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Stabilizing protein pharmaceuticals by imidazolium-type zwitterions

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Abstract

An imidazolium-carboxylate-type zwitterion, OE_2imC_3C, thermally stabilizes the structure in the vicinity of heme in cytochrome *c*. However, it remains unclear whether OE_2imC_3C can thermally stabilize protein pharmaceuticals, which are vulnerable to heating and shaking

during production and transportation. In this study, we investigated the stabilization of the entire structure and function of insulin by OE_2imC_3C. Insulin in the 60 wt% OE_2imC_3C solution did not aggregate under shaking and heating (1,000 rpm and 80 °C) and retained its folded structure. Insulin also exhibited normal function in cell proliferation. In contrast, the secondary structure of insulin in the 40 wt% OE_2imC_3C solution changed, and insulin in the 20 wt% OE_2imC_3C solution aggregated after shaking and heating. Therefore, the 60 wt% OE_2imC_3C solution has potential to stabilize protein pharmaceuticals.

Introduction

The demand for protein pharmaceuticals has recently increased. For example, insulin, a well-known protein pharmaceutical, is widely used as a treatment for diabetes. The global prevalence of diabetes is estimated to be 537 million, with this number expected to rise to 783 million by 2045.[1-3] Antibody drugs are expected to be effective for diseases that are difficult to cure, such as cancer and autoimmune diseases.[4-6] The demand for protein pharmaceuticals is growing annually, and new products are continually being developed.[7]

However, protein pharmaceuticals have complex folded structures. Consequently, they are susceptible to structural alterations from small stresses, such as heating and shaking.[8] For instance, the efficacy of insulin decreases by 14 and 18% when stored at 32 and 37 °C each for

28 days compared with refrigerated storage.[9] Protein denaturation can cause aggregation and thus reduces yield.[10-12] For example, insulin aggregates after heating at 40 °C for 5 min or shaking at 37 °C for 12 h.[13, 14] The resulting aggregation also poses an immunogenic risk.[15] In mice, the injection of aggregated human insulin results in the production of anti-insulin antibodies.[16] The denaturation, aggregation, and immunogenicity-risk have also been confirmed in antibodies. Immunoglobulin G (IgG) is completely denatured and forms a gel-like solution at 55 °C after 25 h.[17] IgG aggregation results in an immune response, characterized by the maturation of dendritic cells and the stimulation of T cells.[18]

Therefore, it is essential to develop new additives that inhibit the denaturation and aggregation of protein pharmaceuticals. For practical applications, a low-toxicity stabilizer that does not require purification before injection is preferable. We previously developed a zwitterion, designated OE_2imC_3C (Figure 1), as a low-toxicity protein stabilizer, and making it a promising candidate for stabilizing protein pharmaceuticals. In previous studies, OE_2imC_3C improved the thermal stability of cytochrome *c*, a non-pharmaceutical protein, with cytochrome *c* remaining stable at 80 °C in 70–80 wt% OE_2imC_3C solutions.[19] However, these evaluations were performed only in the vicinity of heme, without sufficient investigation of the entire protein structure and function. In this study, we evaluated the ability of OE_2imC_3C to stabilize protein secondary structure, prevent aggregation, and preserve function during shaking and heating.

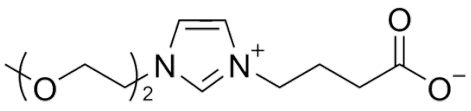

Figure 1 Chemical structure of OE_2imC_3C

Results and discussion

1. The effect of OE_2imC_3C solution on insulin aggregates

In this study, we employed insulin as a model for protein pharmaceuticals. Insulin is insoluble in both water and saline solutions. A popular method for dissolving insulin is to decrease pH by adding HCl. The OE_2imC_3C solution dissolved insulin, under non-acidic environment (pH 8.7–9.6, depending on the OE_2imC_3C concentration). The high hydrogen-bond basicity of OE_2imC_3C [20] is a plausible reason for the dissolution of insulin.

The influence of OE_2imC_3C on insulin aggregation was investigated. The amount of insulin remaining in the supernatant after shaking and heating was measured using the Bradford method[21] (Figure 2). Insulin completely aggregated in a saline solution (dissolved in HCl). In contrast, 60 and 40 wt% OE_2imC_3C solutions stabilized insulin after 1 h of shaking and heating, with remaining high insulin ratios. On the other hand, 20 wt% OE_2imC_3C was ineffective, as most insulin aggregated. Both 40 and 60 wt% OE_2imC_3C effectively inhibited insulin aggregation induced by shaking and heating. IgG, which has a different molecular weight and structure, also showed a similar tendency for aggregation inhibition (Figure S1). These findings suggest that

OE_2imC_3C can function as an inhibitor of protein aggregation.

To investigate the effect of the oligoether side chain (OE_2 group) of OE_2imC_3C, a zwitterion without OE_2 group, C_1imC_3C (Figure S2A), and a diethylene glycol monomethyl ether were subjected. Insulin was dissolved in the equimolar solutions to 60 wt% OE_2imC_3C. Because insulin did not dissolve in a diethylene glycol monomethyl ether solution, it was dissolved by decreasing the pH with HCl. C_1imC_3C solution stabilized insulin after shaking and heating, with remaining high insulin ratio like OE_2imC_3C (Figure S2B). The remaining insulin ratio in the C_1imC_3C solution greater than 1.0 is assumed to be related to the solubility of insulin. The insulin solubility in the C_1imC_3C solution was lower than that in the OE_2imC_3C solution at 0 h, and the absorbance in C_1imC_3C solution increased over time (Figure S2C). The long and low-polar OE_2 group probably interacted with low-polar parts of insulin surface and promoted the insulin solubility. Therefore, the inhibiting aggregation effects of C_1imC_3C and OE_2imC_3C were not significantly different but OE_2imC_3C was presumably suitable from the viewpoint of insulin solubility. On the other hand, a diethylene glycol monomethyl ether solution was ineffective, as most insulin aggregated. From these results, critical effect on the thermal stabilization was derived from not OE_2 group but the zwitterion par. OE_2 group plausibly either does not contribute to the zwitterion solubility in the water because C_1imC_3C is freely miscible in water and long chain polyethylene glycol and diethyl ether are not soluble in water.

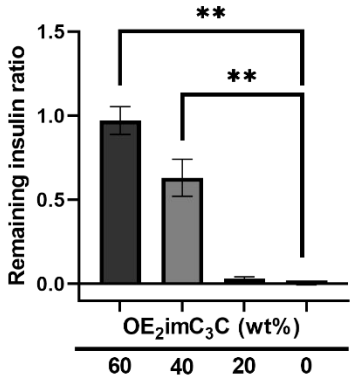

Figure 2 The remaining insulin ratio after shaking at 1,000 rpm and 80 °C for 1h in OE_2imC_3C/saline solutions. Error bars represent standard error. $^{**}p < 0.01$ vs. 0 wt%.

2. Insulin secondary structure

Protein secondary structure was analyzed by Fourier transform-infrared spectroscopy (FT-IR) through the absorbance of the amide I' band, which was primarily attributed to the C=O stretching mode of deuterated peptide groups (1600–1700 cm^{-1}). Here, D_2O was used as the solvent because H_2O absorbs in the same spectral range. Second-derivative IR spectra are used for detailed analyses.[22, 23] Figure 3 shows the second-derivative spectra of insulin before and after shaking and heating. The α-helix and intermolecular β-sheet were evident as negative peaks at approximately 1656 cm^{-1} and 1620 cm^{-1}, respectively.[24] The *a*-helix and intermolecular β-sheet contents qualitatively decreased and increased in D_2O (dissolved with HCl), respectively, after shaking and heating. However, in the 60 wt% OE_2imC_3C solution, the

spectrum after shaking and heating was almost identical to that before shaking and heating, indicating that the formation of intermolecular β-sheet did not occur and the α-helix, which is the native insulin structure, was maintained. The 20 and 40 wt% OE_2imC_3C solutions did not suppress the shift in secondary structures. Calculation of the content of intermolecular β-sheet (Table 1) revealed an increased the content from 0% to 37% in D_2O after shaking and heating. No intermolecular β-sheet was generated in 60 wt% OE_2imC_3C solution, even after shaking and heating. The inhibition of insulin aggregation by 60 wt% OE_2imC_3C was confirmed to be based on the stabilization of the secondary structure.

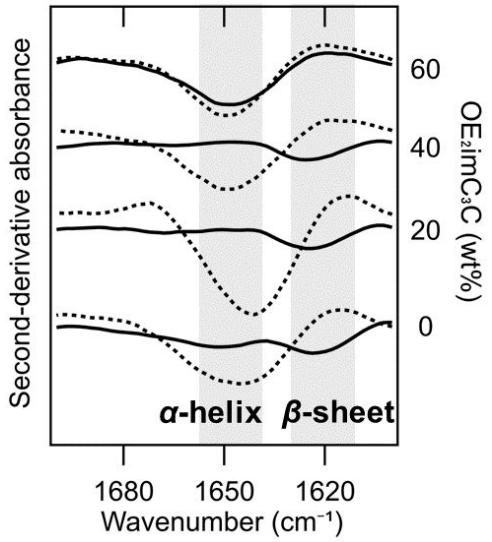

Figure 3 Second derivative FT-IR spectra of insulin before (dashed line) and after (solid line) shaking at 1,000 rpm and 80 °C for 1 h in OE_2imC_3C/D_2O solutions. Insulin in the 0 wt% solution was dissolved with HCl.

Table 1 Intermolecular β-sheet content of insulin before and after shaking at 1,000 rpm and 80 °C for 1 h in OE_2imC_3C/D_2O solutions.

OE_2imC_3C	Intermolecular β-sheet (%)	
(wt%)	Before	After
60	0	0
0*	0	37

*Insulin in the 0 wt% solution was dissolved in HCl

3. Thermal stability of insulin measured by differential scanning calorimetry (DSC)

Protein denaturation is observed as an endothermic peak in DSC charts.[25, 26] The endothermic peaks were not observed in the OE_2imC_3C solutions; instead, exothermic peaks were detected (Figure 4). Exothermic peaks of proteins generally indicate the formation of aggregates.[27, 28] However, insulin aggregation was not observed at 80 °C in the 40 wt% OE_2imC_3C solution (Figure S3). This indicates that the exothermic peaks were not derived from insulin aggregation. A plausible cause of the exothermic peaks is the increased interaction between insulin and OE_2imC_3C. Insulin is initially denatured by heat and then the resulting randomized structure increases the access of OE_2imC_3C to more amino acid residues. OE_2imC_3C has a high hydrogen-bond basicity[20] and can form strong hydrogen bonds with polar residues, which may explain the large exothermic peaks.

The denaturation of insulin was observed from 46 °C as the onset temperature (T_{onset}) in saline (dissolved with HCl) (Figure 4). Nevertheless, the 60 wt% OE_2imC_3C solution enhanced the thermal stability of insulin, shifting T_{onset} to 83 °C. The T_{onset} values for 20 and 40 wt% OE_2imC_3C solutions shifted to around 60 °C. Although we did not observe exothermic denaturation peaks in the OE_2imC_3C solutions, insulin was assumed to be in its natural state before reaching T_{onset}. Therefore, OE_2imC_3C presumably exhibited an efficient stabilizing effect on insulin.

To confirm that OE_2imC_3C stabilizes insulin through direct observation of the denaturation peak, a low-concentration OE_2imC_3C solution (1.2 wt%) was used to avoid the large exothermic peak. However, insulin did not dissolve in the 1.2 wt% OE_2imC_3C solution. Rather, insulin was dissolved in a 60 wt% OE_2imC_3C solution and then diluted to 1:50 (Figure 5). The denaturation temperature of insulin in the 1.2 wt% OE_2imC_3C solution was higher than that in saline (dissolved with HCl), confirming that OE_2imC_3C effectively stabilizes insulin.

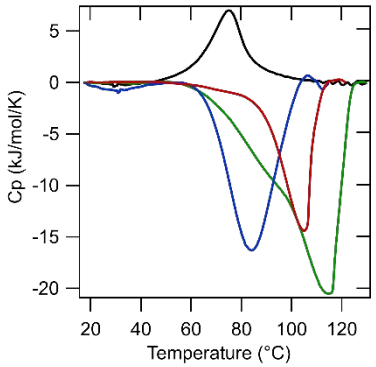

Figure 4 DSC charts of 1 mg/mL insulin in OE_2imC_3C solutions. OE_2imC_3C concentrations: 60 wt% (red line), 40 wt% (blue line), 20 wt% (green line), and 0 wt% (black line, dissolved with

HCl). The scan rate was 120 °C/h.

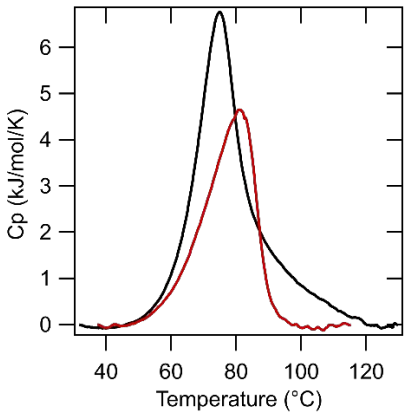

Figure 5 DSC charts of 1 mg/mL insulin in OE_2imC_3C/saline solutions. OE_2imC_3C concentrations: 1.2 wt% (red line) and 0 wt% (black line, dissolved with HCl). The scan rate was 120 °C/h.

4. Insulin function *in vitro*

Insulin plays a role in cell proliferation, and its effect was evaluated in this study. However, an accurate evaluation of insulin function in general culture media is impossible, because the general media contain serum and the serum includes insulin. Here, we chose human promyelocytic leukemia (HL-60) cells because they can proliferate in serum-free media supplemented with insulin, transferrin, and selenium.[29] The shaken and heated insulin in the sample solutions was added to the HL-60 culture medium with 714-fold dilution. Insulin function was disrupted in the saline, as evidenced by reduced cell proliferation after shaking and heating (Figure 6). Insulin function was maintained in the 60 wt% OE_2imC_3C solution, as shown by

unaltered cell proliferation before and after shaking and heating. Insulin function was hindered in the 40 wt% OE_2imC_3C solution, corresponding to reduced cell proliferation. The 60 wt% OE_2imC_3C solution protected the insulin function from shaking and heating.

The number of living cells in the 60 wt% OE_2imC_3C solution before shaking and heating seem to be little bit lower than that in saline (dissolved with HCl) (Figure 6), but this difference was not statistically significant (p = 0.86). Notably, we confirmed that OE_2imC_3C did not affect insulin function. Insulin was added separately with OE_2imC_3C solution to avoid exposure to highly concentrated 60 wt% OE_2imC_3C because less than 40 wt% OE_2imC_3C was inert (see 0 wt% "before" and 40 wt% "before" in Figure 6). Specifically, the insulin dissolved in saline with HCl was diluted 714-fold into the medium, followed by separate addition of the OE_2imC_3C solution. The final concentrations of insulin and OE_2imC_3C was the same as the sample described as 60 wt% "before" in Figure 6, except insulin was not exposed to highly concentrated OE_2imC_3C. Avoiding the exposure to highly concentrated OE_2imC_3C did not increase the number of living cells (see 60 wt% "before" and * in Figure 6). Insulin function was not affected by OE_2imC_3C exposure. OE_2imC_3C cytotoxicity could be another reason for this. Cell viability in serum-containing medium with 0.084 wt% OE_2imC_3C (714-fold dilution of 60 wt% solution) was the same as that in OE_2imC_3C-free medium (p = 0.36, Figure S4). No evidence of OE_2imC_3C cytotoxicity was observed, which is consistent with our previous results.[30, 31] Therefore, we

conclude that the slight decline in the number of living cells in the 60 wt% OE_2imC_3C solution was due to experimental error.

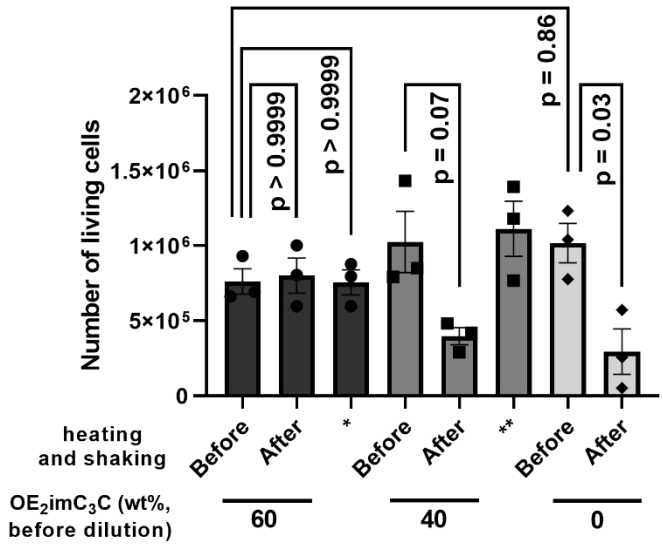

Figure 6 The number of living cells after shaking at 1,000 rpm and 80 ℃ for 1 h in the prepared medium containing insulin in the OE_2imC_3C solution (biological triplicates). */ **: Insulin was dissolved with HCl and diluted 714-fold with medium, followed by the addition of 0.084 or 0.056 wt% OE_2imC_3C (714-fold dilution of 60 or 40 wt% solution). The final concentrations of OE_2imC_3C correspond to the final concentrations used in the comparative experiments. Error bars represent the standard error. Data analysis was performed using one-way ANOVA.

5. Presumed insulin structures in the OE_2imC_3C solutions

A summary of the findings throughout this study is presented in Table 2. The 60 wt% OE_2imC_3C solution inhibited insulin aggregation and preserved its secondary structure.

Additionally, the proliferative function of insulin was preserved. The 40 wt% OE_2imC_3C solution also inhibited insulin aggregation. However, a decrease in the α-helix content and an increase in the intermolecular β-sheet were observed, and insulin function declined after heating and shaking. Therefore, the insulin structure was presumed to be in an intermediate between folded and aggregate states. Similar to saline, the 20 wt% OE_2imC_3C solution did not prevent aggregation nor maintain the secondary structure of insulin.

Table 2 Major secondary structure, function, and presumed state of insulin in the OE_2imC_3C solutions after shaking at 1,000 rpm and 80 °C.

OE_2imC_3C (wt%)	After heating and shaking		
	Major secondary structure (*original: α-helix)	Maintenance of the function	Presumed state
60	α-helix	Yes	folded
40	β-sheet	No	intermediate
20	β-sheet	-[a]	aggregated
0	β-sheet	No	aggregated

-[a] Not studied

Conclusion

The 40 and 60 wt% OE_2imC_3C solutions inhibited insulin aggregation under shaking and heating conditions. In contrast, the 20 wt% OE_2imC_3C solution did not exhibit this inhibitory effect. The secondary structure of insulin was retained in the 60 wt% OE_2imC_3C solution, as

evidenced by the FT-IR analysis. However, a reduction in α-helix content and an increase in intermolecular β-sheet content were observed in the 20 and 40 wt% OE_2imC_3C solutions. The T_{onset} and denaturation temperatures of insulin in the OE_2imC_3C solutions were higher than that in saline, indicating that OE_2imC_3C enhanced the thermal stability of insulin. The 60 wt% OE_2imC_3C solution maintained insulin function after shaking and heating. Thus, OE_2imC_3C was an effective protein stabilizer against shaking and heating, which can occur during transportation.

Materials and methods

1. Materials

Human insulin (recombinant and animal-derived-free) was purchased from Fujifilm Wako Pure Chemical Corp., Osaka, Japan. and used as received. Human IgG was purchased from Equitech-Bio, Inc., Kerrville, TX, USA and used as received. OE_2imC_3C and C_1imC_3C were synthesized as previously described.[32, 33]

2. Preparation of protein solutions and their heating and shaking

Saline was prepared by dissolving 0.9 wt% NaCl in water. Insulin in 0 wt% OE_2imC_3C was dissolved by acidification with HCl (pH < 4) and partially neutralized with NaOH (pH 4.2). The OE_2imC_3C solutions were prepared by diluting with saline. Insulin solutions containing

OE_2imC_3C were prepared via direct dissolution without using HCl. Heating and shaking were conducted at 1,000 rpm and 80 °C using a block shaker (MyBL-100CS, AS ONE Corp., Osaka, Japan).

3. Aggregation ratio of insulin after heating and shaking

Insulin (3.6 mg/mL) was dissolved in OE_2imC_3C solution or saline. The samples were shaken at 1,000 rpm and heated at 80 °C. Bradford reagent was prepared by mixing 5 mg coomassie brilliant blue G-250 in 5 mL methanol, 10 mL of 85 wt% phosphoric acid aqueous solution, and 85 mL of pure water.[21] The insulin samples were diluted 10-fold with pure water and centrifuged at 12,000 rpm for 10 min to precipitate insulin aggregates. The supernatant (10 μL) was mixed with 200 μL Bradford reagent, and the mixture was added to a 96-well plate. Absorbance at 595 nm was measured using a UV-vis plate reader (Epoch, Bio Tek Instruments Inc., Winooski, VT, USA). The amount of insulin remaining after heating and shaking was calculated as:

$$\text{Remaining insulin} = \frac{\text{(Solution absorbance at 595 nm after heating and shaking) - (Solvent absorbance)}}{\text{(Solution absorbance at 595 nm before heating and shaking) - (Solvent absorbance)}}$$

For IgG, the method was essentially the same as that used for insulin, with the following modifications: protein concentration (5.0 mg/mL), solution (PBS), and volume of Bradford

reagent (300 μL).

For heating only, shown in Figure S3, the method was basically the same as for the heated and shaken insulin, with modifications in the insulin concentration (1.0 mg/mL) and the volume of the supernatant (50 μL).

4. FT-IR

Insulin (50 mg/mL) was dissolved in the OE_2imC_3C/D_2O solutions or D_2O. D_2O was used instead of saline because the peak derived from H_2O appears at approximately 1650 cm^{-1}. The samples were shaken and heated at 1,000 rpm and 80 °C for 1 h. The FT-IR spectra (ATR mode) were measured using a Nicolet iS10 (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 20 μL samples. The number of scans was set to 512, with a resolution of 4.0 cm^{-1}. Second-derivative spectra were obtained using the Savitzky-Golay method with 21 points and 3 orders. The intermolecular β-sheet ratio was calculated from the original spectra using the following equation:

$$\text{Intermolecular } \beta\text{-sheet (\%)} = \frac{\text{Integrated intensity of intermolecular } \beta\text{-sheet}}{\text{Sum of integrated intensity of all peaks from 1620 cm-1}} \times 100$$

5. DSC

Insulin (1.0 mg/mL) was dissolved in the OE_2imC_3C solutions (20, 40, and 60 wt%) or saline.

In the case of the 1.2 wt% OE_2imC_3C solution, 50 mg/mL insulin in the 60 wt% OE_2imC_3C was diluted with saline because 1.0 mg/mL insulin was insoluble in the 1.2 wt% OE_2imC_3C solution. DSC was performed using a MicroCal PEAQ-DSC instrument (Malvern Panalytical Ltd., Malvern, WR, UK). Measurements were performed at 10−130 °C heating scan, scan rate of 120 °C/h, and no feedback mode. The progress type of baseline parameters was selected. The none fitting model was applied when only exothermal peaks were present; otherwise, a non-two-state fitting model was used.

6. Cell culture

HL-60 cells were precultured in Roswell park memorial institute (RPMI) 1640 medium (Nacalai Tesque Inc.) containing 10 vol% fetal bovine serum (FBS; Sigma-Aldrich Co., LLC) and 1.0 vol% penicillin–streptomycin-amphotericin B suspension solution (P/S/A; Fujifilm Wako Pure Chemical Co.) at 37 °C under 5% CO_2 humidified atmosphere.

For the cell proliferation experiments with heated and shaken insulin, insulin (3.6 mg/mL) was dissolved in the OE_2imC_3C solution or saline. The samples were shaken and heated at 1,000 rpm and 80 °C for 1 h. Insulin samples were diluted to 5.0 mg/L (714-fold dilution) with RPMI 1640 containing 1.0 vol% P/S/A, 20 mM HEPES/NaOH (HEPES and NaOH was purchased from Tokyo Chemical Industry Co., Ltd. and Kanto Chemical Co., Inc., respectively), 30 nM selenium dioxide (Tokyo Chemical Industry Co., Ltd.), and 5.0 mg/L transferrin (Fujifilm

Wako Pure Chemical), prepared by using 1.7 M HEPES buffer (pH 7.2), 3.3 mg/L selenium dioxide, and 3.6 mg/mL transferrin. Selenium dioxide (3.3 mg/L) was prepared using a 1,000-fold dilution of a 3.3 mg/mL solution. Cells (2×10^5 cells) were cultured in the media in 6-well plates for six days. The number of living cells was counted using hemocytometers.

The toxicity of OE_2imC_3C was investigated using an MTS assay. Cells (1×10^4 cells) were cultured in RPMI 1640 medium containing 10 vol% FBS, 1.0 vol% P/S/A, and OE_2imC_3C in 96-well plates for 6 days. Relative cell viability was obtained from the absorbance of the sample at 490 nm using MTS reagent (Promega Co.). Absorbance was measured using a multi-plate reader (Infinite M Plex, Tecan Group Ltd., Männedorf, Switzerland).

7. Statistical analysis

Data analysis was conducted using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, otherwise noted in the caption. Statistical tests were performed using GraphPad Prism9.

Conflicts of interest

There are no conflicts to declare.

Data availability

All data are available in the main text or the ESI.

Acknowledgements

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Footnotes

† Electronic supplementary information (ESI) available. See DOI:

Data Availability

All data are available in the main text or the supplementary materials.