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Formulation Development of Sustained-Release Microencapsulated IR3535® with Long-Duration Mosquito Repellency

EDUARD CASAS-TERRADELLAS, EMMA BEBBINGTON, TYCHO SPEAKER AND THOMAS P. DOOLEY
LivFul Inc., Cheshire, United Kingdom (E.C.T., E.B., T.P.D.)
Capsulent, Santa Cruz, California, USA (T.S.)

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Synopsis

Bites from pathogen-harboring insects (e.g., mosquitos and sandflies) and noninsect arthropod pests (e.g., ticks) can result in a variety of vector-borne pathogenic diseases. Numerous commercial repellent products containing DEET, IR3535® (Merck KGaA, Darmstadt, Germany), or other repellent active ingredients—but lacking sustained-release technology—are effective at repelling pests from human skin for several hours. To reduce or prevent the potential for arthropod vector-borne diseases, sustained release formulations of repellent active ingredients with long-duration benefits are needed to increase efficacy and user compliance. Here, the authors present the formulation development of a Lewis acid–Lewis base adduct encapsulated emulsion containing 20% w/w IR3535. Stability testing of the lotion was performed at ambient room temperature (20°–25°C) and 40°C. Repellency efficacy on human subjects was tested against *Aedes aegypti* mosquitos in laboratory arm-in-cage studies. Anti-infective properties were assessed against multiple bacterial species. A series of sustained-release lotions of microencapsulated IR3535 was prepared. During formulation development, chemical stability was problematic. However, chemical stability was achieved by buffering the lotion with sodium citrate–citric acid. The microencapsulated formulation manifested a long-duration efficacy of 12–13 hours against *A aegypti* mosquitos in laboratory arm-in-cage studies. The lotion also demonstrated anti-infective properties against multiple bacterial species. This sustained release formulation achieved a long-duration efficacy benefit superior to commercial repellent products, without the unpleasant odor or oily feeling that is customary for DEET-containing products.

INTRODUCTION

Insect-borne and non-insect arthropod pest-borne pathogenic diseases affect millions of people worldwide with acute and chronic conditions. Some of the prominent vector-mediated infectious diseases include malaria, dengue fever, Lyme disease (borreliosis), leishmaniasis, Chagas disease (trypanosomiasis), Zika fever, equine encephalitis, and yellow fever. In lieu of (or in addition to) eradication of, or physical separation from, insects and noninsect arthropod pests, prevention of vector-borne diseases or the symptoms thereof

Address all correspondence to Thomas P. Dooley, tom@tomdooley.org

may be achieved by application to the skin, hair, and/or clothing of an effective long-duration sustained-release repellent formulation.

The repellency efficacy of a topical formulation is a function of: (a) the active ingredient concentration on the skin at a particular time; (b) the rate of skin penetration; and (c) the rate of aromatic evaporation from the skin, in addition to the arthropod species and environmental conditions. An ideal sustained release formulation will reduce the rate of skin penetration and evaporation rate of the active ingredient; maintain a residual concentration of the active ingredient; and prevent the active ingredient from evaporating without entirely sequestering it. Thus, a formulation chemist seeks a "slow" rate of evaporation rather than "no" rate, whereas nonsustained-release conventional formulations exhibit rapid and high volatility without long-duration efficacy.

Sustained-release formulations can fail during development for various reasons, including the three parameters mentioned previously, as well as chemical instability of the active ingredient and/or excipients and physical instability (phase separation, flocculation, viscosity variations, etc.). Achieving an effective topical sustained-release formulation can be challenging, as one must optimize the composition in conjunction with the manufacturing processes (e.g., the sequence of addition of the ingredients, temperatures, and mixing intensity).

Microencapsulation of the common synthetic active ingredient DEET within a topical repellent formulation has been demonstrated to decrease the rate of skin ("transdermal") penetration (1,2). Microencapsulation can also prolong evaporation rates of DEET, thus providing a sustained release mode of action (2). These publications, in conjunction with a related US patent T.J. Speaker, Microencapsulation product and process, US Patent 8,039,015 (2011), have disclosed the microencapsulation of an emulsion using Lewis acid–Lewis base adduct interactions. In the present work, the authors desired to use a similar Lewis acid–Lewis base microencapsulation approach, albeit using another active ingredient, IR3535®. This ingredient has several properties that make it more appealing than DEET to consumers.

IR3535 has been known for more than 2 decades to be an effective and safe mosquito repellent active ingredient. This active ingredient has been included in multiple commercial consumer products in various countries. The efficacy of IR3535-containing topical formulations (e.g., sprays and lotions) against mosquitos has been ranked relative to DEET-containing formulations (3–10). Comparisons of the two active ingredients have also been made using the insect vectors of Chagas disease (trypanosomiasis), (11,12), lone star ticks (*Amblyomma americanum*) (13), and two species of sandflies (*Phlebotomus mascittii* and *Phlebotomus duboscqi*), which are the vectors of leishmaniasis (14). A third species of sandflies, *Phlebotomus papatasi*, has also been tested for IR3535 (15).

In the context of typical commercial formulations, in repellency efficacy comparisons of the same or similar concentrations of active ingredients, IR3535 is less effective or comparable to DEET. For example, the mean complete protection time (CPT) for human subjects in a field trial in the Florida Everglades for 25% IR3535 in ethanol was 3.0 hours versus 5.6 hours for 25% DEET (4). The CPT is the number of minutes (or hours) elapsed between topical application and the first landing and/or probing (biting) of the biting insect. The CPT may be defined as the time of the confirmed first bite (CFB), which represents the first subject within a group of subjects experiencing his/her second bite or confirmatory bite. Although these publications disclose the duration of repellency efficacy for compositions containing IR3535, they do not present comparisons of standard formulations (e.g., in

ethanol or an emulsion) versus sustained-release formulations that are intended to enhance long-duration repellency efficacy.

The repellency of DEET can be greater than that of IR3535. However, the latter has several distinct advantages over DEET for use on human or nonhuman mammalian skin Most notably, IR3535 lacks DEET's strong odor and produces a nonoily/nongreasy feel for preferred cosmetic appeal to consumers. Still, a repellent formulation containing IR3535 with high mosquito repellency (e.g., 12+ hours on human skin) has not been disclosed in prior studies or for commercial products.

A major goal while developing a topical consumer product (e.g., insect repellent lotion) is the establishment of a stable composition with a "formal" and/or "imputed" shelf life of at least 12 months, preferably 18 months, and more preferably 24 or 36 months at ambient room temperature (RT) (20°–25°C). Inherent within this shelf life are multiple relevant parameters, such as: (a) overall chemical stability, an example of which is pH stability, preferably exhibiting no more than 1.0 pH unit variance (decrease or increase) during the imputed or formal shelf life; and (b) active ingredient stability with a variance of no more than 5% to 6% loss of the active ingredient during the imputed or formal shelf life. In other words, if there is an initial nominal active ingredient concentration of 20% w/w, then the loss over time must not exceed 0.05 x 20% w/w or 0.06 x 20% w/w. Thus, a loss greater than 1.0% or 1.2% respectively of the nominal concentration of the product label is unacceptable (i.e., a concentration less than 19.0 or 18.8% w/w respectively).

Whenever sufficient time does not permit the formal establishment of a shelf life, extrapolations of linear regression slopes obtained over shorter time periods at accelerated temperatures—such as 40°C and/or 50°C—can be used as surrogates to establish an "imputed" shelf life. When comparing RT of approximately 20°–25°C to 40°C, the shortened time interval's result at elevated temperature is extrapolated or multiplied by a factor based upon empirical results. One may observe that 40°C accelerates the instability or other failure aspect(s) of the formulation (e.g., reduction in active ingredient, phase separation, flocculation, viscosity changes, etc.) over RT by a factor of, for instance, 3 times. Thus, in this example, if it does not fail at 6 months at 40°C, but fails shortly thereafter, then the imputed RT shelf life would be limited to 6 months times 3, or 18 months.

International regulatory agencies state the allowed tolerances or variances for the content of the active ingredient to be maintained during the entire shelf life of insect repellents or pesticides. WHO guidelines declare an allowed tolerance of 6% for repellent products with a declared nominal content of active ingredient at 20°C ± 2 of 10% to 25% w/w or w/v (16). This 6% tolerance is also accepted by the European Chemicals Agency and most of the other regulatory authorities. However, the US Environmental Protection Agency (EPA) specifies an active ingredient tolerance of only 5%. Thus, the variance from the nominal label amount or concentration of the active ingredient should not exceed 5% or 6% over the designated shelf life of the repellent product. As an example, a product with a nominal concentration of 20% w/w of active ingredient should not have a concentration greater than 21% w/w or less than 19% w/w using the EPA tolerance standard, or a slightly higher tolerance in most jurisdictions outside of the United States.

The authors describe the formulation development and properties of a repellent lotion consisting of 20% w/w IR3535 in a Lewis acid–Lewis base adduct for microencapsulation and carefully selected cosmetic ingredients.

METHODS

FORMULATION DEVELOPMENT

IR3535 was selected as the active ingredient because it has multiple advantages over DEET. Most notably, those advantages include the lack of odor, lack of oily feel on skin, and better toxicological profile. The selected concentration was 20% w/w.

In view of decades of expertise by our scientists in topical formulation development, careful consideration was given to the chemical properties for the selected excipients for inclusion within the microencapsulated emulsions. Consideration was given to the selection of and preferences for cosmetically acceptable fluidic carriers (e.g., water, ethanol, glycerol); suspending and emulsifying agents (e.g., sorbitan oleate, polysorbate 20, lecithin, sodium lauryl sulfate); hydrophobic oils or lipids (e.g., isoeicosane, isopropyl myristate, isopropyl palmitate); viscosity agents (e.g., polyamide-3, cetyl alcohol, carrageenan, cellulose, methyl cellulose, polyethylene glycols, or carbomer homopolymer type A); film formers (e.g., dimethicone, acrylates copolymer); preservatives (e.g., phenoxyethanol, ethylhexylglycerin, parabens); stabilizing agents such as antioxidants (e.g., butylated hydroxytoluene, butylated hydroxyanisole, citric acid); and Lewis acids and Lewis bases for microencapsulation of emulsions (e.g., chitosan, algin, xanthan gum). The International Nomenclature of Cosmetic Ingredients served as a guide during selection of excipients. Fragrance was optional.

From these possible excipients, a "*generic ingredients list*" for IR3535-containing microencapsulated formulations was compiled, including the target concentrations of the ingredients. The list was utilized to conceive a formulation referred to as prototype version 1.0 (Table I). As described in the results section, successive derivatives of version 1.0 resulted in formulations 2.0 and 2.55.

Table I
Generic Ingredients List to Generate Sustained-Release Repellent Formulation Prototype Version 1.0

Category	Chemical name or class	% (w/w)
Active	IR3535	20
Emollient	Acyclic alkane	~2
Emulsifier	Sorbitan fatty acid ester	~1
"	Polysorbate	<1
Encapsulation	Algin + cellulose	<1
"	Chitosan	<1
"	Xantham gum	<1
Thickener	Polyamide	~1
"	Cetyl alcohol	<1
"	Carrageenan	<1
Film former	Silicone	~ 1
"	Nonsilicone	~ 1
pH/antioxidant	Citric acid (optional)	<1
Preservatives	Preservatives	<1
Fragrance	Fragrance (optional)	<1
	Active + excipients	~30
	water	~70

The method of preparation of repellent version 2.0 is the following:

- In a heated mixing container, combine the emollient, the emulsifiers, two of the thickeners (polyamide-3 and cetyl alcohol), and the film former dimethicone (Phase A). Commence heat to 90°C. Stir until melted Phase A is completely homogenous.
- Discontinue heating, and when temperature is below 80°C, add IR3535 (Phase A1) gradually, stirring to incorporate.
- While Phase A (including Phase A1) is cooling, in a separate heated container, prepare Phase C. Add citric acid into the purified water and stir until completely solubilized, then add the trisodium citrate (dihydrate). Once the trisodium citrate is completely solubilized, the pH should be approximately 6.2. Add the final amount of purified water to complete the final weight of Phase C, and begin heating to 50°C.
- In a separate container mix the (dry) encapsulation ingredients and the thickener carrageenan (Phase B), then add Phase B into Phase A slowly and gradually, stirring to avoid local freeze-out of gel. Mix thoroughly.
- When Phase C is at 50°C, add Phase A/B into Phase C. When all Phase A/B has been added to Phase C, recommence stirring. Shear hard and homogenize. Continue to shear with the homogenizer to form creamy white emulsion.
- Add the film former acrylates copolymer (Phase D) and the preservatives (Phase E) into the emulsion sequentially with stirring, while cooling the product.
- If required, add additional purified water for appropriate final weight.
- Check that the pH of the final emulsion is between 6.0 and 6.5. For composition version 2.0, the target pH is 6.2.
- Add (optional) fragrance (Phase F) into the emulsion once the temperature is below 40°C, Mix thoroughly.

TESTING OF STABILITY AND PHYSICAL ASPECTS

This study focused upon repellent compositions that manifest stabilized formulation properties at RT and/or accelerated temperature at 40°C. The formulations were also stability tested at 50°C (data not shown). Weekly assessments of pH were performed. The linear regressions of the time in weeks to arrive at a reduction in pH loss of 1.0 units were determined.

Each formulation was also assessed for viscosity at 25°C using an analogue rotationary viscometer (PCE RVI 1, spindle #4, 12 rpm). The preferred viscosity range for the lotion was 3,000–10,000 cps. The texture and fluid flow pattern of a lotion, as well as a white to off-white color, were also assessed.

The concentration of IR3535 within the lotions stored at 40°C and RT were analyzed by gas chromatography. This was conducted by Stillmeadows Inc. (Sugar Land, Texas) using a gas chromatograph Trace™ 1310 with autosampler, Chromeleon™ (Thermo Fisher Scientific, Waltham, Massachusetts) integration software, Phenomenex ZB-FFAP column (30 m length times 0.32 mm inner diameter times 0.5 μm film thickness). The operating conditions included an injector temperature of 220°C; injection volume of 1.0 μL; split ratio 25; flame ionization detector at 260°C; column (oven) temperatures (initial 100°C, hold 1.0 minute, increase 20°C/min, final 250°C, hold 6.5 minutes, total run time 15 minutes); carrier gas hydrogen, 3.0 mL/min constant flow (by electronic flow control); and make-up gas nitrogen. Integration parameters were measurement type; area; retention

time(s); internal standard methyl undecanoate at 4.3 minutes; and the active ingredient 3-[n-N-butyl-N-acetyl]-aminopropionic acid ethyl ester at 7.4 minutes.

ARM-IN-CAGE MOSQUITO EFFICACY TESTING ON HUMAN SUBJECTS

Repellency efficacy tests (arm-in-cage) were conducted for version 2.0 and prototype version 1.0 against the mosquito species *Aedes aegypti* at Laboratorios Ecolyzer LTDA (Sao Paulo, Brazil). These tests were performed according to the OPPTS 810.3700 methodology (July 7, 2010), which is one of a series of test guidelines established by the Office of Chemical Safety and Pollution Prevention of the EPA, for use in testing pesticides and chemical substances to develop data for submission to the agency. The arm-in-cage procedure for testing of LivFul Inc.'s (Cheshire, United Kingdom) microencapsulated emulsions of 20% IR3535 was approved (May 22, 2018) by the Institutional Review Board for experiments on human subjects.

Mosquitos. There were host-seeking females of *A aegypti*; 55 mosquitos per cage (1 mosquito/1,160 cc) in acrylic cages 40 × 40 × 40 cm (64,000 cc).

Number of subjects. There were 10 subjects. The same volunteer subjects were used as an untreated group (to validate the study and as a mosquito attractiveness control) and as treated volunteer subjects with the repellent lotion group using a different arm. An attractiveness test to the participants was performed before the application of the repellent product at each time point. This procedure was performed to verify whether the participants were suitable for the study. At least five landings/bites were needed to be recorded for acceptance.

Dose applied. The dose was 1 g lotion/600 cm^2 of skin (forearm). The area of the forearm of each participant was calculated according to the formula {[(M1 + M2 + M3 + M4 /4) × C]/600}, where M1 was the forearm circumference at wrist height (cm), M2 and M3 were two equidistant measurements of the forearm circumference between elbow and wrist (cm), M4 was the forearm circumference at the elbow, and C was the forearm length (cm).

Methodology. Before each evaluation with the forearm treated with repellent, mosquito activity evaluations were performed in all test cages. The exposure time of the forearm without treatment was 1 minute inside the cage or until 5 landings or bites. The treated group exposed their arm for 5 minutes starting after 30 minutes from the application of the product and every hour until evidence of first bite. The CPT is the number of minutes (or hours) elapsed between topical application and the first landing and/or probing (biting) of the insect. CFB is the second incidence of an insect probing (biting).

IN VITRO ANTI-INFECTIVE TESTING

To determine whether versions 1.0 and/or 2.0 have anti-infective properties, the bacteriological standard test methodology BS EN 1276:2019 was used at Melbec Microbiology Ltd. (Haslingden, United Kingdom). In brief, BS EN 1276:2019 uses a quantitative suspension of *Pseudomonas aeruginosa* (ATCC 15442), *Escherichia coli* (ATCC 10536 and/or K12 NCTC 10538), *Staphylococcus aureus* (ATCC 6538), and *Enterococcus hirae* (ATCC 10541), with an acceptance criterion of a 5 log10 reduction in viable bacterial counts.

An aliquot of the lotion was directly added to a series of test suspensions of *P aeruginosa*, *E coli*, *S aureus,* and *E hirae* respectively. The suspension was mixed with bovine albumin

(0.3 g/L), which was used as the interfering substance for the test conditions. After 60 seconds contact time, a neutraliser was immediately added to stop the effects of the disinfectant, and a sample of the corresponding mixture was poured into a bacterial medium plate and incubated. After incubation, the number of surviving bacteria was counted and compared against the original culture number. A 5 log (base 10) reduction within all 4 species met the formally established standard, although reductions below this level and/or in less than 4 species may (also) be indicative of antimicrobial activity.

RESULTS

FORMULATION DEVELOPMENT

When the lotion prototype version 1.0—containing 20% w/w IR3535 and according to the generic ingredients list of Table I—was subjected to stability testing at RT and accelerated temperatures (e.g., 40°C and/or 50°C), it manifested rapid reductions from the initial pH over the course of weeks to months. The reductions in pH by 1.0 units occurred within 5.1 weeks at 40°C and 16.4 weeks at RT. A variance of pH of 1.0 units or greater within this predetermined time (i.e., 12 months at RT) was deemed as an undesirable property; it may be a key factor used as a determinant of the imputed or formal shelf life.

The pH drift underscores that some component(s) of the prototype version 1.0 were undergoing chemical hydrolysis or degradation, as evidenced by increased concentration of hydronium ions (H3O+) or protons, detected by a pH meter. Lower pH and/or the presence of degradants within the formulations might be disadvantageous to human or animal skin. Note that none of the initial formulation variants, including version 1.0, contained trisodium citrate, although some variants contained citric acid as an optional acidifier/antioxidant.

According to the manufacturer of IR3535 (Merck KGaA), the ideal pH for IR3535 is between 6.0 and 6.5. A challenge of using IR3535 as the selected active ingredient in these encapsulated repellent formulations is that in order to adequately solubilize, suspend, or emulsify the IR3535 and the other essential excipients, a pH lower than its inherent ideal pH of IR3535 can be desirable. For human skin, a pH of approximately 5.0–6.5 is appropriate, and approximately 5.5 is often preferable or optimal in topical consumer products. These two parameters are in apparent opposition; logic suggests that a low pH might be desirable in the process of producing an IR3535-containing encapsulated formulation, but a higher pH (approximately 5.0–6.5) is desirable for application of the formulation to human skin to avoid unnecessary skin irritation.

It follows that the manufacturing process steps (e.g., sequence of addition of the ingredients, temperatures, and mixing intensity) in preparing the formulations may also impact ingredient solubilization, suspension, emulsification, and encapsulation in a pH-dependent manner. Thus, empirical formulation development and analyses via a reiterative process can improve from an initial prototype formulation toward preferred formulations.

In multiple efforts to ameliorate the reductions in pH and concentration of the active ingredient, attempts were made to stabilize the formulation(s) with: (a) addition of NaOH to increase the initial pH; (b) addition of NaOH to restore (increase) the pH after the formulation had manifested some degree of pH loss; and (c) addition of an antioxidant, with the intent of retarding the chemical instability. None of these three empirical approaches

adequately addressed the chemical instability issue(s). The instability persisted even when NaOH or an antioxidant was included. It is likely obvious to an experienced formulation scientist that the back neutralization of a citric acid–containing solution (or emulsion) with sodium hydroxide is inherently equivalent to the use of a sodium citrate (or hydrates thereof) buffer solution, differing only in order of addition of the component parts. Nevertheless, this approach did not resolve the issue of pH getting lower over time.

The concentration of IR3535 within prototype version 1.0 was assayed at various time points using gas chromatography. The analysis revealed that the degradation of IR3535 at 24 months at 20°C exceeded the WHO and EPA guidelines' allowed tolerances of 5% to 6% for repellent products, with a declared nominal content of active ingredient of 10% to 25% w/w or w/v and stored at 20°C ± 2.

These findings (as previously mentioned) further underscored the problems and confounding variables to be addressed. In summary, the problematic issues faced during formulation development included: (a) most notably rapid pH loss; (b) loss of IR3535; (c) a preference for a lower pH for solubility, suspension, emulsification, and encapsulation during formulation development; (d) preference for human skin of a pH of approximately 5.0–6.5, and more preferably 5.5; and (e) attempts to maintain appropriate pH conditions by the addition of NaOH or an antioxidant not being sufficient. Thus, multiple attributes of the excipient components, concentrations, and sequence of addition (order) were evaluated to optimize the desired formulation.

To prevent the rapid pH reduction manifested by prototype version 1.0 during stability testing (see following section), a formulation version 2.0 was prepared that was essentially prototype version 1.0 but that included trisodium citrate dihydrate (2.024% w/w) plus citric acid (0.095% w/w). This buffered formulation contained a total of 30.63% active ingredient plus excipients, with an initial pH of 6.2. Formulations with two chemical versions of sodium citrate were studied in the dihydrate form and the monohydrate form. To maintain equimolar citrate ion concentrations, less of the monohydrate was required relative to the dihydrate. The dihydrate form was routinely used in commercial consumer products and foods, was less expensive, and may be preferable for the formulation(s).

Formulation variants were also prepared with a concentration range of sodium citrate–citric acid to identify preferred amounts of the buffer. Concentrations ranged from 0.2% to 5.0%. In general, the optimal concentration for chemical stability and physical aspects was approximately 2%. Formulation version 2.55 was identical to version 2.0, except it contained 1.80% trisodium citrate dihydrate: 0.31% citric acid, at initial pH 5.5, and 69.38% water.

TESTING OF STABILITY AND PHYSICAL ASPECTS

A major goal during formulation development was to limit the reduction in pH to no more than 1.0 units within 12 months (and preferably longer) at RT. This desirable increased pH stability was also expected to stabilize the concentration of IR3535, so that it did not exceed 5% to 6% loss over the imputed or formal shelf life. The slopes of the linear regressions (with r2 values >0.9) were extrapolated into imputed (estimated) weeks to reach a pH loss of 1.0 units—thus, from an initial pH of 6.2 downward to 5.2 for version 2.0, or from an initial pH of 5.5 downward to 4.5 for version 2.55.

Table II reveals that linear regression analyses of weekly measurements of the unbuffered repellent lotion prototype version 1.0 manifested rapid reductions in pH by 1.0 units within

Table II
Chemical Stability: Elapsed Time (Weeks) to Reach a pH Reduction of 1.0 Unit[a]

Version	**1.0 -**	**2.0** (Bd2)	**2.0** (Bi2)	**2.55** (20a)
Buffered pH	*na*	6.2	6.2	5.5
Weeks @ 40°C	5.1	27.2	27.8	90.1
Weeks @ RT	16.4	94.3	68.5	384.6

[a]Version 1.0 is the unbuffered prototype; version 2.0 (Bd2) has ~2% sodium citrate:citric acid buffer with sodium citrate monohydrate; version 2.0 (Bi2) is ~2% sodium citrate:citric acid buffer with sodium citrate dihydrate; version 2.55 (20a) is ~2% sodium citrate:citric acid buffer and with sodium citrate dihydrate.

5.1 weeks at 40°C and 16.4 weeks at RT. These times were deemed to be undesirable. However, at 40°C, the linear regression of the slope revealed that the buffered version 2.0 (two variants; Bd2 and Bi2) at initial pH of 6.2 reduced the rate of pH drift by 5.3 times and 5.5 times, respectively. At 40°C the buffered version 2.55 (20a) reduced the rate of pH drift by 17.7 times. At RT, the buffered versions 2.0 (Bd2 and Bi2) reduced the rate of pH drift by 5.8 times and 4.2 times, respectively. Also, at RT, the buffered version 2.55 (20a) reduced the rate of pH drift by 23.5 times.

If the time to arrive at a loss of 1.0 pH unit is selected as one of the criteria for establishing the imputed or formal shelf life, then the sodium citrate:citric acid buffered versions 2.0 (Bd2 and Bi2) can last for at least 1 year prior to a reduction in pH of 1.0 units. Furthermore, version 2.55 (20a) with an initial pH of 5.5 is expected to be stable for multiple years. These results indicate that chemical stability, of which pH is an important marker, has been substantially enhanced by the selected compositions, and especially so at an initial buffered pH of 5.5.

The shelf-life estimates may also be established as a function of the concentration of the active ingredient (e.g., IR3535), but the variance should not exceed 5% or 6% loss of the active ingredient over the duration of the imputed or formal shelf life. The concentration of IR3535 of version 2.0 was assessed at 26 weeks at 40°C and RT and was determined to conform to the acceptable variance. Thus, the sodium citrate–citric acid buffered formulation 2.0 (initial pH of 6.2) stabilized not only the pH but also the active ingredient from hydrolysis/degradation. Surprisingly, as previously noted, the formation of a citric acid buffer system by the addition of sodium hydroxide to a citric acid–containing solution did not stabilize the pH; the formulations formed by inclusion of trisodium citrate (or the hydrates thereof) were effectively stabilized.

EFFICACY OF FORMULATIONS ON HUMANS AGAINST MOSQUITOS IN LABORATORY ARM-IN-CAGE STUDIES

The repellent efficacy against mosquitos was assessed for buffered version 2.0 and the unbuffered prototype version 1.0. A laboratory efficacy test (arm-in-cage) was conducted for the version 2.0 on 10 human participants against the mosquito species *A aegypti*. The dose was 1.0 g of repellent lotion per 600 cm^2 applied to the forearm. Table III demonstrates that none of the participants experienced a CFB (i.e., second bite) prior to 13 hours. At 13 hours into the study, 4 of the 10 participants experienced mosquitos landing and/or biting. Thus, the CPT for the protection against the mosquito species

Table III
Arm-in-Cage Exposure to *A aegypti* Mosquitos Repellent Version 2.0 Tested on 10 Human Subjects (S1–S10)

Hours	Version 2.0									
	Number of bites by *A aegypti*									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0
13	3	0	0	5	0	0	0	2	0	3
14	6	0	0	11	0	0	0	1	0	0

A aegypti was 13 hours (based upon a CFB criterion; i.e., with evidence of a second bite at 14 hours in at least one subject). The unbuffered prototype version 1.0 manifested a CPT of 12 hours using the same experimental method and facility (Table IV). Thus, both microencapsulated formulation version 2.0 and version 1.0 displayed long-duration efficacy, which was superior to the efficacy reported for other commercial products using IR3535 or other active ingredients.

Table IV
Arm-in-Cage Exposure to *A aegypti* Mosquitos Repellent Version 1.0 Tested on 10 Human Subjects (S1–S10)

Hours	Version 1.0									
	Number of bites by *A aegypti*									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0
12	0	0	1	1	0	0	0	3	0	2
13	0	0	1	8	0	0	0	7	0	5
14										

COSMETIC EXPERIENCE OF HUMANS

The lotion versions 2.0 and 2.55 have been applied to the skin of human volunteers for in-life protection against bites from mosquitos and/or flies. The composition versions 2.0 and 2.55 are cosmetically acceptable, quickly dry on the skin, do not have an oily or greasy feel after drying, and do not have an unpleasant aroma (either with or without optional fragrance). Humans have applied the lotions to exposed skin during the daytime and/or at night to protect themselves from mosquitos and/or other biting arthropod pests.

ANTI-INFECTIVE PROPERTIES OF COMPOSITIONS CONTAINING IR3535

In Table V, formulation version 2.0 demonstrates antiseptic properties with 60 second exposures against both *P aeruginosa* and *E coli* in accordance with the BS EN 1276:2019 test method by achieving a 5 log (base 10) reduction in bacterial load. Version 2.0 also demonstrates some antimicrobial activity against both *S aureus* and *E hirae* with a 3 log (base 10) reduction in bacterial load. The results for version 1.0 were slightly better, with 3 of 4 species of microbes exhibiting 5 log killing.

Direct comparisons of potency can be made to known topical antiseptics, such as 0.2% sodium hypochlorite (17), 70% ethyl alcohol (albeit at a higher temperature) (18), 70% isopropyl alcohol (19), 3% hydrogen peroxide (19), and 5% tea tree oil (20). Note that versions 2.0 and 1.0 were close to the effectiveness of a common antiseptic, 70% isopropyl alcohol, but they were slightly less effective. The two sustained-release repellent lotions exhibited 99.9% killing.

DISCUSSION

Chemical stability was a particular challenge of this formulation development effort. The sodium citrate–citric acid buffering achieved chemical stability with regard to pH loss over time and decomposition of IR3535 at both RT and accelerated temperatures (e.g., 40°C). The linear regressions demonstrated that the rate of chemical instability was better ameliorated at an initial pH of 5.5, rather than 6.2, perhaps due to a more optimal buffering capacity with a higher amount of citric acid.

Table V
Anti-Infective Properties of Repellent Versions 1.0 and 2.0[a]

Disinfectant	Log reduction by species (Log10)				Effectiveness (%)
	P aeruginosa	*E coli*	*S aureus*	*E hirae*	
0.2% sodium hypochlorite	>7	>7	>7	>7	99.9999
70% ethanol	7.7	6.7	6.7	7.4	99.9999
70% isopropanol	5.08	5.37	5.36	5.46	99.999
Version 1.0	**>5.32**	**>5.39**	**3.72**	**>5.3**	**99.9**
Version 2.0	**>5.06**	**>5.26**	**<3.80**	**<3.90**	**99.9**
3% hydrogen peroxide	3.66	2.8	1.32	0.18	90
5% tea tree oil (0.001% tween 80)	1.75	5.25	>1	NR	<90

[a]Contact time was 60 seconds. Temperature was 20°C, except for 70% ethanol at an elevated temperature of 34°C.

The repellent formulations have an additional benefit as topical consumer products, namely anti-infective properties against four bacterial species *in vitro*. High potency (i.e., 5 log base 10 order killing) antibacterial effects were observed for 2 or 3 of 4 bacterial strains and moderate potency for the remainder (i.e., >3 log base 10 killing). One may conclude that the 2 versions exhibited 99.9% effectiveness, approaching that of 70% isopropyl alcohol. This anti-infective effect is perhaps due to the preservatives, surfactants (emulsifiers), and/or other excipients known to have anti-infective properties (e.g., acyclic alkane, citric acid, and chitosan), along with perhaps IR3535 (21). Inherent anti-infective properties of a topical repellent formulation may provide coincidental benefits to mammalian skin, such as in the prophylaxis or treatment of infections in compromised skin (abrasions, lacerations, insect bites, etc.).

The mosquito repellency efficacy of version 2.0 in human subjects in the laboratory was 13 hours. Thus, this formulation has the hallmarks of a best-in-class repellent. The authors are unaware of any approved commercial repellent product with 12+ hours of protection.

An alternative experimental and/or confirmatory approach is to test the efficacy of the topical formulations on humans in life. Human volunteers can be exposed in life to mosquitos, flies, and/or sandflies in outdoor environments known to contain the relevant species of biting insects (22). A common test paradigm is to treat a group of subjects' exposed skin surfaces with the repellent formulation, and the elapsed time is recorded in 30- or 60-minute intervals until CFB occurs (i.e., when the first individual within a group of subjects experiences a CFB at the time of the second bite). This represents a minimum limit; some individuals may or are likely to experience protection beyond the CFB threshold. A preliminary study resembling this type of field trial design was performed with the unbuffered prototype version 1.0 in Florida (United States) and yielded an average protection time of 14 hours (data not shown). Note that an average protection time for a group of subjects can or will be a higher number in hours than a CFB threshold, which is a minimum limits test. It is anticipated that the versions 2.0 and 2.55 will display similar in-life efficacy to that of prototype 1.0, as the unbuffered 1.0 and buffered 2.0 and 2.55 versions all have encapsulated IR3535 at a nominal concentration of 20% (w/w).

Regarding repellency of ticks, a pilot study was performed with several human subjects using the prototype version 1.0 at the Arthropod Control Product Test Centre, London School of Hygiene & Tropical Medicine (London, United Kingdom). It yielded an average repellency time of approximately 15–16 hours against *Rhipicephalus sanguineus* (data not shown). The authors anticipate a similar result with buffered versions 2.0 and 2.55. In aggregate, the preliminary results of the field trial with mosquitos and the pilot study with ticks are suggestive of long-duration repellency, consistent with that reported previously using an arm-in-cage experimental method with mosquitos.

Both humans and nonhuman mammals (e.g., horses, cows, dogs, and cats) may benefit from topical application of this formulation. It is expected to be effective immediately, and the duration of effective mosquito repellency may last for 12–13 hours, depending on the species of mosquito, fly, sandfly, black fly, louse, chigger, bed bug, or vector of trypanosomiasis, as well as the noninsect arthropod pest species of tick, chigger (Trombiculidae), spider, or scorpion.

The repellent lotion formulation provides a practical method of treating skin, hair, or clothing of a mammal to repel insects or noninsect arthropod pests. Dilutions of the lotion can also be prepared prior to application to the skin, hair, or clothing. For instance, a lotion could be diluted 1:5, 1:10, 1:50, or 1:100 in water or another solution or solvent (e.g., ethanol or ethanol:water). The diluted repellent solution could be applied immediately to

a human or nonhuman mammal (e.g., horses, cows, dogs, or cats), or clothing. Diluted versions on skin, *per se*, are expected to have lower repellency than the undiluted lotion.

A long-term goal of our formulation development efforts reaches beyond merely protection from the nuisance of biting insects or other pests. The authors desire to demonstrate that once or twice daily applications of the sustained-release IR3535 lotion can prevent the symptoms of vector-borne diseases. The symptoms will be those common to specific and endemic vector-borne diseases (cutaneous erythema, inflammation, skin irritation, fever, headaches, etc.). Diagnostic tests might include immunologic or microscopic tests for pathogens within the blood, or RNA or DNA of the pathogen within the blood. The reduction in symptoms is anticipated within weeks or months of commencing daily topical applications of the repellent. This benefit is expected to last for the duration of the course of protection from biting insects or noninsect arthropod pests.

At present, clinical prophylactic studies are in the planning stages. Consideration is being given to studies of vector-borne diseases, such as malaria, dengue fever, Lyme disease (borreliosis), leishmaniasis, Chagas disease (trypanosomiasis), Zika fever, equine encephalitis, and yellow fever. Current unanswered questions include whether the daily application of the sustained-release formulation (versions 2.0 or 2.55) can reduce the number of patients experiencing clinical symptoms of an endemic insect-borne disease. Meanwhile, can the daily application of the formulations impact quality of life, such as sleep or productivity at work? Can the preventative formulations be demonstrated as superior to standard-of-care topical repellent products containing a commercial DEET or IR3535 formulation?

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REFERENCES

(1) G.B. Kasting, V.D. Bhatt, and T.J. Speaker, Microencapsulation decreases the skin absorption of N,N-diethyl-m-toluamide (DEET), *Toxicol. In Vitro*, **22**(2), 548–552 (2008).

(2) J.I. Karr, T.J. Speaker, and G.B. Kasting, A novel encapsulation of N,N-diethyl-3-methylbenzamide (DEET) favorably modifies skin absorption while maintaining effective evaporation rates, *J. Control Release*, **160**(3), 502–508 (2012).

(3) M.R. Gomes Fernandes, L.C. Lopes, R.S. Iwami, M. Del Grossi Paglia, B. Mateus de Castilho, A. Maicon de Oliveira, I. Fulone, R.S. Leite, and C. de Cássia Bergamaschi, Efficacy and safety of repellents marketed in Brazil against bites from *Aedes aegypti* and *Aedes albopictus*: a systematic review, *Travel Med. Infect. Dis.*, 44, 102179 (2021).

(4) D.R. Barnard, U.R. Bernier, K.H. Posey, and R.D. Xue, Repellency of IR3535, KBR3023, para-menthane-3,8-diol, and deet to black salt marsh mosquitoes (Diptera: Culicidae) in the Everglades National Park, *J. Med. Entomol.*, **39**(6), 895–899 (2002).

(5) J.E. Cilek, J.L. Petersen, and C.E. Hallmon, Comparative efficacy of IR3535 and deet as repellents against adult *Aedes aegypti* and *Culex quinquefasciatus, J. Am. Mosq. Control Assoc.*, **20**(3), 299 –304 (2004).

(6) U. Thavara, A. Tawatsin, J. Chompoosri, W. Suwonkerd, U.R. Chansang, and P. Asavadachanukorn, Laboratory and field evaluations of the insect repellent 3535 (ethyl butylacetylaminopropionate) and deet against mosquito vectors in Thailand, *J. Am. Mosq. Control Assoc.*, **17**(3), 190–195 (2001).

(7) S.P. Frances, D.O. MacKenzie, K.L. Rowcliffe, and S.K. Corcoran, Comparative field evaluation of repellent formulations containing deet and IR3535 against mosquitoes in Queensland, Australia, *J. Am. Mosq. Control Assoc.*, **25**(4), 511–513 (2009).

(8) S. Licciardi, J.P. Herve, F. Darriet, J.M. Hougard, and V. Corbel, Lethal and behavioural effects of three synthetic repellents (DEET, IR3535 and KBR 3023) on *Aedes aegypti* mosquitoes in laboratory assays, *Med. Vet. Entomol.*, **20**(3), 288–293 (2006).

(9) S.K. Nakayama, R. Graf, A.M. Bohlmann, J. Zurlage, H. Epstein, M. Ranzani, and F. Pflüecker, Influence of sex ratio and density on the comparative repellency of IR3535® against caged *Aedes*, *Anopheles*, and *Culex* mosquitoes, *J. Cosmetic Sci.*, **70**(4), 197–207 (2019).

(10) C. Costantini, A. Badolo, and E. Ilboudo-Sanogo, Field evaluation of the efficacy and persistence of insect repellents DEET, IR3535, and KBR 3023 against *Anopheles gambiae* complex and other Afrotropical vector mosquitoes, *Trans. R. Soc. Trop. Med. Hyg.*, **98**(11), 644–652 (2004).

(11) M.M.N. Reynoso, E.A. Seccacini, J.A. Calcagno, E.N. Zerba, and R.A. Alzogaray, Toxicity, repellency and flushing out in *Triatoma infestans* (Hemiptera: Reduviidae) exposed to the repellents DEET and IR3535, *Peer J*, **5**, e3292 (2017).

(12) R.A. Alzogaray, Behavioral and toxicological responses of *Rhodnius prolixus* (Hemiptera: Reduviidae) to the insect repellents DEET and IR3535, *J. Med. Entomol.*, **53**(2), 387–393 (2016).

(13) J.F. Carroll, J.P. Benante, M. Kramer, K.H. Lohmeyer, and K. Lawrence, Formulations of deet, picaridin, and IR3535 applied to skin repel nymphs of the lone star tick (Acari: Ixodidae) for 12 hours, *J. Med. Entomol.*, **47**(4), 699–704 (2010).

(14) T.J. Naucke, S. Lorentz, and H.W. Grünewald, Laboratory testing of the insect repellents IR3535 and DEET against *Phlebotomus mascittii* and *P. duboscqi* (Diptera: Psychodidae), *Int. J. Med. Microbiol.*, **296** Suppl 40, 230–232 (2006).

(15) E.N.I. Weeks, G. Wasserberg, J.L. Logan, J. Agneessens, S.A. Stewart, and S. Dewhirst, Efficacy of the insect repellent IR3535 on the sand fly *Phlebotomus papatasi* in human volunteers, *J. Vector Ecol.*, **44**(2), 290–292 (2019).

(16) Manual on development and use of FAO and WHO specifications for pesticides, First Edition – third revision, World Health Organization, (2016).

(17) K. Rossington, The Science of Chlorine-Based Disinfectant 2014, accessed June 23, 2022, https://www.manufacturingchemist.com/news/article_page/The_science_of_chlorine-based_disinfectant/103112.

(18) S. Tienungoon, P. Pinnil, K.-O. Kreelieng, and S. Onket, Quantitative bactericidal efficacy of alcohol-based handrub in Thailand using modified EN 1276: 2009 method, *Bulletin Dept. Med. Sciences (Thailand)*, **61**(2), 60–72 (2019).

(19) A. Şahiner, E. Halat, and E.A. Yapar, Comparison of bactericidal and fungicidal efficacy of antiseptic formulations according to EN 13727 and EN 13624 standards, *Turk. J. Med. Sci.*, **49**(5), 1564–1567 (2019).

(20) S. Messager, K.A. Hammer, C.F. Carson, and T.V. Riley, Assessment of the antibacterial activity of tea tree oil using the European EN 1276 and EN 12054 standard suspension tests, *J. Hosp. Infect.*, **59**(2), 113–125 (2005).

(21) Z. Iyigundogdu, S. Kalayci, A.B. Asutay, and F. Sahin, Determination of antimicrobial and antiviral properties of IR3535, *Mol. Biol. Rep.*, **46**(2), 1819–1824 (2019).

(22) S.P. Carroll, Prolonged efficacy of IR3535 repellents against mosquitos and blacklegged ticks in North America, *J. Med. Entomol.*, **45**(4), 706–714 (2008).