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INHIBITION OF ISLET AMYLOID POLYPEPTIDE FIBRILLATION BY OKRA SEED EXTRACT-COATED GOLD-NANOPARTICLES

Sirikhwan Manee[1], Miaoyi Wang[2], Faisal Usman[3], Paweena Wongwitwichot[1] and Jasadee Kaewsrichan [*1]

Department of Pharmaceutical Chemistry [1], Drug Delivery System Excellence Centre [3], Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-yai, Songkhla 90112, Thailand.

ARC Centre of Excellence in Convergent Bio-Nano Science and Technology [2], Department of Drug Delivery, Dynamics and Disposition (D4), Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia.

Keywords:

IAPP, Amyloidosis, Polypeptide aggregation, Fibrillation, Gold nanoparticles, Okra seed extract

Correspondence to Author:
Jasadee Kaewsrichan

Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-yai, Songkhla 90112, Thailand.

E-mail: jasadee.k@psu.ac.th

ABSTRACT: In recent work, an ethanolic extract of okra seeds was successfully conjugated onto gold nanoparticles (AuNPs) via a reduction reaction that has been developed by our laboratory. Because of eliciting antioxidant activity and the presence of γ-sitosterol in the extract, such synthesis methodology could be applied. The resulting okra-AuNPs were physically characterized by different methods, including dynamic light scattering (DLS), Zeta potential measurement, UV-Visible spectrophotometry, Transmission Electron Microscopy (TEM), Helium Ion Microscopy (HIM), and Fourier Transform Infrared (FTIR) spectroscopy. The other two biological characterization techniques, such as thioflavin assay and cell viability test, were also performed. Results indicated that the okra-AuNPs were spherical with a mean diameter of around 35.6 nm. A maximum absorption wavelength (λ_{max}) was at 521 nm. A zeta potential was determined to be around -59.2 ± 2.9 mV. Binding between the okra-AuNPs and islet amyloid polypeptide (IAPP) was observed, resulting in IAPP aggregation and reduction of β-sheet contents of the aggregated polypeptides. It seemed that the okra-AuNPs exhibited cytoprotective activity on pancreatic beta cells by inhibiting IAPP fibrillation. Therefore, the okra-AuNPs would be useful as a delivery system for diseases associating with IAPP assembly and amyloid formation.

INTRODUCTION: Islet amyloid polypeptide (IAPP) is generally co-secreted with insulin by pancreatic β-cells for glycemic control. IAPPs is self-assembled to form amyloid fibrils when triggered by aberrant conditions. Such fibrillation is evident to implicate in degenerative diseases of diverse origin, such as Alzheimer's, Parkinson's, and prion diseases, as well as type 2 diabetes [1].

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In fact, the aggregation or deposition of IAPP is found in more than 90% of type 2 diabetes patients that may lead to functional failures of many organs in late stages of the disease [2]. Since the forming of fibrils or protofibrils plays a key role in the disease process, inhibition of amyloid fibrils formation is considered as a key therapeutic perspective toward diabetes and other amyloid-related diseases.

Abelmoschus esculentus (L.) Moench., synonym Okra, is a plant of the Malvaceae family. Its fruit is edible and elicits a wide range of medicinal values, including as an antioxidant [3], an antidiabetic and an anti-lipidemic [4], as well as a neuroprotective agent [5]. Gold nanoparticles are promising for drug delivery systems due to their unique optical,

surface, and electronic properties [6]. There are methods to be used for preparing gold nanoparticles, such as by oxidation-reduction reactions coupled with stabilizing agents, electrochemical reactions [7], photochemical reductions [8], and heat evaporation [9]. Most of them are simple, non-toxic, stable, and biocompatible [10-13] techniques.

To our knowledge, what conjugated to the nanoparticles' surface will define their toxicity and biomedical applications [14, 15]. Based on antioxidant activity [16, 17,] and the capability of reducing metal ions of okra seeds extract [18]; it is possible to coat the extract on gold nanoparticles *in-situ*. Real-time reduction of Au^{3+} by the extract during the coating reaction was investigated. Then, interactions between the synthesized nanoparticles and IAPP were examined *in-vitro*. Results acquired may open the door on green nanotechnology for preparing a drug delivery system to mitigate amyloidosis.

MATERIALS: Okra seed extract was received from the authors of the previous study [16]. Human islet amyloid polypeptide (IAPP) (disulfide bridge: 2–7; MW: 3,906; 37 residues: KCNTATCATQRL-ANFLVHSSNNFGAILSSTNVGSNTY) was obtained as a lyophilized powder from AnaSpec (CA, USA). To prepare a stock solution, the peptide was weighed on a Cubis MSE balance (Sartorius), dissolved in Milli-Q water to a concentration of 200 µmole/L, and used immediately for Thioflavin T (ThT) assay and viability test. ThTdye (Sigma-Aldrich) was dissolved in Milli-Q water to a concentration of 200 µmole/L immediately before use. Propidium iodide (PI, Thermo Fisher Scientific) was dissolved in Milli-Q water to be 1 mg/mL solution and kept at -20 °C with light protection.

METHODS:

Synthesis of Okra-Coated Gold Nanoparticles (Okra-AuNPs): Aqueous solution of chloroauric acid (10 mL, 1 mmole/L) was heated to 80 °C. A 2-mg okra seeds extract dissolved in 1 mL water was added to the heated solution, resulting in that the solution's color was changed from yellow to colorless and to reddish pink, respectively. This indicated the appearance of gold nanoparticles. This solution was continually heated for another 2 h, followed by stirring overnight to complete the reaction. The nanoparticles were purified *via* centrifugation filtration and stored at 4 °C until use.

Physical Characterizations of Okra-AuNPs:

Dynamic Light Scattering: The size and zeta potential of the nanoparticles were determined at room temperature by using a dynamic light scattering device (Zetasizer Nano-ZS, Malvern Instruments).

UV-visible Absorption Spectrum: The UV-visible absorption spectrum of the nanoparticles was obtained by using surface plasmon resonance (SPR) with a UV-visible spectroscopy device (UV-3600 UV-Vis-NIR Spectrophotometer, Shimadzu Instruments).

Fourier Transform-Infrared (FTIR) Spectra: FTIR spectra were acquired at room temperature by using Attenuated Total Reflectance Fourier Transform Infrared Spectrometer (IRTracer-100, Shimadzu) in a range between 4000–400 cm^{-1} at 8 cm^{-1} resolution.

Transmission Electron Microscopy (TEM): Morphology of the nanoparticles was determined by TEM (FEI Technei F20) using the carbon-coated copper grid at an accelerated voltage of 200 kV. For visualization, negative staining with 1% uranyl acetate was performed.

Biological Characterizations of okra-AuNPs:

ThT Assay: ThT (100 µmole/L) was mixed with IAPP (50 µmole/L), okra seed extract (12.5, 25, 50, 100 and 200 µg/mL) or okra-AuNPs of each corresponding concentration of okra extract. For the control, ThT was mixed with the extract or okra-AuNPs without the addition of IAPP. Each mixture in the volume of 100 µL was added to a well of the 96-wells plate (Costar black/clear bottom), and ThT fluorescence read from the plate bottom was recorded every 10 min over 20 h (120 readings) using Flex Station 3 Multi-Mode Microplate Reader (Molecular Devices), with excitation at 440 nm and emission at 485 nm.

Cell Viability: Human pancreatic islets beta cells (β-TC6) were cultured in DMEM with 15% FBS supplement. Wells of a 96-well plate was coated with 70 µL of 70 µg/mL poly-D-lysine for 20 min and washed three times with 100 µL PBS. Then, the cells of ~ 5×10^4 cells per well were seeded and

incubated at 37 ℃ in a 5% CO_2 incubator for 2 days. In parallel, mixed solutions containing IAPP (50 µmole/L) with or without 50 µg/mL okra extract or okra-AuNPs were prepared. Each mixture was added to the cells seeded on a well and incubated for 30 min. PI dye solution (1 µmole/L in complete media) was then added.

The cells were imaged on Operetta High-Content Imaging System (PerkinElmer), utilizing standardized excitation/emission settings for PI with images of 5 areas within a well taken every hour for 20 h. Total cell counts per well were evaluated by phase-contrast mapping within the sampling areas. Cell death overtime was expressed as % PI-positive cells per total cell count.

Cell Morphology: β-TC6 cells were incubated with IAPP for 1 h (fresh) or 24 h (old) in the presence or absence of okra seed extract or okra-AuNPs for 30 min. The treated cells were fixed by using 2.5% paraformaldehyde at 4 ℃ overnight. The samples were dehydrated by centrifugation and the medium was replaced by ethanolic solution, which made gradient concentrations ranging from 20% to 80%. Incubation for 2 h was performed by each exchange.

The sample was air-dried on carbon tape, while the morphology of treated βTC6 cells was visualized by using a helium ion microscope (Orion NanoFab, Zeiss) compared to the untreated control.

Statistical Analysis: Data were expressed as mean ± SD of three times repeats. The statistical analysis using Student's t-test was carried out for pairing the data. A value of $p<0.05$ was considered statistically significant.

RESULTS:

Characteristics of Okra-AuNPs: The UV-visible spectrum of okra-AuNPs was shown in **Fig. 1A**. The maximum absorption wavelength was apparent at 521 nm. The purple color displayed by the resulting product confirmed the formation of gold nanoparticles **Fig. 1B**. Their sizes distributed in a range between 30 and 40 nm with an average size of 35.6 nm. At neutral pH, the nanoparticles showed a zeta potential of -59.2 ± 2.9 mV. The TEM image as shown in **Fig. 2C** indicated a spherical shape of okra-AuNPs.

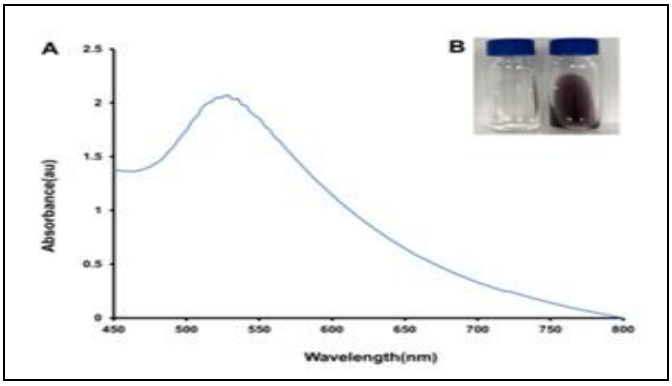

FIG. 1: A, UV–VISIBLE ABSORPTION SPECTRUM OF OKRA-AuNPs; B, COLOR CHANGES IN THE REDUCTION REACTION OF Au^{3+} IONS

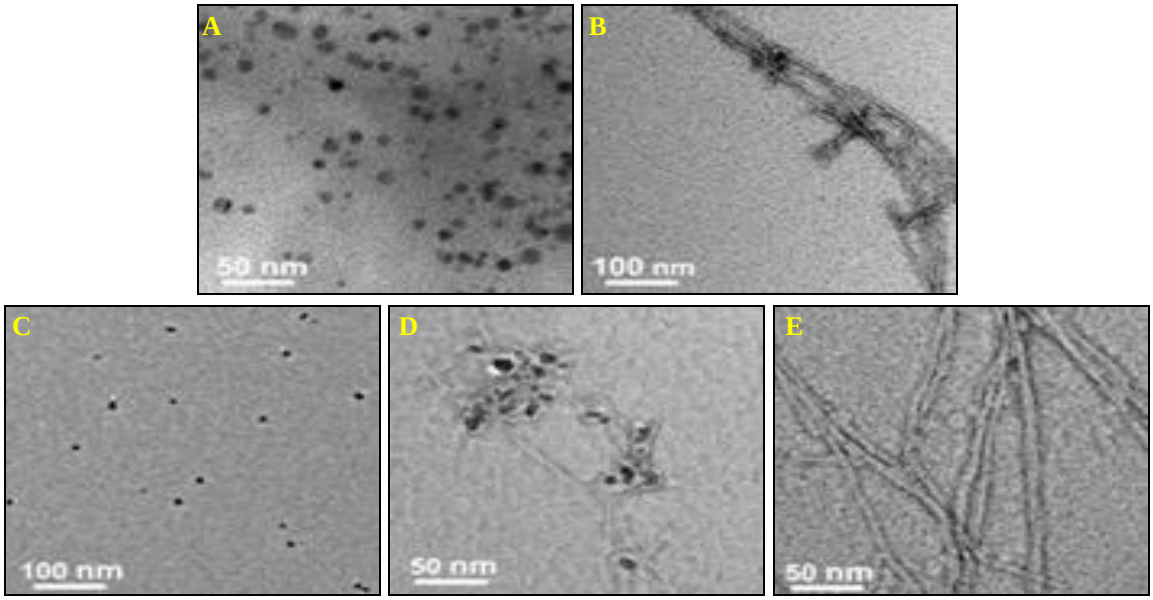

FIG. 2: IMAGES FROM TEM; A, MORPHOLOGY OF OKRA SEEDS EXTRACT; B, AN IMAGE OBTAINED FOLLOWING INCUBATION OF OKRA SEEDS EXTRACT AND IAPP; C, MORPHOLOGY OF THE OKRA-AuNPs; D, AN IMAGE OBTAINED FOLLOWING INCUBATION OF THE OKRA-AuNPs AND IAPP; E, MORPHOLOGY OF IAPP

Inhibition of IAPP Fibrillation: The extent of IAPP fibrillation was determined by ThT assay. IAPP alone exhibited an exponential increase in the fluorescence signal after a lag phase of 7 h **Fig. 3**. This was due to the fibrillation of IAPP into full-length amyloids **Fig. 2E**. By the addition of okra-AuNPs, the fluorescence intensity was extremely reduced. The result indicated a strong inhibitory effect of okra-AuNPs on the IAPP fibrillation. Again, the physical interaction between okra-AuNPs and the IAPP was revealed by TEM results **Fig. 2D**. Okra seed extract alone was unable to inhibit the IAPP fibrillation **Fig. 2B**. The dips of FTIR spectra at wavelength 1625 nm represented the reduction of β-sheets extents in the peptide fibrils **Fig. 4**. Due to Peak-fit analysis, results suggested that okra seed extract and okra-AuNPs reduced the percentage of β-sheets by about 8.5% and 16%, respectively.

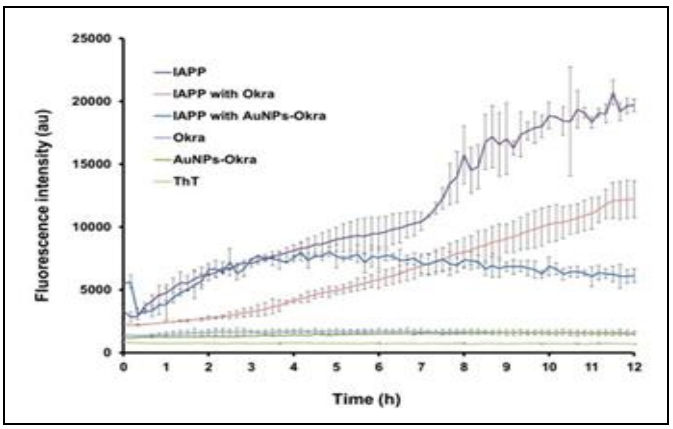

FIG. 3: THT KINETIC ASSAY

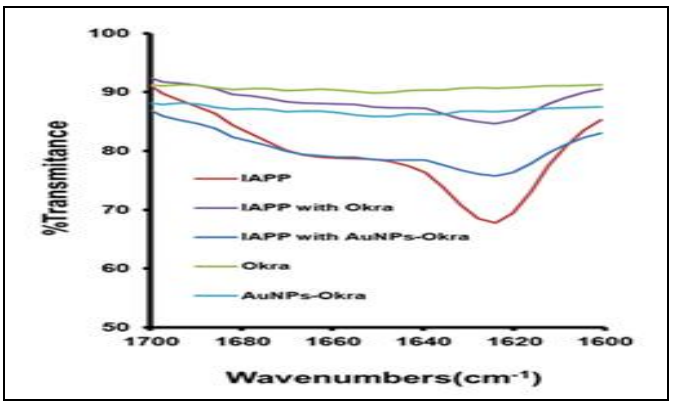

FIG. 4: FTIR SPECTRA AT WAVELENGTHS BETWEEN 1600-1700 nm

Mitigating the Toxicity of IAPP Fibrillation by Okra-AuNPs: The cytotoxicity of IAPP, okra seed extract, and okra-AuNPs on β-TC6 pancreatic beta-cells was investigated. In **Fig. 5**, IAPP was very toxic to the cells regarding the PI signals. Okra seed extract was capable of decreasing the peptide toxicity on those IAPP-treated cells. In addition, most of the treated cells were viable after incubation with okra-AuNPs. It was noted that okra seed extract could recover the cells from IAPP toxicity by about 33.8%. Interestingly, full protection from the IAPP toxicity was indicated by okra-AuNPs treatment. Without incubating with the IAPP, the cells were healthy according to the results of helium ion microscopy (see **Fig. 6**). In contrast, the cells were morphologically damaged when treated with IAPP polypeptide.

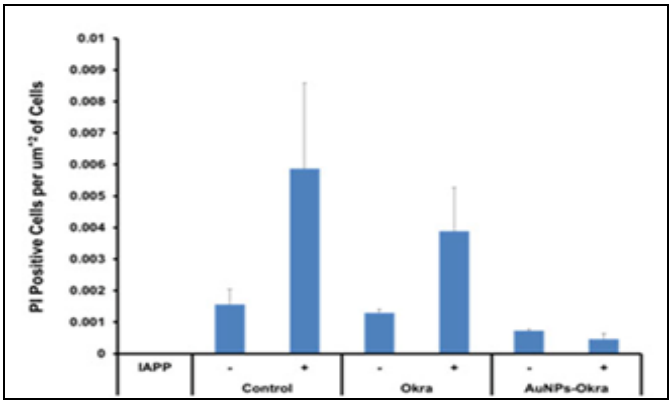

FIG. 5: THE FLUORESCENCE SIGNAL OF PI POSITIVE CELLS

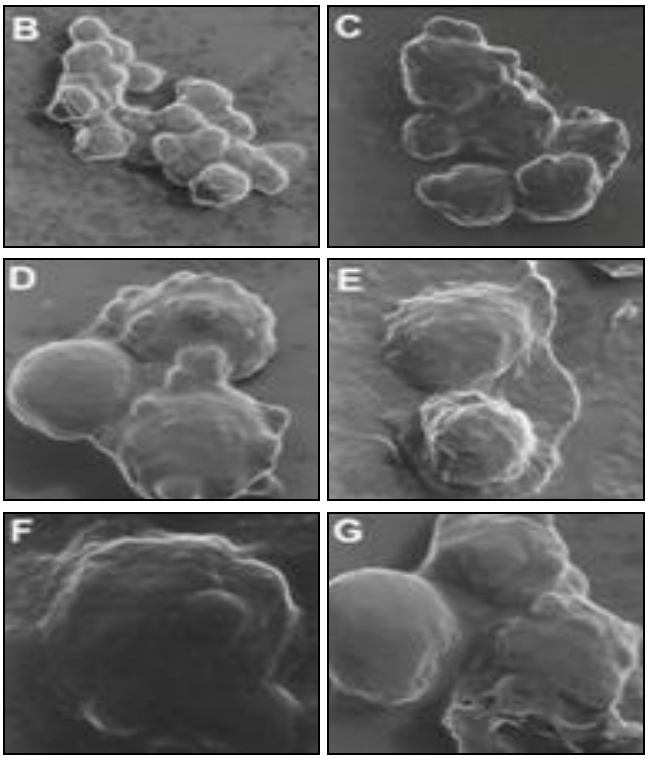

FIG. 6: HELIUM ION MICROSCOPY IMAGES OF B-TC6 CELLS; A, CELLS + OKRA SEED EXTRACT; B, CELLS + OKRA-AuNPs; C, CONTROL CELLS; D, CELLS + IAPP + OKRA SEED EXTRACT; E, CELLS + IAPP + OKRA-AuNPs, F, CELLS + IAPP

DISCUSSION: IAPP is a 37-amino acid residue peptide that is synthesized and co-released with insulin by β-islet cells for functioning in glycaemic control. To our knowledge, self-assembly of IAPP peptide into oligomers and further into long twisted amyloid fibrils, which are rich in β-sheets, may impart toxicity to the surrounding cells and trigger T2D to happen [17].

The surface of gold nanoparticles (AuNPs) not only offers an unusual mode of interaction against amyloid peptides but also enables imaging and stabilizing compounds that immobilized on their surface [15, 18]. The extract from okra seeds has been reported for an antidiabetic activity via α-amylase and α-glucosidase inhibition [19]. Furthermore, it was determined to contain phenolic acids, flavonoids, terpenoids, tannins, β-sitosterol, as well as small chain fatty acids [16]. In this study, it was attractive to prepare okra-AuNPs by conjugating okra seed extract on gold nanoparticles by an *in-situ* reduction reaction.

Upon characterization, the λ_{max} of okra-AuNPs was indicated at 521 nm **Fig. 1A** with sizes ranging between 30 and 40 nm. Indeed, how big they are may depend on chemical properties and phytochemical contents of the used extract [20, 21]. It seemed that β-sitosterol and small chain fatty acids, two major constituents of okra seed extract, would be held responsible for the stability of the synthesized AuNPs [22, 23]. Okra-AuNPs displayed a zeta potential of -59.2 ± 2.9 mV, therefore additionally indicating that they were highly stable.

Inhibition of IAPP fibrillation by okra-AuNPs was evident, as manifested by significant suppression of ThT curve **Fig. 3**. In contrast, okra extract alone showed no inhibitory activity on IAPP fibrillation. As stabilized on AuNPs, the extract could inhibit IAPP fibrillation by truncating the fibrils growth, and okra-AuNPs were embedded in the aggregated IAPP **Fig. 2D**. In regard to the TEM image of **Fig. 2A**, nanomicelles of okra seed extract were apparent. This was suggested by the presence of small chain fatty acids, which responsible for forming nano-micelles [23]. FTIR spectra **Fig. 4** indicated the lowest percentage of β-sheets when treating IAPP with the conjugated AuNPs. This result was further corroborated the ThT data **Fig. 3**. In addition, as revealed by HIM morphology imaging **Fig. 6**, okra-AuNPs mitigated the toxicity of IAPP peptide towards human islets beta cells after 20 h of incubation. The cytotoxicity of IAPP has been associated with the processes of oligomerization and fibrillation of the peptide [24]. The presence of okra-AuNPs in the vicinity might act as nano-sinks in sequestering the fibrillated IAPP on their surface, thereby depleting the peptide concentrations surrounding the islet cells. This was proposed as a mechanism for inhibiting IAPP oligomers' toxicity due to which their binding to the cell membranes was hindered.

CONCLUSION: Preparation of okra coated gold nanoparticles were successfully carried out by using redox reaction. Because IAPP self-assembly, which becomes to belong to twisted amyloid fibrils, was inhibited following incubated with the okra-AuNPs, the gold nanoparticles might be advantageous for use as a delivery system for any neuronal diseases that associate IAPP fibrillation.

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CONFLICTS OF INTEREST: There is no conflict of interest to declare.

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