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ORIGINAL PAPER

Gold nanoparticle-based colorimetric detection of staphylococcal enterotoxin B using ssDNA aptamers

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Abstract Staphylococcal enterotoxin (SE) B is one of the most common serotype of SEs to cause staphylococcal food poisoning. To ensure food safety, rapid and low-cost methods have been constantly developed and applied to detect SEB worldwide. In the present investigation, a panel of aptamers of single-stranded DNA molecules against SEB was obtained by optimizing the procedure of systematic evolution of ligands by exponential enrichment, and five of them were selected for further analyzing the characteristics of sequences and second structures. Afterward, an aptamer-based colorimetric method of SEB detection was carried out by employing unmodified gold nanoparticle probes (AuNPs). Results showed that the main second structures of these aptamers were stem loop and hairpin forms. In addition, applying one of these aptamers (No. 15-1), SEB could be detected at the concentration of 10 ng/mL by AuNPs-based colorimetric method. Moreover, the aptamer also possessed a good selectivity toward SEB and SEC_1. Our work demonstrated that aptamers had their potential applications as a bioprobe for the detection of SEs in food products.

Keywords Aptamer · Systematic evolution of ligands by exponential enrichment · SEB · Gold nanoparticle · Colorimetry

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Abbreviations

AuNPs	Gold nanoparticle probes
BSA	Bovine serum albumin
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
SE	Staphylococcal enterotoxin
SELEX	Systematic evolution of ligands by exponential enrichment
SPR	Surface plasmon resonance
ssDNA	Single-stranded DNA

Introduction

Staphylococcal food poisoning is one of the most commonly erupted foodborne illness that results from the consumption of foods containing staphylococcal enterotoxins (SEs) produced by enterotoxinogenic strains of *Staphylococcus aureus* [1–4]. SEs, with a lot of serotypes, can bring various types of disease symptoms [5]. Besides, they are potent gastrointestinal exotoxins resistant to proteolytic enzymes, high temperature (at 100 °C), and extreme pH values because of their compact tertiary structures, hence retaining their activities in the digestive

tract after ingestion [6–8]. Among the SEs, SEB is one of the most commonly presented serotypes in staphylococcal food-poisoning cases [9, 10], and rapid, accurate, and reliable detection of SEB is necessary and significant to protect public health against SEB.

Currently, immuno-based assays [11, 12], surface plasmon resonance (SPR) assay [13], and biomolecular interaction mass spectrometry [14] are the most important methods to identify SEs. As is well known, the availability of specific and high-affinity antibodies is of paramount importance in an immuno-detection system [15]. However, these antibodies are sensitive to temperature and have limited lifetimes; moreover, there are still several other problems in the production of antitoxic protein antibodies, such as identical antisera cannot be prepared constantly or maintaining hybridoma involves high costs [16].

Aptamers, generated through the systematic evolution of ligands by exponential enrichment (SELEX), are random single-stranded nucleic acid oligomers (ssDNA or RNA) with a specific and complex three-dimensional shape [17]. As an alternative strategy, aptamers possessing high recognition ability toward specific molecular targets have a strong potential application as bioprobes for targeting drug, developing new drugs, and biosensing [18, 19]. What's more, aptamers have many advantages over antibodies, such as more stable, easier modification, easier synthesis, and higher affinity, and they can be fluorescently labeled and do not require experimental animals for synthesis [20, 21]. Due to these properties, a variety of aptamer-based analytical methods including electrochemistry, fluorescence, atomic force microscope, and quartz crystal microbalance have been developed for molecular recognition and detection [22–25]. Moreover, the aptamer-based colorimetric method applying gold nanoparticles (AuNPs) as sensing elements is attracting more and more attention because of the potential to eliminate the use of analytical instruments and observe results by naked eyes [26–28].

In this study, SELEX technique was applied to select ssDNA aptamers specific for SEB. And then, an unmodified AuNPs-based colorimetric method employing the selected aptamers was attempted to detect SEB. To the best of our knowledge, this is the first time the abovementioned method is applied for testing SEB. Compared to the aptamers reported for SEB detection [15, 29], this study provided another rapid and cheap way, and the aptamers acquired could be applied for simultaneous detection of multifarious SEs.

Materials and methods

Purified SEA, SEB, and SEC_1 were kindly provided by Dr. Jiang from Academy of Military Medical Sciences (Beijing, China). Taq DNA polymerase and dNTPs were obtained from Zomanbio Co., (Beijing, China), and pEASY-T1 cloning vector was purchased from TransGen Biotech Co., (Beijing, China). All other reagents were of analytical purity.

ssDNA original random library and primers

The ssDNA original library containing a random sequence of 40 nucleotides was flanked by two constant regions (5′-ACCGACCGTGCTGGACTCT-$(N)_{40}$-AGTATGAGCG AGCGTTGCG-3′). A forward primer (P1: ACCGACCGT GCTGGACTCT) and a reverse primer (P2: CGCAAC GCTCGCTCATACT) were used to generate double-strand DNA, and a stem loop primer (P3: GCTAACGCGGTGG GACTTCCTAGTCCCACCGCGCGCAACGCTCGCTCA TACT) was used for single-strand DNA (ssDNA) generation together with the primer P1. All oligonucleotides were synthesized and purified with polyacrylamide gel electrophoresis (Genscrip Biological Co., Nanjing, China).

Preparation of ASIAN library by unequal-length strand PCR

In the SELEX process, the original ssDNA library was applied as the starting pool for the first selection round. After the first selection round, every other selection round needs the last selection round product as "starting pool," which was called ASIAN library in this study. Herewith, we employed the method of unequal-length strand PCR to prepare ssDNA of ASIAN library.

With primers of P1 and P3, the unequal length of ssDNA molecules was prepared by PCR as follows: 25 μL of PCR mixture contains 2.5 μL of 10× PCR buffer, 2 μL of 2.5 mM dNTPs, 10 pmol each primer, 1 pmol template, and 1 U Taq DNA polymerase. The mixture was thermally cycled 30 times through 94 °C for 45 s, 60 °C for 90 s, and followed by 5 min extension at 60 °C. The PCR products were analyzed by 8 % polyacrylamide gel electrophoresis (PAGE) with 7 M urea, and the lower band DNAs of interest were purified from the gel for the next round of selection.

The amount of separated ssDNA was measured by urea denatured PAGE silver staining. Five different concentrations of origin ssDNA with the same volume and equivoluminal DNA loading buffer (8 M Urea, 20 mM EDTA, 5 mM Tris–HCl (pH 7.5), 3.7 mM bromophenol blue) were subjected to urea denatured 8 % PAGE according to Laemmli [30]. The gels were stained with silver and then analyzed by the software Quantity One [31].

In vitro selection of DNA aptamers with specific binding to SEB

The SELEX procedure was applied with reference to Wen et al. [32]. For the initial selection, 2 nmol ssDNA original

library was heated to 100 °C in 1 mL binding buffer (20 mM HEPES-Na, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.35) for 10 min and cooled at 4 °C immediately for 5 min to facilitate the equilibration of different conformations. A certain amount of SEB was then added and incubated at 37 °C for 60 min with shaking. SEB·ssDNA complexes were separated from free ssDNA by pressured filtration with mixed cellulose membrane treated by 0.4 M KOH previously. After that, the mixed cellulose membrane was washed by 1 mL binding buffer for 5 times and cut into pieces; 100 µL of elution buffer (7 M urea, 0.5 M NH_4Ac, 7 mM SDS, 1 mM EDTA, pH 8.0) was added to the pieces and heated at 100 °C for 10 min and then centrifuged at 7,500 rpm/min for 10 min at 4 °C, and the supernatant was collected. The SEB·ssDNA was extracted by chloroform and then ethanol precipitated. The precipitated ssDNA was dissolved in 100 µL of deionized water and amplified by unequal-length strand PCR using the forward primer and step loop primer as described in "Preparation of ASIAN library by unequal-length strand PCR" section.

Cloning and sequencing

After 15th round of aptamer selection, the collected ssDNA was amplified by PCR with primers P1 and P2. Each 25 µL of parallel PCR mixtures contained 2.5 µL of 10× PCR buffer, 2 µL of 2.5 mM dNTPs, 10 pmol each primer, 1 U Taq DNA polymerase, and an appropriate amount of template ssDNA. PCR conditions employed were as follows: an initial heat activation step at 94 °C for 5 min and 10 cycles of 94 °C for 45 s, 68 °C for 45 s, and 72 °C for 45 s, and followed by 5 min extension at 72 °C. The products were purified with Miniquick Purification Kit (ZomanBio, Beijing, China) and subsequently cloned into *Escherichia coli* (DH5α) using the pEASY-T1 vector system. Ten clones were picked and sequenced. Their secondary structures were investigated by RNA structure [33].

Preparation of AuNPs and detection of SEB by aptamer-based colorimetric analysis

AuNPs (~12.74 nm in diameter) were prepared by the citrate reduction of $HAuCl_4$ [34]. Aptamer-based colorimetric detection of SEB was performed as follows: First, 120 µL of AuNPs was mixed with 2 µL of 132 µM aptamer No.15-1 (aptamer No.15-1 was selected from the other four according to simulation results between aptamers and SEB by Visual Molecular Dynamics software, data not shown), and then, the solution was allowed to react for 10 min at room temperature. Secondly, different concentrations of SEB in deionized water were added to the prepared solution, and the solution was allowed to react for another 15 min at room temperature. Finally, 80 µL of 2.75 M NaCl was added to develop color. The absorbance spectrum was measured using UV–VIS spectrometer (UV1700, SHIMADZU). The concentration of SEB was quantified by the absorption ratio (A_{620}/A_{520}) [35]. The specification of aptamer was confirmed by adding BSA, SEA, and SEC_1 in a similar way [36].

Results and discussion

Preparation of ASIAN library using special stem loop primers

Generally, specific aptamers can be obtained through several rounds of SELEX screening, and the success of the SELEX screening depends on the preparation of high purity ssDNA of ASIAN library. Asymmetric PCR and biotin–streptavidin separation are usually employed to prepare ssDNA, but the products acquired appear as a smear of band and not of high purity [37]. By contrast, unequal-length strand PCR using special stem loop primer is cheap, does not need any special modification of primers, and yields good-quality products [38, 39].

Herewith, the stem loop primer P3 contained two parts: the longer 5′ reverse repeat sequence with the ability to form stem loop structures and the 3′ complement sequence of the template (Fig. 1a). Because of the higher T_m of the stem loop primer, advanced structures still remained the same when the Taq DNA polymerase worked at the

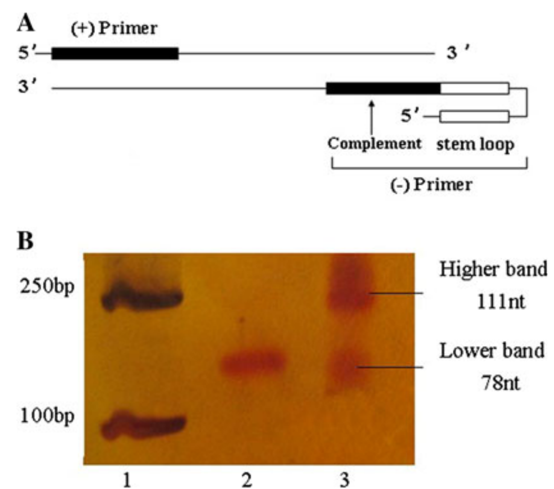

Fig. 1 Unequal-length strand PCR for the preparation of ASIAN library ssDNA using a stem loop primer. **a** Schematic of PCR product using the P1 primer and the stem loop primer. In the 5′ terminus of (−) primer, there is a GC-rich reverse repeat sequence that can form stem loop structures and prevent the (+) strand elongation. **b** Strand separation analysis in a urea denatured PAGE. *Lane 1*, DL2000 DNA Marker; *lane 2*, the ssDNA of the native library; *lane 3*, the unequal-length ssDNA product

temperature range of 55–65 °C and thus prevented the elongation of the (+) strand. As was shown, PCR products had two bands with different migration rates in 7 M urea denatured 8 % PAGE (Fig. 1b). The lower band, which would be employed as the single-strand template, was 33nt shorter than the upper one because of the formation of stem loop structure. Just as implied by Fig. 1b, a large quantity of ssDNA with high purity could be recovered, and the ASIAN library could be applied for the next round of screening.

In vitro selection of ssDNA with specific binding to SEB

Selection processes of ssDNA aptamers binding to SEB were as described in "In vitro selection of DNA aptamers with specific binding to SEB". The starting pool of ssDNA (2 nmol, approximately 10^{14} sequences) contained a 40-base central random region and two constant regions. A total of fifteen rounds of SELEX were performed. The original ssDNA was diluted to different concentration in the same proportion and analyzed by 7 M urea denatured 8 % PAGE. And with the gradient reduction in the molar volume of original ssDNA, the color of ssDNA bands faded and the width became narrow gradually (Fig. 2a). The gray value of each lane was measured by Quantity One software, and correlation between molar volume and gray value was implemented by software Origin 8.0 (Fig. 2b). The results revealed that there was a good linear relationship and high consistency between them, which further confirmed the possibility of this method for quantification of ssDNA.

Sequence and structure analysis of the selected aptamers

High-affinity ssDNA were obtained by the 15th round of selection, and ssDNA from the 15th round were amplified to dsDNA and then cloned. Afterward, ten clones were further selected and sequenced. Among them, sequences of five clones (Table 1) were consistent with the original library which contained a random sequence of 40 nucleotides flanked by two constant regions. RNA structure results showed that the second structures of aptamers were mainly stem loop and hairpin. Stem loop might be the structural basis for aptamers binding to target molecules [40, 41]. The stem might play a stabilizing role, and the loop was directly bound to ligands. In aptamer No. 15-1 (Fig. 3), G·T mismatch can be found, and this mismatch often occurred in the normal double helix at the turning

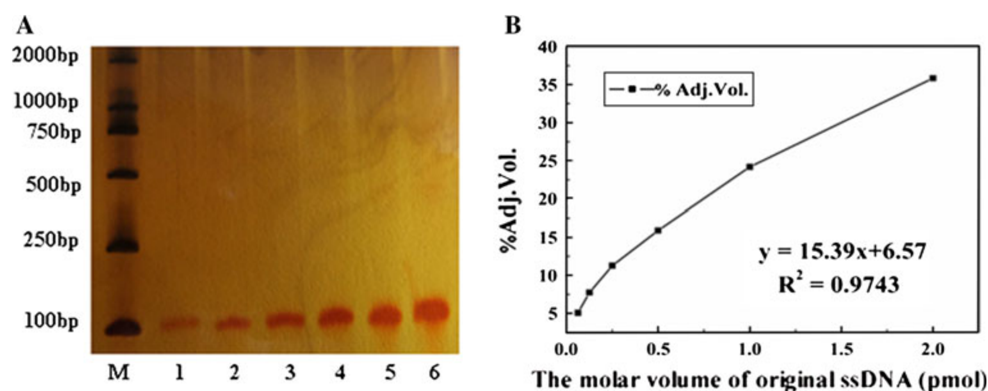

Fig. 2 **a** The different molar volume of the original library in 7 M urea denatured 8 % PAGE and silver-staining results. M: DL2000 DNA Marker. *Lane 1–6*: The molar volume of the original library is 0.0625, 0.125, 0.25, 0.5, 1, 2 pmol. **b** Correlation between the molar volume of ssDNA and gray value

Table 1 Sequences of the aptamers obtained by SELEX

Aptamers	Sequence
2-1	ACCGACCGTGCTGGACTCTTTCGGAGGTGATACCCTGAAAGCAC GCCACTCAAATAGTTCGAGCGAGTATGACGAGCG
10-1	ACCGACCGTGCTGGACTCTTGGTTTTTTTAGTTGTTTGTTTAGAAA TATGTTTGTTGGGAGTATGAGCGAGCGTTGCG
14-1	ACCGACCGTGCTGGACTCTGACCTCATTTGTTGGTTCTGTCATGTT TATTTTTTATTATAGTATGAGCGAGCGTTGCG
15-1	ACCGACCGTGCTGGACTCTATTCATTACCCATCCGAGAACTGGTG GTCATATCTCTACAAGTATGAGCGAGCGTTGCG
15-2	ACCGACCGTGCTGGACTCTGTGTGAACATTTTTAGTACATTCACT ACTTCGTACGATCAAGTATGAGCGAGCGTTGCG

point of stacked base pairs, which easily lead to the closure of rings.

Detection of SEB by aptamer-based colorimetric analysis using AuNPs as probe

It has been reported that unfolded short ssDNA can adsorb onto the surface of AuNPs without any modification, and such ssDNA-treated AuNPs are more stable than untreated ones in the presence of a given high concentration of salt [35]. Based on this, a colorimetric method using AuNPs aggregation was developed to detect target molecules [42]. The mechanism of the colorimetric method to detect SEB was as follows. Because of the negative capping agent's electrostatic repulsion against van der Waals attraction between AuNPs [43], the AuNPs were dispersed and sta-

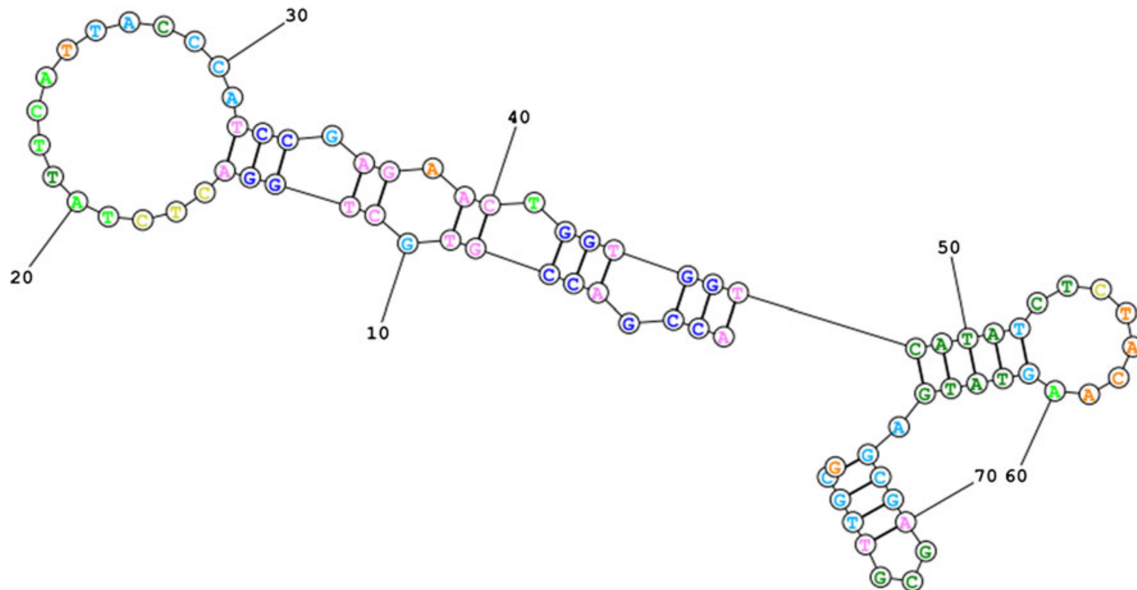

Fig. 3 The secondary structure of aptamer No.15-1 predicted by RNA structure

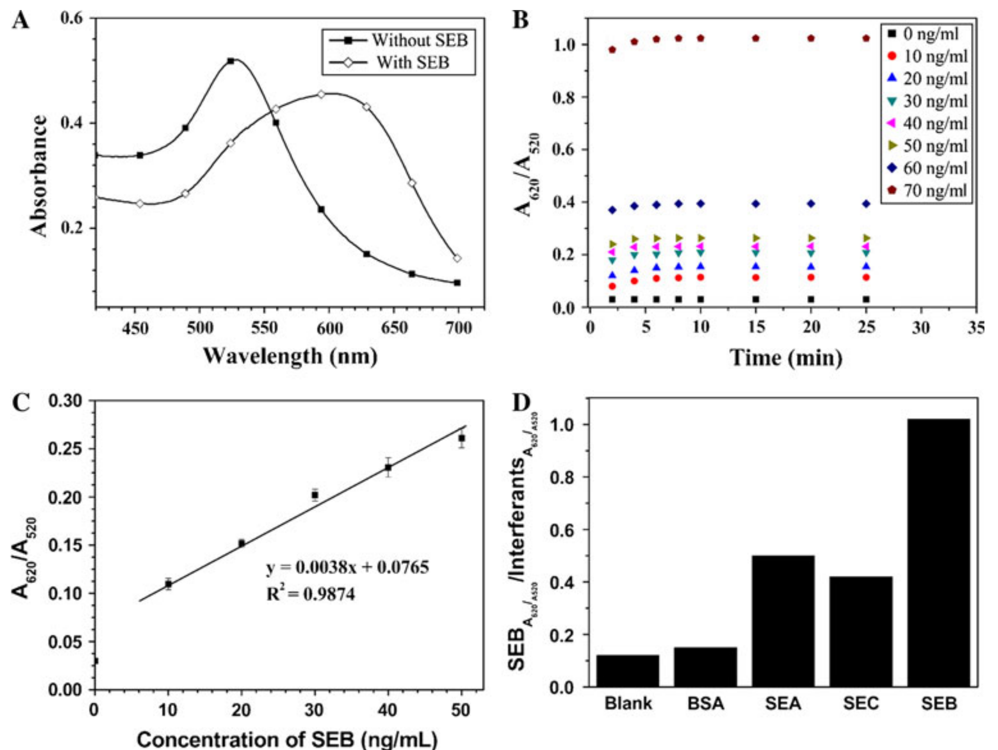

Fig. 4 **a** UV–Vis absorption spectra of AuNPs in the absence and presence of SEB. **b** Plot of changes of absorption ratio (A_{620}/A_{520}) in the presence of various amounts of SEB. **c** Plot of A_{620}/A_{520} versus the concentration of SEB. **d** The ratio of A_{620}/A_{520} of different interferents to that of SEB in the presence of each interferent at a concentration of 50 ng/mL

ble. However, the addition of enough salt could lead to the aggregation of the AuNPs, which brought the color of AuNPs solution from red to blue. After adding aptamers into AuNPs, aptamers would adsorb onto the surface of the AuNPs, so there is no color change and aggregation of AuNPs after adding high concentration salt. But in the presence of SEB, SEB could bind with aptamers in competition with AuNPs. This could in turn bring the color change of AuNPs solution when adding salt. As shown in Fig. 4a, in the absence and presence of SEB in AuNPs solution treated with aptamer (No. 15-1), once 2 M NaCl was added, the UV–Vis spectrum of AuNPs had a red-shifted. The peak at 520 nm (the dispersed AuNPs) decreased, while the peak at 620 nm increased (the aggregated AuNPs). And the absorption ratio (A_{620}/A_{520}) increased with the increasing SEB concentration and the binding time of the aptamer and SEB (Fig. 4b), the absorption ratio reached a maximum at 10 min and then reached a constant value. Applying the measured absorption spectra, the absorption ratio at 620 and 520 nm was found to correlate with the concentration of SEB (Fig. 4c). The linear regression equation is $y = 0.0038x + 0.0765$, and the R^2 is 0.9874. According to the linear range of the curve, the working range to detect SEB was assigned to a concentration between 10 and 50 ng/mL. Because the colorimetric analysis is simple, cost-effective, and conjugated easily with biomaterials, it will be a promising method to detect target molecules.

The selectivity of aptamer (No. 15-1) to SEB was evaluated by measuring the absorption ratio value, A_{620}/A_{520} to some common interferents such as BSA, SEA, and SEC_1 at a concentration of 50 ng/mL. The data derived from Fig. 4d showed that the absorption ratio at 620 and 520 nm in the presence of SEB was larger than those of blank or other proteins. However, it also revealed that the aptamers obtained had an obvious cross-reaction with SEA and SEC_1, indicating that the aptamers selected had potential applications as a bioprobe for the detection of SEs in food products.

Conclusions

In summary, aptamers toward SEB were successfully obtained by employing SELEX system, and second structures of the aptamers were mainly stem loop and hairpin according to RNA structure web server. What's more, with the aptamer-based colorimetric method applying AuNPs as probe, SEB could be easily detected. The results also implied the good selectivity of aptamers toward SEA and SEC_1. Obviously, the colorimetric method would provide a new possibility for the detection of SEs in food products.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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