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A supramolecular formulation of icariin@sulfonatoazocalixarene for hypoxia-targeted osteoarthritis therapy

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ABSTRACT

Osteoarthritis (OA) is the most prevalent joint disease and icariin is a promising drug for its treatment. However, the clinical use of icariin is hindered by poor water solubility, low bioavailability, and non-specific release and biological distribution. Herein, sulfonated azocalix[4]arene (SAC4A) with enhanced water solubility, recognition capacity, and designed responsiveness was used to improve the efficiency of icariin for OA therapy. SAC4A, a macrocycle with well-defined molecular weight and structure, could encapsulate and enhance water solubility of various drugs. In addition, SAC4A enables hypoxia-responsive release of loaded drug. Compared with icariin treatment, supramolecular complex icariin@SAC4A significantly relieved OA symptoms of rats, including more regular bone morphology and structure, and lower degree of cartilage damage. Moreover, the supramolecular formulation demonstrated various advantages, including easy preparation, hypoxia-triggered release, and small size that conducive to drug penetration.

Osteoarthritis (OA), a disease with complex etiology, occurs in the knee joint, hip joint, spine and other parts with heavy load [1,2]. The pathological features of osteoarthritis include chondromalacia, fibrosis, osteophyte formation, subchondral bone remodeling and synovial degeneration [3,4]. The pathological microenvironment of OA encompasses low pH, hypoxia, and overexpression of reactive oxygens and inflammatory cytokines [5–8]. Presently, the treatment of early OA is mainly based on anti-inflammation and analgesia [9]. However, long-term take non-steroidal anti-inflammatory drugs orally would cause side effects such as gastric mucosal lesions and cardiovascular events, and cannot prevent the further process of OA [10]. In recent years, icariin, a flavonol glycoside compound, has exhibited positive effects in the treatment of OA [11]. Icariin can inhibit inflammatory factor-induced synovitis, promote osteogenesis, inhibit the degradation of cartilage extracellular matrix, and promote the proliferation of chondrocytes. Therefore, it could relieve pain, prevent subchondral bone lesion, and delay the destruction and repair the lesions of cartilage [12–14]. In short, icariin effectively treats osteoarthritis, especially in the early stage, but its clinical use is hindered by low bioavailability caused by poor water solubility, and bad biodistribution caused by lack of targeting and controlled release [15–17].

Various types of drug carriers, including polymer nanoparticles, inorganic nanoparticles, and hydrogel-based nanoparticles, have been developed for icariin delivery [18–22]. For example, gelatin nanoparticles enhanced icariin's solubility and enable sustained release [23]. Niu group reported exosomes that loading icariin in exosomes improved its bioavailability [24]. Li reported targeted delivery and controlled release of icariin in nanoparticles functionalized with Arg-Gly-Asp-peptide, using near-infrared light to induce osteogenic differentiation in mesenchymal stem cells [20]. Overall, most icariin delivery systems have improved solubility and achieved a degree of sustained release. However, the icariin delivery systems that have *in vivo* validation for treating OA and the control of release in response to the microenvironment have not been reported. Herein, we disclosed a macrocyclic carrier for icariin delivery based on host-guest complexation, exhibiting

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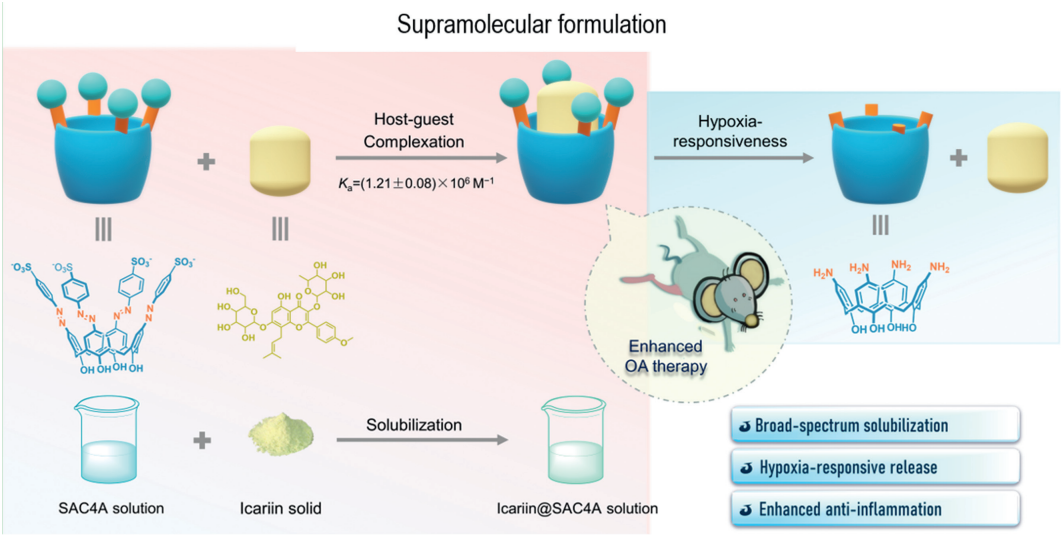

Scheme 1. Schematic illustration of the design of supramolecular formulation icariin@SAC4A and its enhanced therapy for OA.

exceptional solubility, responsiveness to hypoxia stimuli, and facilitates deep penetration into cells and tissues.

Host-guest complexation is a novel approach for constructing drug delivery system, providing quantitative drug encapsulation and molecular-level protection [25–30]. Besides, the dynamic and reversible features of host-guest interactions endow the complexes sensitively responding to disease-specific microenvironments for a rapid drug release [31–34]. Unlike traditional nanocarriers, macrocycles not only show operational simplicity but also possess well-defined molecular structures and molecular weight, which can guarantee the batch-to-batch consistency [35,36]. The unique properties of tunable cavity size and easy modification empower macrocyclic hosts with fascinating molecular recognition, thereby quantitatively loading a wide range of drug guests [37–40]. The constructed host-guest formulation can provide many benefits, such as ease-of-preparation, improved water solubility and stability, molecular-level protection, sensitive response to biostimuli, traceless release, and adaptability to different drugs [41–44]. The host-guest drug delivery system offers an effective avenue to icariin delivery, with the design of macrocyclic carriers as its pivotal cornerstone.

In this work, sulfonated azocalix[4]arene (SAC4A) was synthesized to improve the efficiency of icariin for OA therapy. To improve the bioavailability of icariin, a drug-carrier with good solubility and stimulus response is needed. Calix[4]arene has a wide application in drug delivery for its loading cavity [27,45,46]. The modification of azo groups deepens the cavity of calix[4]arene, thus enabling SAC4A to provide stronger binding affinity towards drugs. Furthermore, SAC4A's sensitivity to the hypoxic inflammatory microenvironment, mediated by azo groups, enables precise and rapid drug release at the site of inflammation [47]. Sulfonate groups were decorated at the upper rim to increase the solubility and solubilization capacity. SAC4A improved the solubility of icariin approximately 4.4×10^3-fold by simple mixing its water solution with icariin solid at 50 °C for 30 min. SAC4A also improved the solubility of triamcinolone acetonide acetate, dexamethasone and budesonide under mild conditions through simple preparation. The host-guest formulation, icariin@SAC4A, achieved various advantages: (i) quantitative loading and reproducibility, (ii) simple and repeatable preparation, (iii) improved water-solubility and bioavailability of hydrophobic drugs, (iv) controlled release *via* endogenous hypoxia stimuli. Finally, the supramolecular formulation icariin@SAC4A effectively inhibited the OA process (Scheme 1).

We firstly investigated the exact cavity-loading pattern *via* host–drug inclusion and quantified host–drug binding constant (K_a). The independent gradient model based on hirshfeld partition (IGMH) analysis [48] were performed with Multiwfn (Version 3.8(dev), release date: 2023-Sep-30) [49]. Molecular graphs were visualized by the VMD program [50]. Icariin match well with the cavity of SAC4A were further validated by computational simulations (Fig. 1A). There are hydrogen bond interaction, including C–H···π, O–H···O, O–H···N, and C–H···N, between icariin and SAC4A (Fig. 1B). High K_a between SAC4A and icariin is necessary to guarantee an efficient solubilization to icariin and prevent premature dissociation. SAC4A exhibited high K_a for icariin of $(1.5 \pm 0.3) \times 10^6$ L/mol by fitting fluorescence titration data according to a 1:1 competitive binding stoichiometry (Fig. 1C and Fig. S1 in Supporting information). Binding affinities lower than 10^5 L/mol are considered insufficient because of dilution in the body [31]. Thus, the icariin@SAC4A complex is sufficiently stable to resist the dissociation caused by body fluid dilution. To facilitate monitoring, we employed the fluorescent dye Rhodamine B (RhB) instead of icariin. The binding affinity of RhB@SAC4A was reported to be 1.20×10^6 L/mol [51], which was equivalent to that of icariin@SAC4A. No remarkable fluorescence regeneration of RhB@SAC4A was observed upon the addition of important biological species (Fig. 1D), indicating the robust complexation stability of icariin@SAC4A with excellent selectivity towards biologically co-existing species, which could avoid the unwarranted off-target dissociation in organisms. Hypoxia-responsiveness of SAC4A was demonstrated by its reduction within 8 min after adding sodium dithionite (SDT) [51]. Fast kinetics means the loaded drug could be quickly released when SAC4A is reduced to NH_2C4A. The fluorescence of RhB could be quenched by complexation with SAC4A but not with NH_2C4A (Fig. 1E), which thermodynamically proves the feasibility of hypoxia-triggered release. The release kinetics of the formulation were evaluated (Fig. 1F) and obtained a rate constant of 2.3 min^{-1} according to the *quasi*-first-order reaction decay model, as well as a half-life of 0.30 min (Fig. S2 in Supporting information).

Then, the general applicability of SAC4A for the solubilization of various drugs was investigated. Acetate triamcinolone acetonide, dexamethasone, and budesonide are all glucocorticoids with potent anti-inflammatory effects [4,6]. The water solubility of icariin is as low as 34 μmol/L (25 °C). The complexation of SAC4A solubilized icariin showed that the solubility of icariin was 1.5×10^5 μmol/L,

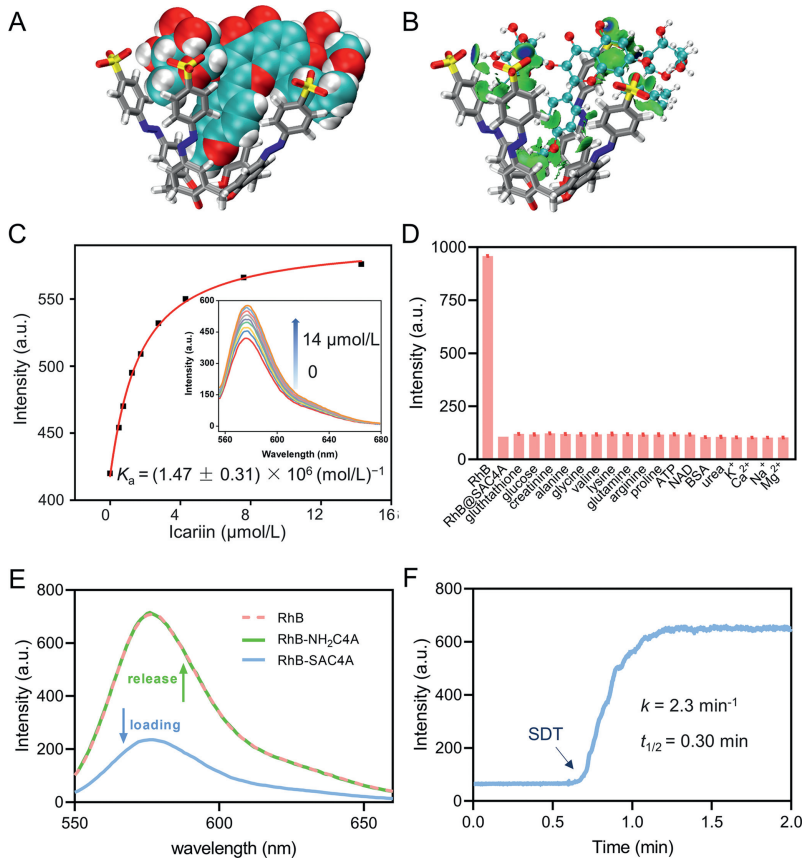

Fig. 1. (A) Optimized binding geometry of the icariin@SAC4A complex at the B3LYP/6–31G(d)/SMD (water) level of theory with Grimme's D3 dispersion correction. (B) IGMH analysis of interaction between icariin and SAC4A, the figure was generated by Multiwfn and visualized by VMD, $\delta g^{inter} = 0.005$ a.u. Isosurfaces colored according to a blue-green-red scheme, blue indicates a strong attraction, green indicates an attraction, and red indicates a repulsion. (C) The associated titration curve at $\lambda_{em} = 576$ nm fitted according to a 1:1 competitive binding stoichiometry. Inset: competitive fluorescence titration of RhB@SAC4A (0.50/0.50 μmol/L) reporter pair with icariin (up to 14 μmol/L) in PBS (10 mmol/L, pH 7.4) at 25 °C, $\lambda_{ex} = 540$ nm. (D) Competitive release of RhB from the RhB@SAC4A complex (1.0/5.0 μmol/L) in PBS (10 mmol/L, pH 7.4) at 25 °C in response to various representative components of blood. RhB alone was used as a control. All results are expressed as mean ± standard deviation (SD) ($n = 3$). (E) Fluorescence spectra of RhB (1.0 μmol/L) before and after addition of NH_2C4A (2.0 μmol/L) and SAC4A (2.0 μmol/L) in PBS (10 mmol/L, pH 7.4), 25 °C, $\lambda_{em} = 576$ nm. (F) Release kinetics of RhB@SAC4A (2.0/12 μmol/L) after addition of sodium hydrosulfite (1.0 mmol/L SDT, a mimicking reagent of azoreductase) in PBS (10 mmol/L, pH 7.4) at 25 °C. The release kinetics of RhB was reasonably deduced to that of icariin because of their comparable binding affinities.

Table 1
Solubilization effect of SAC4A on various drugs.

Drugs	Water solubility (μmol/L)	Enhanced solubility by SAC4A (μmol/L)	Solubilization ratio
Icariin	$(3.4 \pm 0.1) \times 10^1$	$(1.5 \pm 0.1) \times 10^5$	$(4.4 \pm 0.1) \times 10^3$
Triamcinolone acetonide acetate	4.5 ± 0.1	$(2.3 \pm 0.1) \times 10^4$	$(5.2 \pm 0.1) \times 10^3$
Dexamethasone	3.0 ± 0.2	$(1.2 \pm 0.1) \times 10^5$	$(3.9 \pm 0.1) \times 10^4$
Budesonide	48 [52]	$(7.5 \pm 0.2) \times 10^4$	$(1.6 \pm 0.1) \times 10^3$

which increased by approximately 4.4×10^3-fold. Notably, the solubility of triamcinolone acetonide acetate, dexamethasone and budesonide is 34, 3.0 and 48 μmol/L (25 °C), respectively, which was increased by 5.2×10^3, 3.9×10^4 and 1.6×10^3-fold, respectively. The binding affinities of SAC4A towards triamcinolone acetonide acetate and dexamethas were reported to be 3.8×10^6 and 3.3×10^5 L/mol [26], and the binding affinity of SAC4A and budesonide was detected to be $(7.2 \pm 1.3) \times 10^6$ L/mol (Fig. S3 in Supporting information). These excellent solubilizing effects are originating from the excellent water solubility of SAC4A and strong binding affinity with various drugs. The interactions between SAC4A and a neutral molecule may include π-π stacking, hydrogen bonding and hydrophobic interactions, which enables the high binding affinity of SAC4A and a neutral molecule (Table 1) [47].

Collagenase-3 (MMP13) is a major catabolic factor involved in cartilage degradation. MMP13 activity is crucial for OA development and progression, and is a promising target for therapeutic intervention. Fig. 2A showed that the MMP13 expression of lipopolysaccharide (LPS) treated chondrocytes was obviously higher than that in normal group, and MMP13 expression decreased after treating with icariin@SAC4A. This result indicated that icariin@SAC4A had the potential to inhibit cartilage degradation. Col II is the major component of the cartilage matrix. Degra-

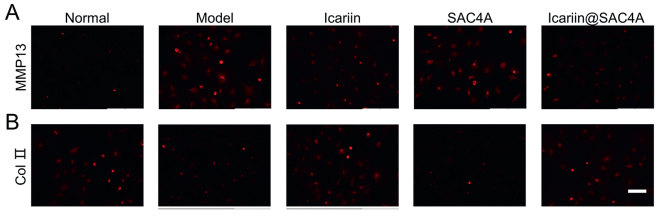

Fig. 2. Fluorescence image of (A) MMP13 and (B) Col II staining after icariin, SAC4A and icariin@SAC4A treatment, respectively. Model group was stimulated by LPS. Scale bar: 100 μm.

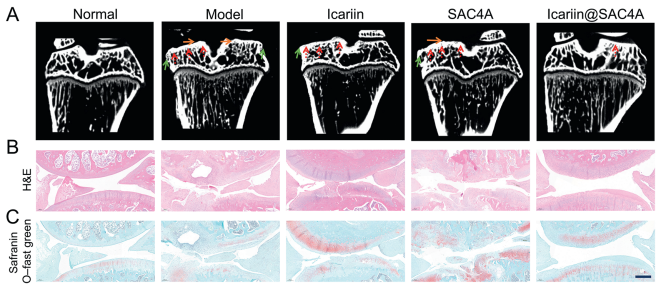

Fig. 3. (A) Micro-CT corona scanning graph of different treatment group. Red arrows indicate sclerosis of subchondral bone, orange arrows indicate the rough surface of tibial plateau, green arrows indicate osteophyte proliferation. (B) H&E staining images and (C) safranin O–fast green images of joints treated with different modalities. Scale bar: 500 μm.

dation and reduction of Col II, which are regarded as typical pathological changes, are frequently observed in OA cartilage. Col II expression of chondrocytes was decreased after treated with LPS. With the treatment of icariin or icariin@SAC4A, Col II expression of chondrocytes can be recovered (Fig. 2B). These results demonstrated that icariin@SAC4A can inhibit the expression of MMP13 and promote the production of Col II, indicating its potential for the OA therapy.

Following arthritis induction, PBS, icariin, SAC4A and icariin@SAC4A were delivered into arthritic knees *via* intra-articular injection every week. At the study endpoint, the knee joints in different treatment groups were assessed by micro-CT to analyze bone morphology and bone microstructure. Rats in the Model group showed that hyperplasia of osteophytes around the tibial plateau and sclerosis of subchondral bone. These symptoms in the icariin treatment group were alleviated (Fig. 3A). The treatment effect of the icariin@SAC4A group was more significant. The surface of the tibial plateau in this group was smooth, without obvious surrounding osteophyte proliferation, and the sclerosis of subchondral bone was also significantly reduced. 3D-reconstruction images showed the similar results with coronal scan (Fig. S4 in Supporting information). Furthermore, the therapeutic efficacy of icariin@SAC4A was analyzed by histological examination. H&E stained images of knee joints from the OA model group showed severe bone destruction, irregular surface, reduced chondrocyte, reduced staining of cartilage matrix, and disruption of Tidemark, which are typical symptoms of OA (Fig. 3B). Treatment with icariin only moderately suppressed these symptoms. In comparison, knee joints of rats treated with icariin@SAC4A showed more regular cartilage structure, including smooth surface of cartilage, increased chondrocyte, slightly reduced staining of cartilage matrix, and intact Tidemark. Safranin-O staining was then performed to assess cartilage degradation and extracellular cartilage matrix (ECM) changes. Rats in the Model group exhibited severely damaged cartilage, synovial hyperplasia, destruction of cartilage surface, and exposure of subchondral bone. Icariin treating group exhibited better structure of cartilage and ECM than the Model group (Fig. 3C). Icariin@SAC4A treatment significantly relieved OA symptoms, including smoother and more complete cartilage surface, less damaged cartilage, and significantly increased subchondral stroma. Quantification by Mankin score [53] showed the lower score of icariin@SAC4A treatment group compared with icariin and SAC4A treatment alone, indicating the lower degree of cartilage damage (Fig. S5 in Supporting information). Collectively, icariin@SAC4A has a certain therapeutic effect on OA and can significantly inhibit its progress.

In this work, SAC4A was used to improve the efficacy of icariin for OA therapy *in vivo* from improving the water solubility and hypoxia triggered release. Attributed to excellent water solubility of SAC4A (153.0 mmol/L) [54] and strong binding affinity with icariin, the solubility of icariin was increased by 4.4×10^3-fold. Besides, strong binding affinity is beneficial to the stability of icariin@SAC4A with excellent selectivity towards biologically co-existing species, and avoiding off-target leakage. Hypoxia-triggered drug release can reduce toxic side effects on normal tissues. Finally, SAC4A improved the efficacy of icariin, and icariin@SAC4A efficiently alleviated the progression of OA. Notably, the solubility of triamcinolone acetonide acetate, dexamethasone and budesonide was also increased by 5.2×10^3, 3.9×10^4, and 1.6×10^3-fold, respectively. SAC4A is a universal solubilizing platform to various drugs, besides, the spontaneity nature of host-guest recognition enables SAC4A-drug complex to be formulated simply and reproducibly. Just as important, icariin@SAC4A is a small-size (1−2 nm) drug formulation and maybe more conducive to diffusion and deep penetration than large nanoparticles. Additionally, small-molecules are easy to manufacture and store, which usually makes them affordable for pharmaceutical production and patients. SAC4A fulfills the essential requirements for a successful drug-delivery system from the viewpoints of both scientific research and manufacture.

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.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.109752.

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