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Fluorescent quantum dots–zika virus hybrid nanoconjugates for biolabeling, bioimaging, and tracking host-cell interactions

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A B S T R A C T
The earliest possible diagnosis and understanding of the infection mechanisms play a crucial role in the outcome of fighting viral diseases. Thus, we designed and developed for the first time, novel bioconjugates made of Ag-In-S/ZnS (ZAIS) fluorescent quantum dots coupled with ZIKA virus via covalent amide bond with carboxymethylcellulose (CMC) biopolymer for labeling and bioimaging the virus-host cell interactions mechanisms through confocal laser scanning microscopy. This work offers relevant insights regarding the profile of the ZIKA virus-nanoparticle conjugates interactions with VERO cells, which can be applied as a nanoplatform to elucidate the infection mechanisms caused by this viral disease.

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1. Introduction

Despite advances in all areas of knowledge, including health and natural sciences and engineering, the current pandemic outbreak of coronavirus (SARS-CoV-2/COVID-19) poses one of the greatest challenges in the history of humankind. Besides the enormous impact on public health, this outbreak has caused humonous losses to the global economy [1]. Similarly, but on a smaller scale, the Zika virus (ZIKV) epidemic in Brazil also had a great impact on public health. The ZIKV infection rapidly spread across Brazil and to more than 50 other American countries. Although typical ZIKV infections are associated with an acute exanthematous disease, it is also associated with microcephaly and fetal abnormalities during pregnancy [2,3]. Thus, it is a general consensus that the earliest possible diagnosis of the virus infection amalgamated with the understanding of the virus-host cell interactions is crucial for hampering viral dissemination and the patient outcome. In this scenario, emerging nanotechnologies combined with biology and medicine (termed as nanomedicine) can offer an arsenal of weapons for battling against viral diseases. A new class of hybrid nanostructures comprising inorganic nanomaterials and macromolecules have been developed associated with biomolecules, drugs, and virus for targeted applications in nanomedicine [4,5]. These integrated nanosystems encompass the best characteristics of each component for performing designed multiple functions in biomedical applications, which could not be achieved separately. Therefore, fluorescent inorganic semiconductor named as quantum dots (QDs) with unique electronic and optical properties have been used in nanomedicine. QDs are versatile nanomaterials because their photoluminescence emission band can be tunable from the UV to the IR regions by the proper selection of the chemical composition and size of the nanoparticles. More recently, “Cd-free” QDs (e.g., AgInS, CuInS, AgInS/ZnS) produced by green aqueous colloidal routes have been preferred due to the lower toxicity and facile process aiming at biomedical and environmental applications. Additionally, QDs synthesized under hydrophilic conditions using biopolymer ligands (e.g., carboxymethylcellulose, chitosan) permits direct chemical conjugation with biomolecules and inactivated pathogens for biolabeling and bioimaging applications via targeting specific cell-receptor sites [6–8].

Herein we report a novel strategy for the synthesis and characterization of hybrid nanostructured materials made of Ag-In-S/ZnS...
Fig. 1. (A) UV–vis (inset: schematic representation of ZP and Dn of QD in water). (B) HRTEM images of the inorganic core with lattice fringes (zoomed). (C) EDX spectrum. (D) Histogram of size distribution. (E) AFM 3D image with line profile. (F) XRD pattern.
(ZAIS) QDs directly stabilized by carboxymethylcellulose as biocompatible polymer ligand and covalently biofunctionalized with ZIKV virus. They demonstrated to be effective for biolabeling and bioimaging virus-host cell interactions in vitro. These findings deepen our understanding of complex mechanisms of infection of RNA viruses and serve as a preliminary resource for developing potential rapid diagnosis and therapeutic approaches.

2. Material and methods

To avoid redundancy, essential information is described in this section, and all of the materials and standard procedures are detailed at Electronic Supplementary Material.

2.1. Design of bioconjugation

CMC (carboxymethylcellulose) was selected as capping ligand for QDs and N-Ethyl-N’-[3-dimethylaminopropyl]carbodiimide hydrochloride-mediated (EDC) chemistry was chosen for conjugation. The carboxylate groups from CMC and amino groups from viruses (protein-based capsid of the ZIKV virus) formed amide bonds using EDC (sulfo-NHS, N-hydroxysulfosuccinimide sodium salt), as a “zero-length” crosslinker, at pH 6.8–7.4, temperature < 37 °C.

2.2. Synthesis and characterization of nanoconjugates

ZAIS QDs were synthesized via an eco-friendly aqueous route using CMC as a stabilizing agent and salts of metals and sulfide precursors. Next, The ZIKV was bioconjugated to the ZAIS QDs using EDC and sulfo-NHS producing hybrid nanoconjugates (ZIKV_ZAIS).

ZAIS were comprehensively characterized using ultraviolet–visible and photoluminescence spectroscopy (PL), high-resolution transmission electron microscopy (HRTEM) coupled with energy-dispersive X-ray spectra (EDX), X-ray diffraction (XRD), atomic force microscopy (AFM), Fourier transformed infrared spec-
troscopy (FTIR), photoelectrons X-ray spectroscopy (XPS), zeta potential (ZP), and dynamic light scattering (DLS).

African green monkey kidney cell line (VERO) was selected for the biological studies because it is usually employed to research the infective entry of *Flavivirus* in cells [9] and previous reports that showed that VERO cells were susceptible to infection by ZIKA virus [10]. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) protocols were performed to evaluate the *in vitro* cytotoxicity of ZAIS. Moreover, the nanoconjugates were evaluated as fluorescent bioprobes for *in vitro* biolabeling and bioimaging using confocal laser scanning microscopy (CLSM).

### 3. Results and discussion

UV–visible spectroscopy of ZAIS QDs revealed a broad featureless absorption spectrum, with a long tail extending out into the visible region (Fig. 1A), which is characteristic of Ag-In-S ternary systems. This is mostly associated with the size distribution of nanocrystals and the presence of sub-bandgap transitions arising from the intrinsic point defects (vacancies, interstitials, etc.) [11].

The surface charge of ZAIS nanocolloids was determined by zeta potential analysis (ZP = −51.5 ± 3.4 mV). The value is consistent with the predominance of negatively charged surface due to the

![Fig. 3. VERO cell uptake: (A) ZAIS coupled to Zika virus (ZIKV_ZAIS) after 5, 10, and 20 min incubation; (B) controls (ZAIS and ZIKV) experiments for internalization (adsorption time = 15–20 min) ([ZAIS] ~ 50 nmol L^{-1} and ZIKV ~ 2.8 × 10^7 PFU mL^{-1}, scale bar = 10 μm). Fluorescence profiles of QD green emission: (C) with the time of adsorption for ZIKV_ZAIS, and (D) comparison to controls (drawing not to scale). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
carboxylic groups (R-COO⁻) of anionic CMC ligand at physiological pH (pKa ~ 4.3) [11], which promoted the electrostatic stabilization of the nanosystems. The hydrodynamic sizes (Dh) of the colloidal QDs were evaluated by DLS analysis. After synthesis, the sum of the contribution of QD inorganic core with the CMC organic shell and its interactions with the surrounding medium resulted in Dh = 44.0 ± 1.5 nm. Fig. 1A (inset) summarizes ZP and Dh data.

TEM-EDX analyses were performed to access the morphological features, sizes, and elemental composition of the quaternary ZAIS QDs nanocolloids stabilized by CMC biopolymer, which demonstrated the formation of fairly monodispersed nanoparticles with a spherical-like shape (Fig. 1B). The lattice fringes obtained by electron diffraction patterns through HRTEM images (inset, Fig. 1B) evidenced the nanocrystalline characteristics (regular spacing) of ZAIS QDs. The histogram of the nanoparticle size distribution (Fig. 1D) indicated an average size of 4.3 ± 0.7 nm. EDX analysis (Fig. 1C) confirmed the presence of the chemical elements Zn, Ag, In, and S of ZAIS QDs in addition to elements from CMC (C, O, and Na), grid (Cu and C), and EDX detector (Si). High-resolution XPS spectra (Fig. S1) showed all of the elements of QDs with identified oxidation states Zn²⁺, Ag⁰, In⁰, and S²⁻, proving the formation of quaternary nanoalloys (Ag-In-Se/ZnS). FTIR-ATR spectra of CMC polymer in comparison to ZAIS (Fig. S2) indicated changes in the intensities of bands assigned to carboxylic/carboxylate groups due to the formation of the M²⁺-COO⁻ complexes as monodentate and bidentate chelates, as well as changes in OH groups/hydrogen bonds associated with coordination with metal ions/stabilization of QDs.

Typical 3D AFM image presented spherical nanoparticles embedded in the “dry” polymer matrix with the ZAIS conjugate size of approximately 5.2 nm. XRD pattern of ZAIS exhibited three broad peaks, located in between the diffraction reflections of the AgInS₂ and ZnS crystals, which are an indication of the formation of a quaternary alloy [12]. The average size of the nanocrystal was 3 nm, calculated using Scherrer’s equation. The dimension of nanoconjugates estimated by AFM was relatively higher than that measured from TEM because it corresponds to the sum of the inorganic core and polymer shell. Conversely, the nanocrystal size (XRD) was smaller than that of the nanoparticle inorganic core (TEM), in agreement with the literature [13].

To evaluate the potential use of ZAIS conjugates as fluorescent biological nanoprobes, 3D excitation-emission contour curves, quantum yield (QY), and lifetime decay curve were obtained. The 3D plots (Fig. 2A) indicated that ZAIS QDs behaved as active fluorophores suitable for bioimaging (e.g., cell-virus interactions) with a broad range of excitation wavelengths and emission in the green–red visible window, which are mostly ascribed to intrinsic crystallographic and surface defects [11]. ZAIS showed QY of 5%, which has already proven suitable as fluorescent nanoprobes for bioimaging applications [4,14]. The relaxation profile (Fig. S3) followed a bi-exponential decay with an average PL lifetime (τ₀) of 366 ± 62 ns. The longer lifetimes of QDs compared to organic fluorophores favor the tracking of biological processes (continuous, long-term, and enhanced sensitivity) [15,16].

The cytocompatibility in vitro of ZAIS QDs for biological applications was assessed using MITT protocol, where the cell viability responses (≥95%) towards VERO cells after 24 h of incubation are presented in Fig. 2B. These results proved that the QDs nanoconjugates were cytocompatible for biomedical applications. Therefore, ZAIS nanoconjugates were tested as fluorescent nanoprobes for VERO cells through cellular internalization evaluated by CLSM, where the results of cellular uptake and Mean Fluorescence Intensity (MFI) are shown in Fig. 2C and 2D, respectively. The ZAIS nanoconjugates presented a green emission coherent in PL spectroscopy experiments. The results demonstrated the internalization of ZAIS nanoconjugates just after 5 min of incubation, with a significant enhancement of the emission intensities with time, which is vital as they attested their activity as fluorescent bioprobes for tracking cellular events.

For biolabeling and bioimaging virus-host cell interactions in vitro, ZIKV_ZAIS conjugates were incubated with VERO cells. Fig. 3A indicates the green fluorescence of ZIKV_ZAIS in the VERO cell line localized predominantly at the cytoplasm. The fluorescence images presented a relative gradual increase of emission with the time of incubation (5–20 min), which were used to estimate the kinetics profile of interactions of ZIKV_ZAIS bioconjugates with VERO cells using “Mean Fluorescence Intensity” (Fig. 3C).

VERO cells treated with “unbound” ZIKV and ZAIS QDs were used as controls (images, Fig. 3B, MFI values, Fig. 3D) showing less fluorescence than ZIKV_ZAIS after 15–20 min of adsorption. The difference (higher than three times) was associated with the presence of receptors to ZIKV in VERO cells that favored the endocytosis of the nanoparticle-virus conjugates, which can provide a powerful tool for biolabeling and detection of ZIKV virus as well as tracking virus behavior in situ.

4. Conclusions

ZAIS QDs were produced with suitable physicochemical and optical properties to be conjugated with ZIKV and applied as a cytocompatible biomarker. The QDs conjugated with ZIKV presented higher entry to VERO cells in comparison to reference QD (ZAIS), indicating the possibility of labeling and detection of ZIKV for tracking the viral infection processes including virus entry and transport in cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.matlet.2020.128279.

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