a, the extracted

https://doi.org/10.1021/acsomega.0c06334
ACS Omega 2021, 6, 17776−17781

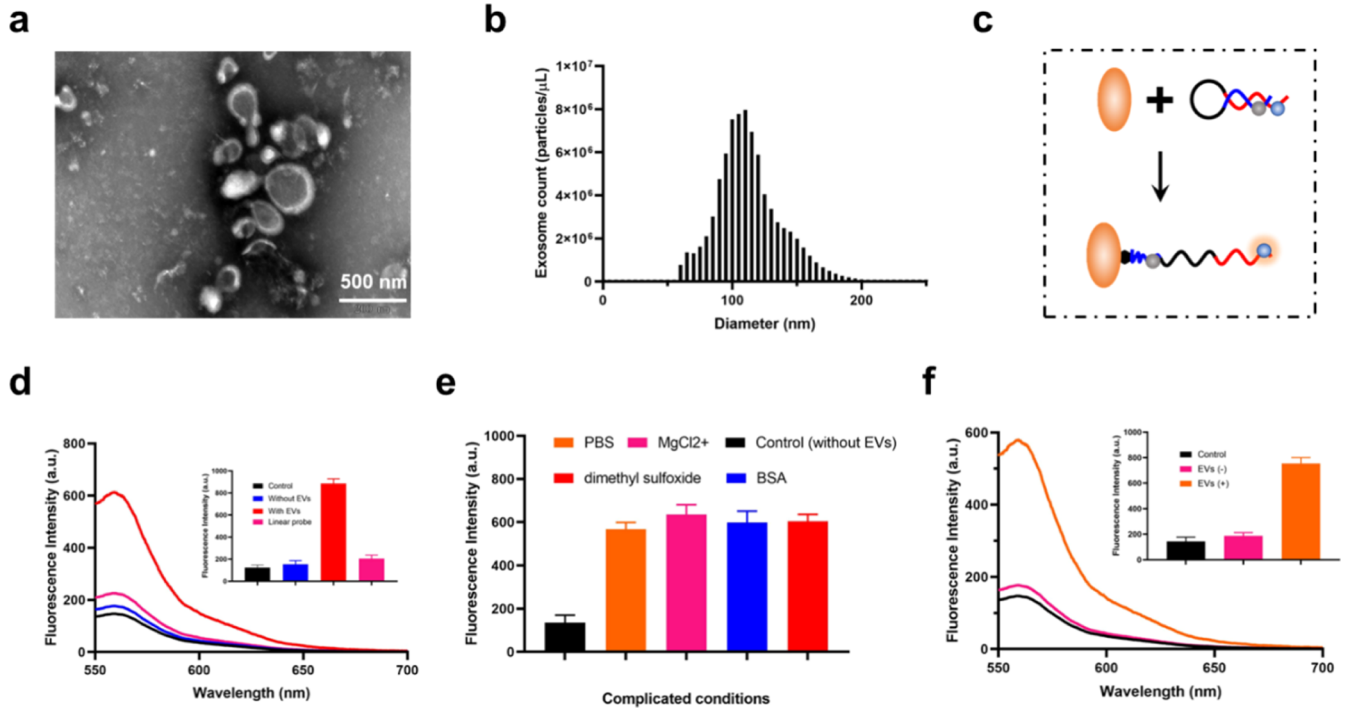


Figure 1. Validation of the proposed allosteric probe-initiated cascade amplification system for circulating EV identification. (a) TEM result of extracted EVs. (b) NTA result of the extracted EVs. (c) Illustration of the fluorescence assay to investigate to recognition of EVs by probes. (d) Fluorescence spectrum of the allosteric probes incubated in the presence or absence of EVs. The inset shows a histogram of the obtained fluorescence intensity. (e) Fluorescence intensity of the allosteric probes incubated in the presence or absence of EVs in complicated conditions, including PBS, BSA, $MgCl^{2+}$, and dimethyl sulfoxide. (f) Fluorescence spectrum of the allosteric probe-initiated cascade amplification system in the presence or absence of circulating EVs.

EVs exhibited a morphology of a sphere with double concave. The size of the extracted EVs in the TEM result is about 120 nm, which is included in size distribution of exosomes. The NTA result demonstrated that the diameter distribution of extracted EVs ranges from 50 to 250 nm (Figure 1b).

To study whether the designed allosteric probe could specially recognize the target EVs and thus lead to the exposure of the signal amplification initiator, a fluorescence assay was performed, as illustrated in Figure 1c. As shown in Figure 1d, the obtained fluorescence intensity at 570 nm of allosteric probes incubated with target EVs was significantly higher than that of probes without target EVs, suggesting that the existence of EVs could initiate the allosterism of allosteric probes and thus lead to the exposure of the signal amplification initiator. In order to study whether allosteric probes could be unfolded by an interference probe and thus to induce the two-cycle SDA, we also designed a linear probe which could totally hybridize with the allosteric probe. From the result, we have observed miniscule fluorescence enhancements when a linear probe existed compared with control group, indicating that linear probes showed little interference on allosteric probes. In addition, the probe could maintain a high stability even in a more complicated environment with BSA, $MgCl^{2+}$, and dimethyl sulfoxide (Figure 1e). Selectivity of the designed allosteric probe was also investigated through incubating it with CD9 proteins. As shown in Figure S1, the obtained fluorescence intensity of allosteric probes is much higher in the CD63 group than in the CD9 group, indicating a favorable selectivity of the method for CD63 positive exosome identification. We then investigated whether the allosteric probe could initiate signal amplification after recognition of target EVs (Figure 1f). Eventually, we observed no significant fluorescence enhancements when the target EVs were absent compared with the control group with hairpin structure probes alone. On the contrary, fluorescence intensity was greatly enhanced when EVs were present, which is almost seven times higher than that when EVs were absent, demonstrating that the existence of EVs could initiate the following allosteric probe-based cascade amplification system to continuously cleave fluorescence and quenching moiety pairs for the fluorescence generation. The signal amplification process in the sensing system was also studied and verified through PAGE analysis (Figure S2).

Sensitivity of the Proposed Method for Circulating EV Detection. Before the investigation of sensitivity, we optimized the experimental conditions of the method including reaction buffer, the concentration of a hairpin probe, the amount of lambda exonuclease, and reaction times. From the result shown in Figure 2a, the reaction buffer 2 (NEBuffer 2, the reaction buffer of DNA polymerase I; lambda exonuclease reaction buffer) induced a much higher fluorescence intensity compared with the reaction buffer 1 (CutSmart buffer, the reaction buffer of Nb.BtsI) and 3 (lambda exonuclease reaction buffer). In the experiments investigating the concentration of hairpin probes, the obtained fluorescence intensity gradually increased with the concentration of allosteric probes from 50 to 300 nM and no more fluorescence intensity increase was observed with more concentration (Figure 2b). Therefore, the concentration of allosteric probes was determined as 300 nM. In addition, the amount of lambda exonuclease and reaction time were determined as 1U (Figure S3) and 40 min (Figure S4), respectively.

https://doi.org/10.1021/acsomega.0c06334
ACS Omega 2021, 6, 17776−17781

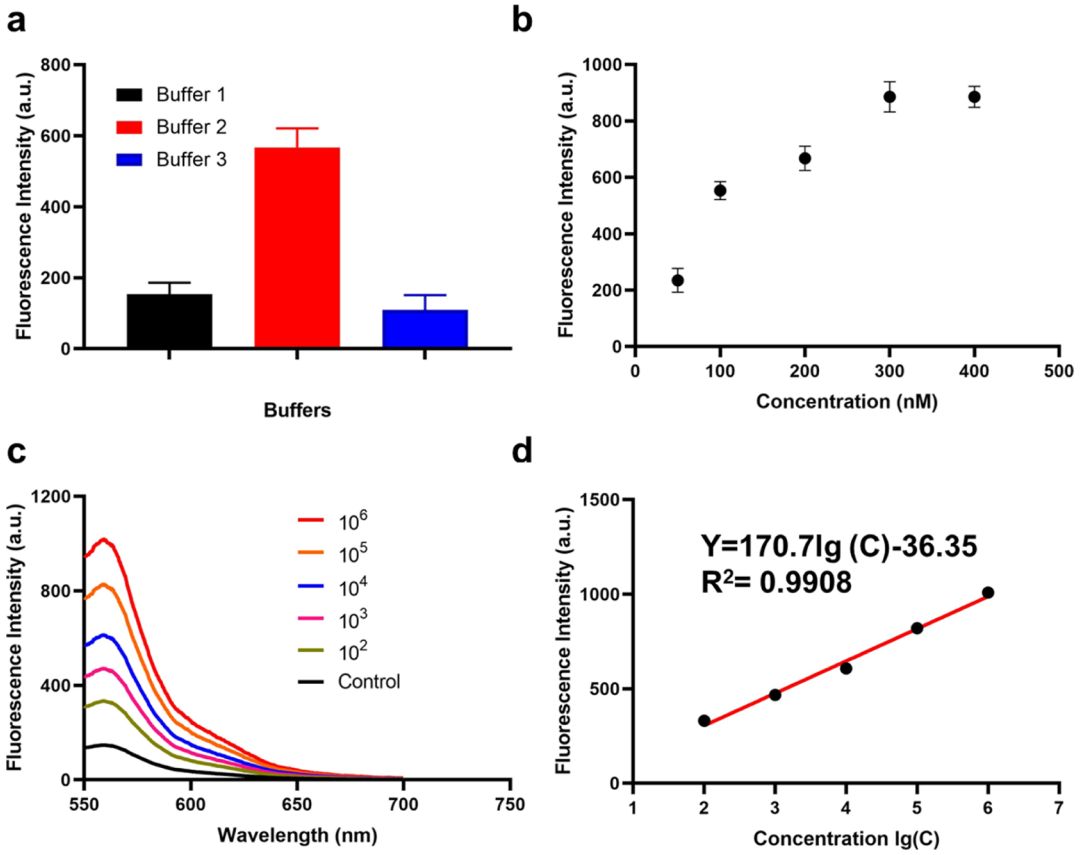


Figure 2. Sensitivity of the proposed allosteric probe-initiated cascade amplification system for circulating EV identification. (a) Optimization of the reaction buffer. (b) Fluorescence intensity of the proposed allosteric probe-initiated cascade amplification system with different concentrations of allosteric probes. (c) Fluorescence spectrum of the proposed allosteric probe-initiated cascade amplification system with different concentrations of extracted EVs. (d) Correlation of the obtained fluorescence intensity and the concentrations of extracted EVs.

Under the optimized experimental conditions, we measured the fluorescence intensity of the proposed method for EV detection with different concentrations ranging from 10^2 to 10^6 particles/μL (the EV samples were from the diluted extracted EVs with 1× PBS buffer). As shown in Figure 2c, the fluorescence intensity showed obvious increase with the increase of the concentrations of EVs from 10^2 to 10^6 particles/μL. Finally, a linear correlation was obtained between the standardized fluorescence intensity and the logarithm of EV concentrations. The obtained equation of correlation was Y = 170.7l g (C)-36.35 with the correlation coefficient R^2 equal to 0.9908 (C was the concentration of the extracted EVs and Y was the obtained fluorescence intensity) (Figure 2d). Compared with some of the former proposed strategies, the method exhibited an evenly matched sensitivity and detection ranges down to 10^2 particles/μL. The more important fact is that the method exhibits 5 orders of magnitude dynamic range, facilitating the accurate quantitative analysis of extracted EVs even under complicated experimental conditions. To the best of our knowledge, the sensitivity of the method is mainly derived from the two factors: (i) recognition of EVs by allosteric probes convert the amounts of EVs to the nucleic acids detection and (ii) dual cycles of the amplification process could continuously generate fluorescence signals. Afterward, we investigated the repeatability of the method for 10 duplicated detections and obtained a relative standard deviation of 4.0%, suggesting that the proposed method had a good reproducibility (Figure S5).

Measurement of Circulating EVs from Clinical Samples for Pancreatitis Diagnosis. The differential quantification of EVs in the human plasma is recognized as a potential predictor for diseases diagnosis. Herein, we studied the relationships of the amounts of EVs from blood samples with the pancreatitis diagnosis. We first collected blood samples from 15 pancreatitis patients and 15 health volunteers and quantified the EVs in these samples through the proposed method. From the result in Figure 3a, the calculated quantification of EVs in the pancreatitis patients' groups was

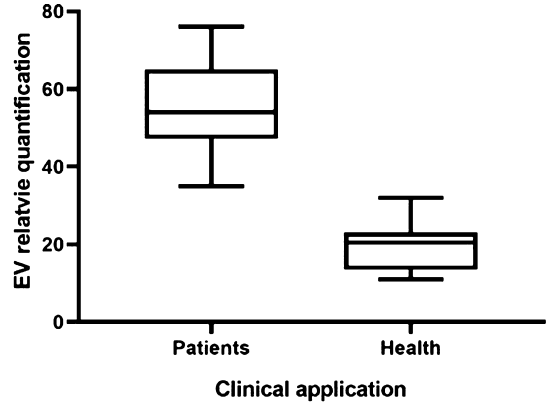


Figure 3. Measurement of circulating EVs from clinical samples for pancreatitis diagnosis.

https://doi.org/10.1021/acsomega.0c06334
ACS Omega 2021, 6, 17776−17781

much higher than that in the health group, indicating a potential difference of the amounts of EVs between the two groups.

Through the allosteric probe-initiated cascade amplification system, the method could realize sensitive quantification of exosomes in a wash-free way. Compared with some previously reported exosome detection methods (Table 1), this method has advantages such as (i) accurate identification from the carefully designed allosteric probe; (ii) a favorable sensitivity from the cascade amplification process; and (iii) wash-free process, which simplifies the procedures.

Table 1. Comparisons of the Previously Proposed Exosome Detection Methods[a]

titles	signal amplification process	sensitivity	wash-free	refs
the method	cascade amplification	10^2–10^5	yes	
AcmPLA	RCA	10^3–10^6	no	12
CISPR-Cas-based strategy	trans-cleavage activity of Cas12	10^3–10^6	no	16
AID-Cas	multiple enzyme; Cas12a	10^2–10^6	yes	17

[a]RCA, rolling circle amplification.

CONCLUSIONS

We have proposed an allosteric probe-initiated cascade amplification sensing system and successfully applied it for accurate identification and sensitive quantification of circulating EVs. In the proposed method, the presence of target EVs could trigger the allosterism of the designed allosteric probe and thus initiate the following dual cycle signal amplification. Eventually, the method exhibited a large dynamic detection range of 5 orders of magnitude and favorable sensitivity. The comparison of the method with some of the previously reported methods suggested the following highlights: (i) allosteric probes ensure a wash-free way for EV detection and greatly simplified the detection procedures. (ii) Dual signal amplification cycle ensured the sensitivity down to 10^2 particles/μL of EVs. We believe that the method could provide a new way for early diagnosis of pancreatitis.

EXPERIMENTAL SECTION

Chemicals and Materials. All the related nucleic acids (Table S1) were all purchased from Takara Biotechnology Co. Ltd. (Chengdu, China) and purified by HPLC. The related enzymes, including DNA polymerase I, Nb.BtsI, lambda exonuclease, and deoxyribonucleoside 5′-triphosphate mixture (dNTPs), were purchased from New England Biolabs (Beverly, MA). Human non-small-cell lung carcinoma (NSCLC) A549 cell lines and pancreatitis-associated cell lines were obtained from Cell Bank, Shanghai Institutes for Biological Sciences (Shanghai, China). Whole blood samples were derived from the healthy volunteers and the adult pancreatitis patients at Chenjiaqiao Hospital affiliated to Chongqing Medical and Pharmaceutical College (Shapingba, Chongqing, China), and this research was approved by the ethics committee of Chenjiaqiao Hospital affiliated to Chongqing Medical and Pharmaceutical College.

Construction of a Cascade Amplification System for Circulating EV Detection. All oligonucleotides were diluted with 1× PBS buffer to 10 μM for the preparation of stock solutions. The hairpin probes were diluted to 10 μM with 1× hybridization buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.0) and incubated for 5 min at 95 °C and then cooled to room temperature to get a perfect hairpin structure. For EV detection assay, 0.25 μL of linear probes (10 μM) was added into the polymerization solution (25 μL) containing different concentrations of target EVs, 5 U of DNA polymerase I, 2.5 μL of 10× CutSmart buffer, 8 U of Nb.BtsI, 2.5 μL of 10× NEBuffer 2, 1 U of lambda exonuclease, 2.5 μL of 10× lambda exonuclease reaction buffer, 300 nM hairpin probes, 150 μM dNTPs, and 20 U of RNase inhibitor and then incubated for 45 min at 37 °C to perform the quencher-free cascade amplification reaction.

Fluorescence Measurement and Gel Electrophoresis. 25 μL of reaction products was diluted with ultrapure water to 60 μL. The fluorescence spectra measurements were performed on a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan) with an excitation wavelength of 310 nm, an excitation slit of 5.0 nm, and emission slits of 5.0 nm. The fluorescence intensity at the emission wavelength of 365 nm was used for data analysis.

EV Extraction. Cancer cells were cultured and grown to exponential growth phase. For EV isolation, we washed the cells three times with PBS buffer and then collected the culture medium for EV isolation by ultracentrifugation according to the standard differential centrifugation separation protocols. Finally, TEM and NTA were performed to characterize these isolated EVs (details of the EVs extraction are given in Supporting Information).

Real Sample Analysis. For clinical applications, the EVs from human serums of eight healthy persons and eight pancreatitis patients were extracted. The extracted total EV concentrations were directly quantified by using the proposed method.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c06334.

Details of the sequences; fluorescence intensity of the method for CD9 and CD 63 protein recognition; fluorescence intensity of the allosteric probe-initiated cascade amplification system with different incubation times; fluorescence intensity of the allosteric probe-initiated cascade amplification system with different Lamda exonuclease concentrations; and fluorescence intensity of the allosteric probe-initiated cascade amplification system for ten duplicate detection (PDF)

AUTHOR INFORMATION

Corresponding Author

Linhong Ning − *Department of Gastroenterology, Chenjiaqiao Hospital Affiliated to Chongqing Medical and Pharmaceutical College, Chongqing 401331, China;* orcid.org/0000-0002-3305-190X; Email: ninglinhong780629@163.com

Authors

Yuan Zhou − *Clinical Medical College of Chongqing Medical and Pharmaceutical College, Chongqing 401331, China*

https://doi.org/10.1021/acsomega.0c06334
ACS Omega 2021, 6, 17776−17781

Feng Yu − *Department of Gastroenterology, Chenjiaqiao Hospital Affiliated to Chongqing Medical and Pharmaceutical College, Chongqing 401331, China*
Heng Cheng − *Department of Gastroenterology, Chenjiaqiao Hospital Affiliated to Chongqing Medical and Pharmaceutical College, Chongqing 401331, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c06334

Author Contributions

[§]Y.Z. and F.Y. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We really appreciate the staff of Department of Gastroenterology in Chenjiaqiao Hospital affiliated to Chongqing Medical College.

■ REFERENCES

(1) Bu, H.; He, D.; He, X.; Wang, K. Exosomes: Isolation, Analysis, and Applications in Cancer Detection and Therapy. *ChemBioChem* **2019**, *20*, 451−461.
(2) Hon, K. W.; Abu, N.; Ab Mutalib, N. S.; Jamal, R. Exosomes As Potential Biomarkers and Targeted Therapy in Colorectal Cancer: A Mini-Review. *Front. Pharmacol.* **2017**, *8*, 583.
(3) Jalalian, S. H.; Ramezani, M.; Jalalian, S. A.; Abnous, K.; Taghdisi, S. M. Exosomes, new biomarkers in early cancer detection. *Anal. Biochem.* **2019**, *571*, 1−13.
(4) Zhang, W.; Peng, P.; Kuang, Y.; Yang, J.; Cao, D.; You, Y.; Shen, K. Characterization of exosomes derived from ovarian cancer cells and normal ovarian epithelial cells by nanoparticle tracking analysis. *Tumor Biol.* **2016**, *37*, 4213−4221.
(5) Cheshomi, H.; Matin, M. M. Exosomes and their importance in metastasis, diagnosis, and therapy of colorectal cancer. *J. Cell. Biochem.* **2018**, *120*, 2671.
(6) Xiao, Y.; Li, Y.; Yuan, Y.; Liu, B.; Pan, S.; Liu, Q.; Qi, X.; Zhou, H.; Dong, W.; Jia, L. The potential of exosomes derived from colorectal cancer as a biomarker. *Clin. Chim. Acta* **2019**, *490*, 186−193.
(7) Greenberg, J. A.; Hsu, J.; Bawazeer, M.; Marshall, J.; Friedrich, J. O.; Nathens, A.; Coburn, N.; May, G. R.; Pearsall, E.; Mcleod, R. S. Clinical practice guideline: management of acute pancreatitis. *Can. J. Surg.* **2016**, *59*, 128−140.
(8) van Dijk, S. M.; Hallensleben, N. D. L.; Santvoort, H. C. V.; Fockens, P.; Besselink, M. G. J. G. Acute pancreatitis: Recent advances through randomised trials. *Gut* **2017**, *66*, 2024.
(9) Chen, J.; Xu, Y.; Lu, Y.; Xing, W. Isolation and Visible Detection of Tumor-Derived Exosomes from Plasma. *Anal. Chem.* **2018**, *90*, 14207−14215.
(10) Chiriaco, M. S.; Bianco, M.; Nigro, A.; Primiceri, E.; Ferrara, F.; Romano, A.; Quattrini, A.; Furlan, R.; Arima, V.; Maruccio, G. Lab-on-Chip for Exosomes and Microvesicles Detection and Characterization. *Sensors* **2018**, *18*, 3175.
(11) Picciolini, S.; Gualerzi, A.; Vanna, R.; Sguassero, A.; Gramatica, F.; Bedoni, M.; Masserini, M.; Morasso, C. Detection and Characterization of Different Brain-Derived Subpopulations of Plasma Exosomes by Surface Plasmon Resonance Imaging. *Anal. Chem.* **2018**, *90*, 8873−8880.
(12) Zhao, X.; Luo, C.; Mei, Q.; Zhang, H.; Zhang, W.; Su, D.; Fu, W.; Luo, Y. Aptamer-Cholesterol-Mediated Proximity Ligation Assay for Accurate Identification of Exosomes. *Anal. Chem.* **2020**, *92*, 5411−5418.
(13) Heinemann, M. L.; Ilmer, M.; Silva, L. P.; Hawke, D. H.; Recio, A.; Vorontsova, M. A.; Alt, E.; Vykoukal, J. Benchtop isolation and characterization of functional exosomes by sequential filtration. *J. Chromatogr. A* **2014**, *1371*, 125−135.
(14) Wang, Z.; Zong, S.; Wang, Y.; Li, N.; Li, L.; Lu, J.; Wang, Z.; Chen, B.; Cui, Y. Screening and multiple detection of cancer exosomes using an SERS-based method. *Nanoscale* **2018**, *10*, 9053−9062.
(15) Yu, X.; He, L.; Pentok, M.; Yang, H.; Yang, Y.; Li, Z.; He, N.; Deng, Y.; Li, S.; Liu, T.; Chen, X.; Luo, H. An aptamer-based new method for competitive fluorescence detection of exosomes. *Nanoscale* **2019**, *11*, 15589−15595.
(16) Zhao, X.; Zhang, W.; Qiu, X.; Mei, Q.; Luo, Y.; Fu, W. Rapid and sensitive exosome detection with CRISPR/Cas12a. *Anal. Bioanal. Chem.* **2020**, *412*, 601−609.
(17) Zhao, X.; Zeng, L.; Mei, Q.; Luo, Y. Allosteric Probe-Initiated Wash-Free Method for Sensitive Extracellular Vesicle Detection through Dual Cycle-Assisted CRISPR-Cas12a. *ACS Sens.* **2020**, *5*, 2239−2246.