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Reduction diameter of $CaCO_3$ crystals by using poly acrylic acid might improve cellular uptake of encapsulated curcumin in breast cancer

Abstract

Although curcumin has many biological activities like antioxidant, anti-inflammatory, anti-tumorigenic anti-coagulant, anti-bacterial and anti-carcinogenic agents it is not widely used for cancer treatment because of its poor aqueous solubility, low adsorption, rapid metabolism and finally it is very sensitive to light. A strategy to encapsulate it in a nanosized delivery carrier is still needed. Since the rate of cellular uptake depends mainly on size and shape of nano-carrier. For this reason, our target is to produce polylectrolyte multilayer capsules depending on characteristic of template such as shape, size and charge. $CaCO_3$ has been chosen as a template because there is no histological evidence for its toxicity after core removal with ethylene di-amine tetra-acetic acid (EDTA) and it is safer in handling than other templates. One important issue is how to get smaller $CaCO_3$ template in range of nanosized scale bar while keeping spherical shape and non-toxic as main target during preparation. In this study, polyacrylic acid (PAA) as a biodegradable polymer was used for shape and size of $CaCO_3$ control during fabrication. Zeta potential was used for identifying ionic properties of polymer adsorption after each coating layer. Curcumin was loaded into capsules after core removal and adsorption was measured by spectrophotometry. Cytotoxicity and cellular internalization were measured by using 3,-4,5 dimethyithiazol-2,5 diphenyl tetrazolium bromide (MTT) assays and transmitted light fluorescence microscopy in empty capsules and in curcumin-loaded capsules. Encapsulation of curcumin inside carrier doped with PAA has resolved two issues: PAA has improved the size and shape of carrier and the carrier could be used as container for curcumin encapsulation.

Keywords: curcumin, calcium carbonate, poly(acrylic)acid, cytotoxicity, nanosized delivery carrier

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Abbreviations: NP, nanoparticles; CHI, chitosan; MLNP, multilayer nanoparticles; PSS, poly (sodium 4-styrene-sulfonate); PAH, poly (allylamine hydrochloride); EDTA, ethylene di-amine tetra-acetic acid; PAA, polyacrylic acid; MTT, 3,-4,5 dimethyithiazol-2,5 diphenyl tetrazolium bromide; FRS, Fetal Bovine Serum; DMSO, Fetal Bovine Serum; TEM, Transmission Electron Microscopy; FITC, fluorescence isothiocyanate;

Introduction

In the current days, the strategy of cancer nano-therapy based on designing bio nano engineering carriers, have ability to overcome issues of previous cancer chemotherapy.[1,2] The intelligent nano-therapies are being designed in order to be accumulated at targeted sites, to be prolonged in blood circulation and to reduce drug resistance.[3] In spite of these desirable features, certain drawback related to nanoparticles template should be addressed especially for $CaCO_3$ templates such as aggregation state, uncontrolled template crystallization and control $CaCO_3$ dimension.[4,5] Initially, $CaCO_3$ nanoparticles that are fabricated for drug delivery application should be functionalized by many alternate layers forming finally multilayer nanoparticles (MLNPs) after core removal. In this case, the multilayers can increase efficiency of nanoparticles by controlling properties like thickness, composition, roughness, porosity offering good vehicles for biomedical and pharmaceutics application.[6] However the optimization of MLNPs depends mainly on designing of $CaCO_3$ template. Hanafy[4] studied control $CaCO_3$ template by integration polymers inside $CaCO_3$ matrix during it is fabrication by using biodegradable polymers such as polyacrylic acid (PAA) and chitosan (CHI) and non-biodegradable polymers such as poly (sodium 4-styrene-sulfonate) (PSS), poly (allylamine hydrochloride) (PAH). The final result showed that the integration of polymers (either biodegradable or non-biodegradable) inside $CaCO_3$ template can control $CaCO_3$ crystallization providing in most case, homogenous population. Also, the control $CaCO_3$ dimension toward elongated like rode shape was studied. Since PAH is a weak polymer can prolonged at alkaline pH providing interior supporter inside $CaCO_3$ matrix.[7] To accept advantage of designing suitable template for biomedical application, the control $CaCO_3$ matrix needs other investigation. Since, this network matrix-type is important for the final capsule building against environmental conditions, such as temperature and humidity. Moreover, this network type might give capsule mechanical improvement to store it for long time. Additionally, the network matrix might be important for capsule structure in blood stream.[8] The obtained capsule quality is closely related to the quality of prepared template. In our previous work, PAA provided $CaCO_3$crystals with diameter ranged as 400 nm-600 nm. Our recent attempt is to reduce diameter of $CaCO_3$ down 400nm with keeping spherical shape and non-toxic as the main target during preparation. Three concentrations of PAA (1,2 and 3 mg/1 ml) are used and zeta potential surface were measured in case of template, during alternate coating and after core removal. The second aim is to investigate the cellular cytotoxicity of encapsulate curcumin in breast cancer by using MCF-7 cell lines.

Material and methods

Chemicals

The suppliers of the chemicals were as follows: protamine salt, grade III (PRM) from Sigma, USA: Dextran sulfate sodium salt from Leuconostoc spp., poly acrylic acid (PAA) from sigma: Calcium chloride dehydrate 99,99% ($CaCl_2$-$2H_2O$) from Aldrich, USA: Sodium carbonate (Na_2CO_3) from Merck, Germany; ethylenediaminetetraacetic acid disodium salt dihydrate 99+% from Sigma, USA. Curcumin, Dulbecco's modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), L-glutamine, Penicillin/Streptomycin, Trypsin thiazolyl blue tetrazolium bromide, Dimethyl sulfoxide (DMSO) and MTT were purchased from Sigma-Aldrich (Milan, Italy).

Polymers preparation condition

The concentration of used PAA introduced after several experiments. The final three concentrations are considered as following (1mg, 2mg &3mg/1ml d.w).

Fabrication of $CaCO_3$ Particles

Calcium carbonate particles ($CaCO_3$) were fabricated with the same molar used by Volodkin et al. Briefly, 750µl of PAA polymer was taken in glass bottle and mixed well with 615 µl of 0.33 M Na_2CO_3 then 615 µl of 0.33M $CaCl_2.H_2O$ was added rapidly under magnetic starrier for 30 second and the solution left for 3 minutes without stirring for sedimentation time. $CaCO_3$ particles were collected by centrifuge at 5000rpm for 1 minute and washed three times by using Mill Q Water. The procedure results in highly homogeneous, spherical $CaCO_3$ nanoparticles. PAA used in three concentrations; (1mg/ml, 2mg/ml and 3mg/ml). Calcium carbonate was fabricated in two conditions; PAA mixed with $CaCL_2$ and PAA mixed with Na_2CO_3. The modification was studied by zeta potential.

Fabrication of PRM/DEX multilayer on PAA doped $CaCO_3$ template

Protamine and dextran were assembled on the core template alternatively in 0.5M NaCl solution for 20 minutes (3min. sonication+17min. shaking) followed by three times washing in milli Q water. The concentration of each polymer was 5mg/ml. The excess of polyelectrolyte polymers was removed by centrifugation at 3000 rpm for 6 min. After assembling the layers, capsules were obtained by dissolving the core in 0.2 M. EDTA (pH 5.5) solution under shaking for 30 min. followed by washing three times by using 0.2M.EDTA (pH 7.2). Capsules were centrifuged under low speed 800rpm /15 min. to prevent aggregation and they were washed three times in milli Q water. Furthermore the obtained capsules were kept in PBS pH 7.3.

Transmission Electron Microscopy (TEM)

For TEM analysis, 10µl of each sample suspension was deposited on the copper grid and air- dried before measurement. Copper grids sputtered with carbon films were used to support the sample. Samples were analyzed by a JEOL JEM 1011 operating at 100 kV, coupled with a GATAN camera ORIUS SC600 with a resolution of 7 Megapixel. The GATAN camera is controlled by Digital Micrograph.

Electrophoretic Mobility

The electrophoretic mobility of particles and LbL-coated nanoparticles was measured by using a Malvern Nano ZS90 (Malvern Instruments, UK). The mean of five successful running were taken for particles and after adsorption of each layer.

Cellular uptake

Breast cancer cell lines (MSF-7) were seeded onto sterilized cover slip in 6 multiwell microplates (5000 cells/well). The cells were grown in 2 ml DMEM High Glucose (4,5g/l) supplemented with 5% L-Glutamine, 10% fetal bovine serum, 5% penicillin streptomycin and 5% sodium pyruvate in a humidified atmosphere with 5% CO_2 at 37 °C. After 24 hours, the encapsulated curcumin and free capsules (40 µl) were added to each well and incubated in a humidified atmosphere of 37 °C, 5% CO_2. MCF-7 cells were fixed by 4% paraformaldehyde, then washed by PBS, pH 7.2 (phosphate buffer saline). Cells were stained by DAPI (nuclear stain) for 30 min and then washed twice by PBS, pH 7.2. Cellular uptake was analyzed after 24 hours by transmitted light fluorescence microscopy.

Cytotoxicity Assay (MTT)

MSF-7 cell lines were seeded (10,000 cells/well) in 96-well flat bottom microplates with 100 µL of medium. The cells were allowed to attach to the bottom of the dish for 24 h at 37 °C and then exposed to different treatments: (40 µl) of free capsules and encapsulated curcumin. Then cells were incubated for 24 hours. Afterwards, the cells were washed with PBS, pH 7.2 and incubated with MTT solution (5 mg/mL) for 4 hours, and the formazan precipitate was dissolved in 100 µL dimethyl sulfoxide, and then the absorbance was measured in the spectrophotometer reader at 570 nm. The cell viability ratio was calculated by the following formula:

Cell viability ratio (%) = [sample absorbance-blank absorbance/ control absorbance-blank absorbance]×100.

Results and discussion

Poly (acrylic) acid (PAA) is a hydrophilic polymer[9] that adopts random coil conformation in solution. These coils have swelling properties under ionic and salt strength leading to extend chain conformations in alkaline solution (Figure 1).[7]

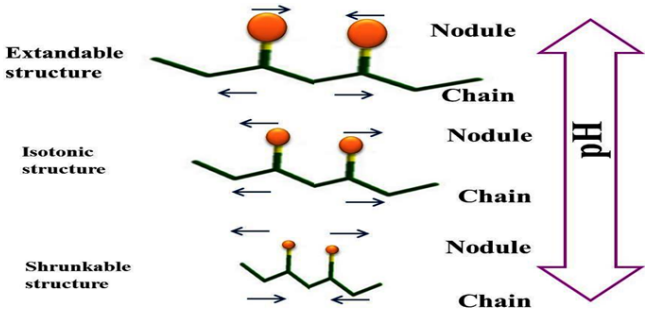


Figure 1 Coils of PAA in different pH.

The reason of these prerties is due to the COOH side chain which is pH responsive i.e. protonated (COOH) at pH ≤5. While it is de-protonated (COO^-) at pH >5.[10] In aqueous media of appropriate pH and ionic strength, the carboxylic groups ionize and develop fixed charges on the polymer network, generating electrostatic repulsive forces responsible for pH-dependent causes swelling or de-swelling of the hydrogel structure.[11] In this study, PAA is initially integrated into matrix of $CaCO_3$ crystal during fabrication and then is entrapped inside the nanocapsules after core removal giving them with unique

properties.[3] They are negatively charged related to charge of polymer. This method might produce detached particles, improve morphological properties, give rise to a more homogenous population and reduce the hexagonal shape from population, being calcite and vaterite the main shapes in $CaCO_3$ crystallization (Figure 2).

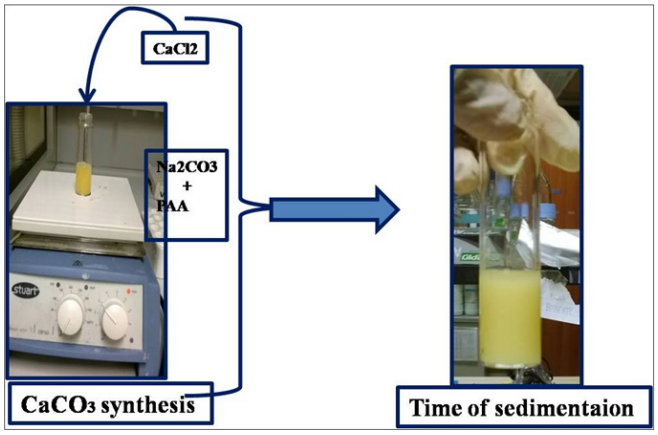


Figure 2 $CaCO_3$ fabrication in the presence of PAA with Na_2CO_3.

In figure 3, Zeta potential measurements indicate that PAA entrapped $CaCO_3$ crystal has presented good potential surface in case of mixing PAA with Na_2CO_3 compared to $CaCL_2$-PAA. This behavior is related to influence of ionic strength of Na_2CO_3 on PAA chain resulting in strong negatively charged. In addition, the negatively charged is not only affect nuclei agglomeration inside crystal matrix but it is extended to influence layers that were adsorbed up to its surface forming changeable adsorption values for each alternate layers (Figure 3).

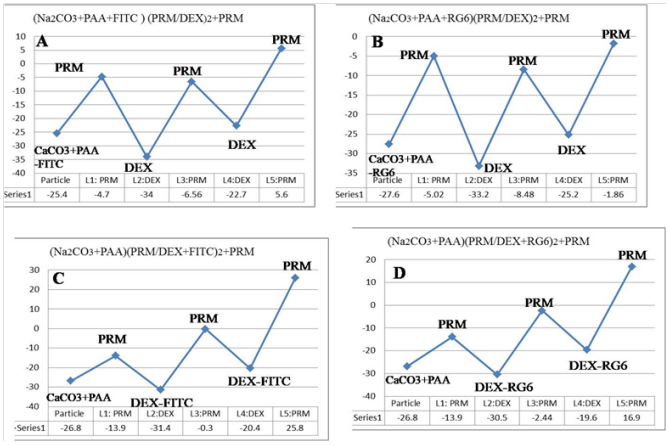


Figure 3 Zeta potential measurements. A) PAA-FITC mixed with Na_2CO_3, B) PAA-RG6 mixed with Na_2CO_3, C) PAA alone mixed with Na_2CO_3 and FITC with layers, D) PAA alone mixed with Na_2CO_3 and RG6 added to layers.

Protamine/dextran layers showed different adsorption values each layers confirming that the moieties of network matrix (inside cavity and into the surface) play vital role in ions stability that could affect adsorbed layer assembled into $CaCO_3$ surface and also that was integrated into matrix. Hanafy[4] & Volodkin[5] investigated that, responsiveness of MLNPs with desirable size range can be achieved by ionic strength, pH of the solution, polyelectrolytes concentration and type of integrated polyelectrolytes. The final layer of adsorbed protamine/dextran showed good potential surface and adsorption in case of integrated PAA alone inside $CaCO_3$ matrix. While this adsorption was influenced by integrated fluorescent molecules such as rhodamine (RG6) and fluorescence isothiocyanate (FITC) attached with PAA inside $CaCO_3$ matrix. However adsorbed dextran exhibited in all case good adsorption compared to protamine.

In figure 4, Zeta potential measurements showed no good potential with poor adsorption in case of adding PAA to $CaCL_2$. Meaning that PAA kept its chain with no more configurations for its hydrogen protons giving less negatively charged. Although PAA-$CaCL_2$ was mixed later with Na_2CO_3, PAA is not answer ionic strength of Na_2CO_3 strongly compared to previous case. This evidence may be related to reaction of PAA with Ca^{2+} ions that could minimize de-protonation of PAA. However adsorbed layer of dextran is still expressed on its good potential surface in all case (Figure 4).

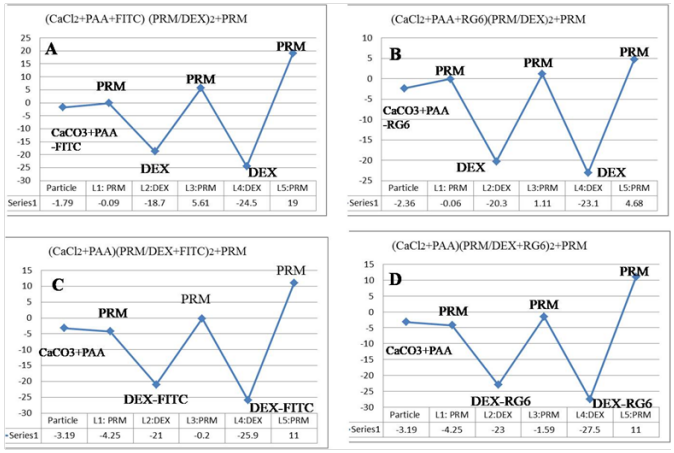


Figure 4 Zeta potential measurements. A) PAA-FITC mixed with $CaCL_2$, B) PAA-RG6 mixed with $CaCL_2$, C) PAA alone mixed with $CaCL_2$ and FITC with layers, D) PAA alone mixed with $CaCL_2$ and RG6 added to layers.

In figure 5, TEM photomicrograph showed semi spherical shape with many rounded structures like nucleoli. As it is expected PAA could form a hydrophilic corona. In this case, swelling coils of PAA polymer on water makes it favourable for protection because PAA adsorbs water many more times than its weight in alkaline pH.[12] Diameters of MLNPs were ranged from 86nm-250nm. This means that PAA has strong effect on electrostatic stability of particles preventing their growth (Figure 5).[4]

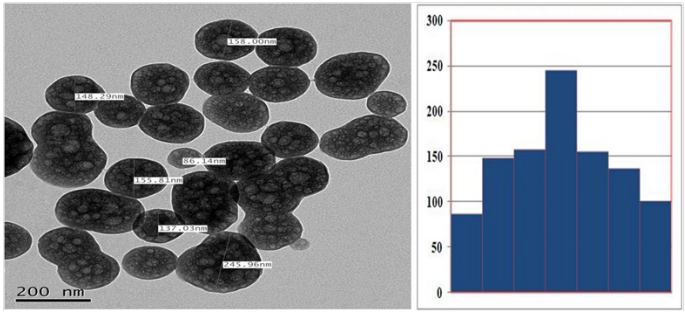


Figure 5 TEM photomicrograph showed morphology of nanoparticles and their diameter.

In figure 6, MLNPs are readily up-taken by MCF-7 cells as confirmed by transmitted light fluorescence images. After 24 h of MLNPs incubation, aggregates of curcumin are still present

inside cells in large quantity as demonstrated by an intense green fluorescence. This evidence revealed that the nanoparticles (NPs) size is an important parameter that determines the mechanism of their cellular uptake. In particular, NPs smaller than approximately 200 nm are internalized typically via receptor-mediated endocytosis whereas, for larger NPs other mechanisms are involved.[13] Meaning that cellular uptake might increase in case of less diameter leading to high accumulation of NPs inside cytoplasm and could penetrate to other organelles such as nucleus and mitochondria[14].This surprising observation suggests that the optimal size of $CaCO_3$ template strongly depends on content of $CaCO_3$ matrix (Figure 6).

MTT assay allows assessing the ability of viable cells to reduce the tetrazolium salt of MTT reagent by NADH to insoluble formazan product, forming purple needle-shaped crystals in the cells. After these crystals have dissolved in organic solvent, a purple colour is generated.[5] In figure 7, the intensity of the purple colour, measured spectroscopically, reveals how many MCF-7 cells are viable[15]. The MTT assay conducted for encapsulated curcumin demonstrated a high cytotoxicity of the drug on MCF-7 in both cases (1mg& 2 mg/ml) PAA. But 2mg/ml PAA showed higher reduction in growth of MCF-7. This could reveal to high restored capacity of encapsulated curcumin compared to 1mg/ml PAA. While there is no cytotoxicity could be mentioned in both cases of free MLNPs with (1mg & 2mg/ml) PAA. From previous mention, cellular uptake behavior is largely influenced by particle size, the control of diameter and shape of template plays an important role to improve drug delivery carrier (Figure 7).[16,17]

In figure 8, PAA ions have strong effect on the stability of multilayer nanoparticles. This could confirm that protamine penetrated pores of $CaCO_3$, has reacted with PAA inside $CaCO_3$ matrix. Meaning that network matrix could be formed inside the cavity of $CaCO_3$. Like this matrix can provide good mechanical support in blood stream, and also at long storage. Additionally, they can give active group for reaction with encapsulated cargo molecule, increasing efficiency and drug capacity (Figure 8).[18-21]

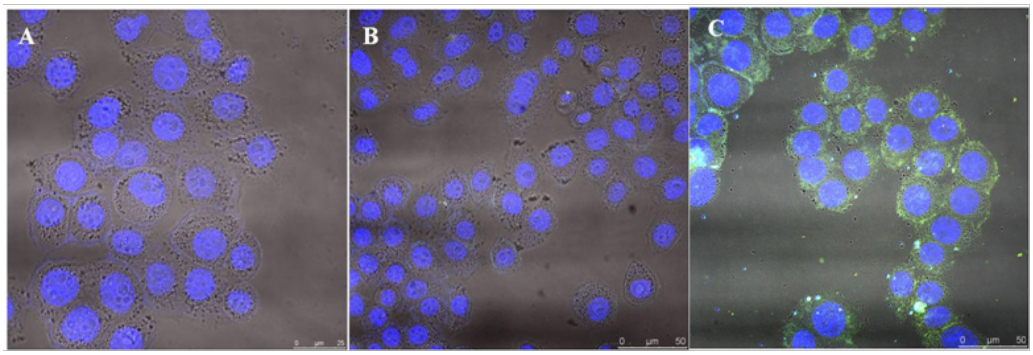


Figure 6 Cellular uptake of breast cancer.A) Control MCF-7 breast cancer cell line, B) Free capsules with no fluorescent molecules, C) Encapsulated curcumin.

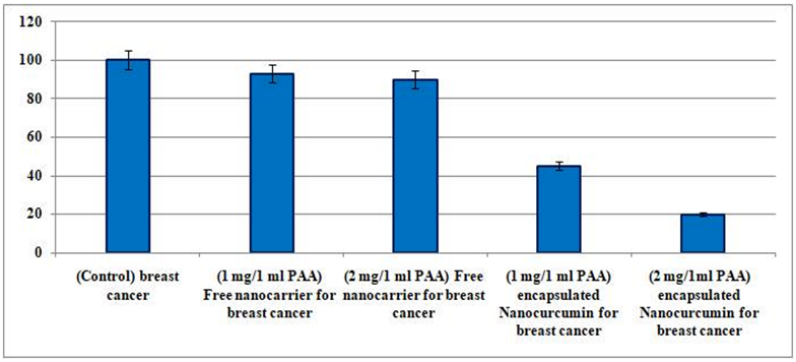
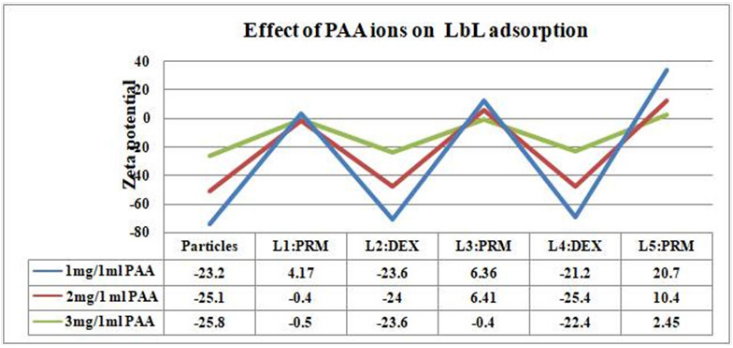


Figure 7 Cellular cytotoxicity of free capsules and encapsulated curcumin.



	Particles	L1:PRM	L2:DEX	L3:PRM	L4:DEX	L5:PRM
1mg/1ml PAA	-23.2	4.17	-23.6	6.36	-21.2	20.7
2mg/1 ml PAA	-25.1	-0.4	-24	6.41	-25.4	10.4
3mg/1ml PAA	-25.8	-0.5	-23.6	-0.4	-22.4	2.45

Figure 8 Influence of PAA ions on stability of multilayer nanoparticles.

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Conclusion

Nanotechnology has solved the issue of low viability of curcumin by encapsulating it inside nanocarriers. Poly acrylic acid (strong negatively charged) is used to affect nucleation of $CaCO_3$ during fabrication. Carriers that were produced, have small size and spherical shape. No evidence for toxicity of PAA doped carrier was observed as demonstrated by MTT assay on breast cancer cell line. Curcumin has good influence on breast cancer cell line (MCF-7) after 24 hours of incubation.

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Conflict of interest

Authors declare that there is no conflict of interest.

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