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Review

Biofabrication with insect cells

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ABSTRACT

Insect cells may be preferred over mammalian cells for biofabrication because of several bioprocess benefits including tolerance to fluctuations in the external environment, low secretion of and sensitivity to toxic by-products and ease of genetic modification. Insect muscle cells, in particular, are functionally promising *in vitro* but have yet to find a purpose outside of basic research. Insect muscle cell development and physiology are well-documented and myogenic cell populations from a variety of species and tissue types have been propagated *in vitro*. Muscle cells can be easily isolated from insect embryos or metamorphosing stages, separated from contaminating cell types and triggered to differentiate *via* administration of insect-specific hormones. The abilities of insect cells to grow under ambient conditions, survive and function (i.e., contract) for extended periods of time without fresh nutrients and to exhibit powerful contractions present an attractive alternative to mammalian cell culture in the context of advanced manufacturing processes. Moreover, insect cells are less costly to produce at large-scale, lowering barriers to commercialization. Bioactuation devices, cultured meat and ingestible vaccines have been identified as promising areas of application for insect cell cultivation, with others likely to emerge. Some of the next steps to advance insect cell-based technologies include the design of control systems to regulate *in vitro* contractions, adaptation of tissue engineering techniques for invertebrate cells, scaling insect cell and tissue formation to meet the needs of these broader applications, and evaluation of food nutrition and safety.

KEYWORDS: insect cell culture, insect muscle cells, tissue engineering, bioactuation, cellular agriculture, cultured meat, vaccine production, robotics, advanced manufacturing.

INTRODUCTION

Insect cells are often preferred over mammalian cells in biotechnology industries, such as recombinant protein production, due to a number of bioprocess advantages such as greater tolerance to fluctuations in the external environment (e.g., pH, temperature), lower secretion of and sensitivity to toxic by-products (e.g., ammonia, lactate), more scalable production (e.g., serum-free medium adaptation, suspension culture) and ease of immortalization [1, 2]. Further, when coupled with the baculovirus expression vector system, insect cells are efficient at manufacturing complex proteins and performing post-translational modifications while retaining high expression levels [1]. Insect cells have also remained as key subjects for basic research as they are relatively simple to isolate, culture and maintain, coupled with the diversity of insect species giving rise to a myriad of cells with interesting properties [3]. Furthermore, there is significant literature surrounding insect cell physiology and numerous research tools (e.g., antibodies, cell lines, growth medium) are available to facilitate insights.

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The majority of biotechnology innovations are applied to the fields of human medicine and conventional agriculture. However, in recent decades, potential uses of *in vitro* cells, tissues and organs have expanded to include future food systems and biomaterial and device fabrication. Given their unique properties, insect cells may be specifically well-suited for these newer and novel applications of biotechnology. This review first summarizes the history and progression of insect cell culture and details literature relevant to insect fat body and muscle cell isolation, culture and maintenance. We also present cultured meat, edible vaccines and bioactuation devices as opportunities for insect cells to transcend beyond the research laboratory.

History

The earliest documentation of cell culture is attributed to Harrison who, in 1907, cultured frog embryonic tissues – and observed differentiation of epidermal, muscle and nerve cells – within droplets of frog lymph, maintained for up to four weeks [4, 5]. Five years later, Glaser and Chapman reported the first insect cell culture study in which the progression of a viral disease was examined in caterpillar hemocyte cultures [6, 7]. A significant milestone was achieved in the 1960s when long-term (i.e., more than one year) cultures were established for multiple cell strains derived from *Opodiphthera eucalypti* (emperor gum moth) ovarian tissue [8]. Success was ascribed to the use of an original medium formulation (i.e., Grace's Insect Medium) composed of salts, amino acids, sugars, organic acids, vitamins, antibiotics and *O. eucalypti* insect plasma [8, 9]. Derivatives of this medium are still widely used to culture insect (e.g., dipteran, lepidopteran) and crustacean cells [10]. As of March 2020, over 1,000 insect cell lines had been established from over 150 distinct species [11].

Common applications of insect cell cultivation include fundamental biology, virology, pesticide development and pharmaceutical manufacture, advances of which are reviewed elsewhere [6, 9]. One noteworthy application lies in the potential of insect antimicrobial substances [12]. Fat body tissues of multiple insect species are known to secrete antimicrobial peptides to oppose infection [3]. These peptides could be a powerful tool against antimicrobial resistance as, due to the specific modes of action (e.g., membrane disruptive activity) and broad activity, microbes are less likely to develop resistance to these substances compared to traditional antimicrobials [12]. Another prominent achievement was the commercialization of a recombinant influenza vaccine (i.e., Flublok) produced within engineered insect cells. Approved by the Food and Drug Administration (FDA) in 2013, FluBlok holds multiple advantages over the conventional egg-based influenza vaccines, including the absence of allergens, broader protection and higher antigen yield [13, 14].

Most established insect cell lines are derived from embryonic or ovarian tissues and the most frequently used lines are S2 (*Drosophila melanogaster*; embryonic), Sf9, Sf21 (*Spodoptera frugiperda*; ovarian) and BTI-TN-5B1-4 (*Trichoplusia ni*; ovarian) [6]. Although reports of muscle-specific insect cell culture are relatively scarce, existing findings point to interesting new directions for development. Since the 1970s, insect muscle cells have been successfully isolated from multiple insect orders (e.g., Diptera, Hymenoptera, Lepidoptera) and tissue types (e.g., dorsal vessel, embryonic, leg, ovarian) [15-20]. While the majority of studies focus on primary cultures, a few continuous cell lines have been established (e.g., IPLB-Tex2, NISES-AnPe-426, R1-R7) [15-17]. Insect muscle cells can be classified *in vitro* by their spindle-like morphology, differentiation upon administration of molting hormone (i.e., ecdysone) and spontaneous contractions. Several studies demonstrate the capacity for insect muscle cells to survive and function *in vitro* over multiple months in the absence of medium refreshment [21-24].

Advances in cell culture and tissue engineering have prompted the emergence of novel applications of biofabrication, of which the vast majority of efforts are focused on mammalian cell types. Here we aim to review the unique properties of invertebrate cells, with emphasis on insect muscle and fat body cells, and elucidate the relevance of these cells towards unconventional approaches to

meat production, vaccine manufacture and robotics. While these activities are in their infancy at present, the view towards the benefits of these cells' sources and the diversity of cell types, suggests important opportunities ahead to integrate insect cells into a wide range of advanced manufacturing concepts and needs.

Insect muscle development and physiology

Much of the research surrounding insect muscle development and regeneration is derived from studies on the dorsal longitudinal muscles (i.e., flight muscles) of *D. melanogaster* [25]. During embryogenesis, a set of mesodermal cells gives rise to myogenic progenitor cells and fusion-competent myoblasts [26]. Myogenic progenitor cells asymmetrically divide to produce muscle founder cells and adult muscle precursor cells [27]. One founder cell will fuse with 2-25 fusion-competent myoblasts to generate larval muscle [28]. Adult muscle precursor cells remain quiescent through embryogenesis and aid in the formation of adult muscle tissue. During metamorphosis, most adult muscles form de novo from adult muscle precursor cells and imaginal discs [29]. In select cases, such as specific flight muscles in *D. melanogaster* and *Manduca sexta*, adult muscles do not form de novo and instead form by fusion-competent adult-specific myoblast fusion with larval muscle templates [30, 31]. Adult muscles are identifiable due to the expression of myosin heavy chain, as in vertebrate muscles [32]. Details of this process as well as the formation of other insect somatic muscles and information on insect satellite cell populations have been reviewed extensively elsewhere [25].

Insect muscle can be classified as skeletal or visceral muscle, both of which are striated. Skeletal muscles are elongated and parallel while visceral muscles are often formed from a lattice [33]. Though vertebrate skeletal muscles are largely consistent in structure, invertebrate muscle features (e.g., filament structure, nuclei distribution) vary widely between species, developmental stage and muscle type [33, 34]. Also in contrast with vertebrate muscle, the innervation of insect muscle fibers is polyterminal and muscle contractions are evoked by multiple local endplate potentials rather than propagating action potentials [33]. Moreover, while vertebrate muscles are limited to a maximum contraction strain of 50%, select invertebrate muscles (e.g. insect dorsal vessel, larval intersegmental and visceral muscles) can perform "supercontractions" of up to 76% [33, 35].

Insect muscle cell isolation and culture

Insect muscle cells have been cultivated in primary cultures to investigate myogenesis and *in vitro* differentiation since the early 1970s [36, 37]. Insect species of the Diptera (e.g., *D. melanogaster*) and Lepidoptera (e.g., *Antheraea pernyi*, *Ctenoplusia agnate*, *M. sexta*) orders are common cell donors for these studies [17, 19, 37, 38]. Myogenic cells are most frequently isolated from insect embryos although they have also been isolated from larval dorsal vessel, ovarian and pupal leg tissue [19, 20, 37, 39]. To obtain myogenic primary cultures, embryos or tissues are homogenized and transferred to a culture vessel with growth medium (Figure 1A). Growth medium typically consist of modified formulations of basal medium (e.g., Grace's, Leibovitz's L-15, Schneider's) and are supplemented with fetal bovine serum ranging between 10-20%, although a couple of serum-free medium have been successfully employed [16, 24]. The cell population is often heterogeneous although myogenic purity can be increased by embryo staging, tissue selection, medium additives (e.g., insulin) or substrate coatings (e.g., protamine) [36, 37, 40, 41]. Common medium additives include insulin, which increases myotube formation and protein synthesis, and ecdysone, which often triggers differentiation [19, 40]. Observed timelines for *in vitro* cell elongation, fusion, striation and contraction vary widely between studies (Table 1). Most studies observed the formation of spontaneously contractile cells over the first few weeks in culture and contractions were reported to last over multiple weeks or months (Table 1). A few myogenic cell lines have been established, all originating from embryonic cells. IPLB-Tex2 (*T. exiguum*) and NISES-AnPe-426 (*A. pernyi*) spontaneously immortalized while R1-R7 (*D. melanogaster*) were genetically immortalized *via* Ras^{V12}, an oncoprotein [15-17]. One notable study induced myogenic differentiation in the popular S2 cell line by administration of neocarzinostatin, a drug that induces double-strand breaks in DNA, albeit

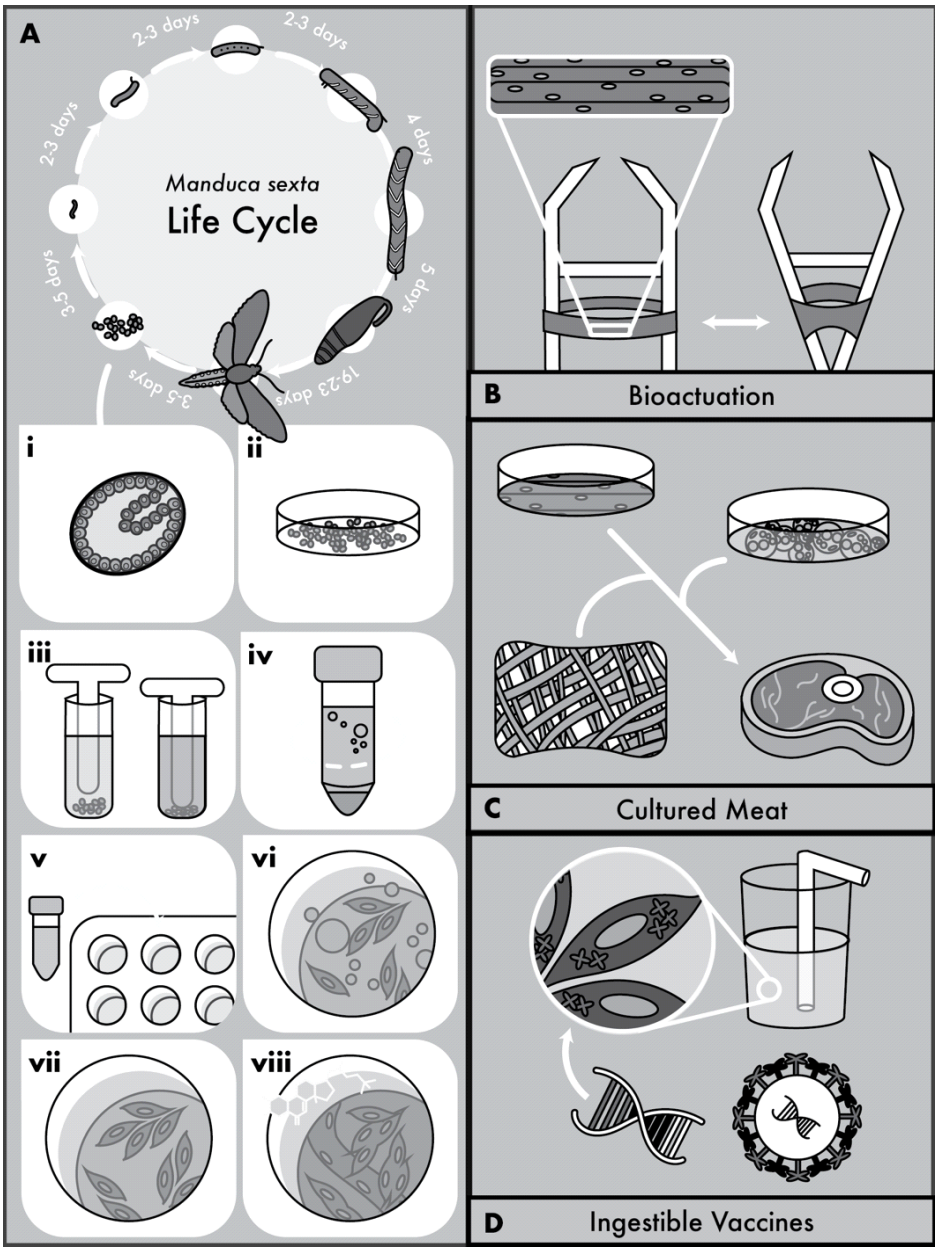

Figure 1. (**A**) Embryonic isolation from *M. sexta* for myogenic cell culture as described in [22]. (i) Eggs harvested and staged at 19 hours, at which time gastrulation and germ band elongation is underway but myogenic differentiation is not complete. (ii) Eggs counted, sterilized and rinsed with growth medium. (iii) Eggs gently homogenized to release cells into the growth medium. (iv) Cell suspension centrifuged to separate yolk cells from the adherent cell (e.g., fibroblast, myoblast, neuron) populations. (v) Cell culture plates coated with proteins (e.g., concanavalin A, laminin, protamine) to increase myogenic cell adhesion [19, 41]. (vi) Cell pellet resuspended and plated on the protein-coated surface. (vii) After an incubation period, non-adherent cell types removed. (viii) Cells treated with molting hormone (i.e., ecdysone) to trigger myogenic differentiation. (**B**) Bioactuation devices: *In vitro* insect muscle constructs coupled with substrates to power actuation *via* spontaneous or electrically induced contractions. (**C**) Cultured meat: Insect muscle cells, fat body cells and edible scaffolding material integrated to generate biofabricated meat for human consumption. (**D**) Ingestible vaccines: Insect cells engineered to express antigens from human pathogens (e.g., influenza virus) and formulated into food products to immunize against infectious disease in a convenient and effective manner.

Table 1. Procedural details and results from select studies of *in vitro* culture of myogenic insect cells.

Reference	**[36]**	**[37]**	**[43]**	**[44]**
Insect strain	-	WT Oregon-R	WT Oregon-R	WT Oregon-R
Culture type	Primary	Primary	Primary	Primary
Cell line	-	-	-	-
Species	*Dasychira plagiata; Malacosoma disstria; Trichoplusia ni*	*Drosophila melanogaster*	*Drosophila melanogaster*	*Drosophila melanogaster*
Order	Lepidoptera	Diptera	Diptera	Diptera
Common name	Pine tussock moth; Forest tent caterpillar, cabbage looper	Common fruit fly	Common fruit fly	Common fruit fly
Tissue	Pupae head, thoracic muscle	Gastrulae	Gastrulae	Gastrulae
Stage	2-3 days	1-3 hours, 9 hours	30-180 min. post-gastrulation (4-6.5 hours)	35 min. post-gastrulation
Basal medium	Yunker modified Wyatt-Grace's	Schneider's	Modified Schneider's	Modified Schneider's
Animal serum	10% Fetal bovine serum	15% New-born calf serum	18% Fetal calf serum	18% Fetal calf serum
Antimicrobials	Penicillin, streptomycin sulfate	Penicillin, streptomycin sulfate	Penicillin, streptomycin	Penicillin, streptomycin
Additives	10% Whole chicken egg ultrafiltrate, 1% bovine plasma albumin	Glutathione (1 mg/mL)	-	-
Substrate	Sykes-moore tissue culture chamber; cooper vessel	Tissue culture plastic	Cover glass slip	Tissue culture plastic
Seeding density	-	1 embryo/5 uL	3000 cells/mL	300 cells/0.3 mL
Incubation temperature (°C)	27	25	26	26
Doubling time	-	-	-	-
pH	-	-	6.78	6.78
Atmosphere	-	7% Carbon dioxide	Humidified air	Humidified air
Medium change intervals	4 days	-	weeks	-
Subculture ratio	-	-	-	-

Table 1 continued..

Reference	**[36]**	**[37]**	**[43]**	**[44]**
Subculture intervals	-	-	-	-
Muscle cell length	70-100 um	30-50 um	-	8-141 um
Contraction rate/force	-	1-30 contractions/minute	-	-
Time of first observed contraction	6 days	20 hours	-	-
Time of first myoblast division	-	-	-	5 hours
Time of elongation	-	-	-	12 hours
Time of fusion	24 hours	-	-	-
Time of striations	3 days	-	-	-
Length of culture	3 days	-	-	-

Reference	**[40]**	**[41]**	**[45]**	**[16]**
Insect strain	WT Oregon-R	P2 Oregon-R	-	-
Culture type	Primary	Primary	Primary	Continuous
Cell line	-	-	-	IPLB-Tex2
Species	*Drosophila melanogaster*	*Drosophila melanogaster*	*Periplaneta americana*	*Trichogramma exiguum*
Order	Diptera	Diptera	Blattodea	Hymenoptera
Common name	Common fruit fly	Common fruit fly	American cockroach	Trichogrammid wasps
Tissue	Gastrulae	Gastrulae	Embryonic thoracic & abdominal muscle somites	Embryonic
Stage	35-50 min. post-gastrulation	2-3 hours	11 days	-
Basal medium	Modified Schneider's	-	Leibovitz's L-15, Yunker's modified grace's	IPL-52B; ExCell 400
Animal serum	18% Fetal calf serum	-	5% Horse serum	-
Antimicrobials	Penicillin, streptomycin	-	Penicillin, streptomycin	-
Additives	Insulin (0-11.1 mU/mL)	-	Ecdysone (10 ug/mL)	Ecdysone (0.1-10 ug/mL)

Table 1 continued..

Reference	**[40]**	**[41]**	**[45]**	**[16]**
Substrate	Tissue culture plastic	Protamine-treated (0.1 mg/mL) tissue culture plastic	Tissue culture plastic	-
Seeding density	1 embryo/3 mm^2	20 embryos/60 mm dish	180K cells/ 50 x 12 mm dish	-
Incubation temperature (°C)	26	26	29	27
Doubling time	-	-	-	50 hours
pH	6.78	-	-	-
Atmosphere	Humidified air	Humidified air	-	-
Medium change intervals	-	-	-	-
Subculture ratio	-	-	-	1:2
Subculture intervals	-	-	-	2-4 weeks
Muscle cell length	-	-	39-50 um	-
Contraction rate/force	-	-	-	-
Time of first observed contraction	-	15 hours	17-29 days	3 days (after ecdysone treatment)
Time of first myoblast division	-	-	1 day	-
Time of elongation	-	-	2 days	-
Time of fusion	-	-	4-5 days	-
Time of striations	-	-	18-20 days	-
Length of culture	-	-	35-40 days	120 subcultures

Reference	**[17]**	**[19]**	**[42]**	**[39]**
Insect strain	-	-	-	-
Culture type	Continuous	Primary	Continuous	Primary
Cell line	NISES-AnPe-426	-	S2	-
Species	*Antheraea pernyi*	*Manduca sexta*	*Drosophila melanogaster*	*Thysanoplusia intermixta*
Order	Lepidoptera	Lepidoptera	Diptera	Lepidoptera
Common name	Chinese oak silkworm	Tobacco hawkmoth	Common fruit fly	Chrysanthemum golden plusia

Table 1 continued..

Reference	**[17]**	**[19]**	**[42]**	**[39]**
Tissue	Embryonic	Pupal leg muscle	Embryonic	Larval dorsal vessel
Stage	48 hours	P2	Late stage	-
Basal medium	MGM-448	Modified L-15-grace's	Schneider's drosophila; 0.5% polypeptone	TC-100
Animal serum	10% Fetal bovine serum	10% Fetal bovine serum	10% HI Fetal bovine serum	20% HI Fetal bovine serum
Antimicrobials	-	-	Penicillin, streptomycin	Penicillin, streptomycin
Additives	-	Ecdysone (1 ug/mL)	Ecdysone (0.5 uM); Neocarzinostatin (20 nM)	-
Substrate	-	Glass coated with concanavalin A (200 ug/mL) + laminin (2 ug/mL)	Tissue culture plastic	
Seeding density	-	-	1E4 cells/24-well	-
Incubation temperature (°C)	25	26	27	25
Doubling time	-	-	-	-
pH	-	6.2	-	-
Atmosphere	-	-	-	High humidity
Medium change intervals	3 weeks		3-4 days	2 weeks (50% change)
Subculture ratio	1:2		1:5	-
Subculture intervals	2-3 months		3-4 days	-
Muscle cell length	-		-	-
Contraction rate/force	5-35 contractions/ minute	-	1 Hz	
Time of first observed contraction	4 months	4 days	-	7 days
Time of first myoblast division	-	-	-	-
Time of elongation	-	2 days	12 hours (after drug treatment)	-
Time of fusion	-	4 days	-	-
Time of striations	-	-	-	-
Length of culture	5+ years; 15 subcultures	-	-	18 days

Table 1 continued..

Reference	**[38]**	**[27]**	**[22]**	**[23]**
Insect strain	-	twi(promoter)-actin-GFP; apME-NLS::GFP; apME-NLS::dsRed	-	-
Culture type	Primary	Primary	Primary	Primary
Cell line	-	-	-	-
Species	*Ctenoplusia agnate*	*Drosophila melanogaster*	*Manduca sexta*	*Manduca sexta*
Order	Lepidoptera	Diptera	Lepidoptera	Lepidoptera
Common name	Moth	Common fruit fly	Tobacco hawkmoth	Tobacco hawkmoth
Tissue	Larval dorsal vessel	Embryonic	Embryonic	Embryonic
Stage	Last instar	3-4 hours	19-22 hours	19-22 hours
Basal medium	TC-100	Schneider's	Modified L-15-Grace's	Modified L-15-Grace's
Animal serum	10% Fetal bovine serum	20% Fetal bovine serum	12% Fetal bovine serum	12% Fetal bovine serum
Antimicrobials	Penicillin, streptomycin	Penicillin, streptomycin, gentamicin	Penicillin, streptomycin, amphotericin B	Penicillin, streptomycin, amphotericin B
Additives	-	Insulin (1 mU/mL)	Ecdysone (20 ng/mL); Methoprene (250-1000 ng/mL)	Ecdysone (20 ng/mL)
Substrate	Tissue culture plastic; PDMS thin film; agarose gel	Tissue culture plastic	Tissue culture plastic	Tissue culture plastic; PDMS mold
Seeding density	-	2.5E5 cells/cm^2	5 embryos/cm^2	2.7E4 cells/cm^2
Incubation temperature (°C)	25	18	26	15-37
Doubling time	-	-	-	-
pH	-	6.9	6.5	5.5-7.5
Atmosphere	-	-	Humidified air	Humidified air
Medium change intervals	-	-	-	-
Subculture ratio	-	-	-	-
Subculture intervals	-	-	-	-
Muscle cell length	-	-	-	-
Contraction rate/force	.24 Hz	-	-	2 kPa

Table 1 continued..

Reference	**[38]**	**[27]**	**[22]**	**[23]**
Time of first observed contraction	-	-	5 days	-
Time of first myoblast division	-	-	-	-
Time of elongation	-	-	-	-
Time of fusion	-	8-24 hours	10 days	-
Time of striations	-	12 days	-	-
Length of culture	-	-	75 days	months

Reference	**[15]**	**[20]**	**[46]**	**[24]**
Insect strain	Act5C>UAS-Ras(V12), UAS-GFP	-	-	Act5C>UAS-Ras(V12), UAS-GFP
Culture type	Continuous	Primary	Primary	Continuous
Cell line	R1-R7	-	-	R3
Species	*Drosophila melanogaster*	*Bombyx mori*	*Ctenoplusia agnate*	*Drosophila melanogaster*
Order	Diptera	Lepidoptera	Lepidoptera	Diptera
Common name	Common fruit fly	Domestic silkworm	Moth	Common fruit fly
Tissue	Embryonic	Ovarian	Larval dorsal vessel	Embryonic
Stage	8-24 hours	Fifth instar	Final stage	8-24 hours
Basal medium	Schneider's	Grace's	TC-100	Schneider's; ExCell 405
Animal serum	10% Fetal bovine serum	10% HI Fetal bovine serum	20% Fetal bovine serum; 5% hemolymph	10% Fetal bovine serum
Antimicrobials	Penicillin, streptomycin	Penicillin, streptomycin, amphotericin B, gentamycin	Penicillin, streptomycin	Penicillin, streptomycin
Additives	Ecdysone (1 ug/mL)	-	-	Ecdysone (500-1000 ng/mL)
Substrate	Tissue culture plastic	Tissue culture plastic	Copolymeric gel-grafted surface	Chitosan films, sponges
Seeding density	-	2E5 cells/mL	-	7.5E4-3E5 cells/cm^2

Table 1 continued..

Reference	[15]	[20]	[46]	[24]
Incubation temperature (°C)	22	26	25	19
Doubling time	-	72 hours	-	-
pH	-	-	-	-
Atmosphere	-	-	-	-
Medium change intervals	2 weeks	1 week	2 days	1 week
Subculture ratio	1:2-1:4	1:2	-	1:2-1:5
Subculture intervals	7 days - 4 weeks	5-10 days	-	1-2 weeks
Muscle cell length	-	40 um	-	68-143 um
Contraction rate/force	-	-	.18 Hz	-
Time of first observed contraction	-	1 month	-	-
Time of first myoblast division	-	-	-	-
Time of elongation	24 hours (after ecdysone treatment)	7 days	-	-
Time of fusion	-	-	-	-
Time of striations	-	-	-	-
Length of culture	60+ subcultures	4 months	-	-

at low efficiency, as only 1-2% of cells were multinucleated [42]. The existing literature provides robust protocols for isolation, culture and maintenance of functional myogenic insect cells. One area that is lacking is the application of tissue engineering techniques for invertebrate cells, which would advance the development of larger, three-dimensional constructs.

Insect fat body physiology and cell culture

The insect analog of vertebrate adipose tissue is an organ called the fat body, which also performs endocrine, metabolic and immunity functions [47]. In insect species (e.g., cockroaches, crickets) that do not pupate (i.e., hemimetabolous), fat body tissue generated in larval animals persists through adulthood while in pupating (i.e., holometabolous) species (e.g., flies, moths), larval fat body disintegrates during metamorphosis and adult fat body tissue is rebuilt from dissociated larval fat cells or developed *de novo* [48]. Fat body tissue primarily consists of trophocytes although hemoglobin cells, mycetocytes, oenocytes and urocytes have been identified in some species [47, 49]. Like muscle, in *D. melanogaster* the fat body is derived from the embryonic mesoderm which gives rise to fat body progenitor cells that proliferate and form the three domains of larval fat body: the dorsal fat-cell projections, the lateral fat body and the ventral commissure [48]. Insect fat body plays multiple roles in the storage and synthesis of carbohydrates (e.g., glycogen, trehalose), lipids (e.g., triglycerides) and proteins (e.g., diapause proteins, vitellogenin) [49]. Mobilization of these energy storages is regulated by adipokinetic hormone, insulin and hypertrehalosemic hormone signaling [48].

Over 60 cell lines have been established from the fat body tissue of various insect species and it was one of the first insect tissues to be cultured [11, 47]. Fat body cells can be isolated *via* explant culture by anaesthetizing an animal (generally female animals contain more fat body tissue) with carbon dioxide and/or ice, dissecting the fat body tissue away from the trachea tissue and performing pre-plating to remove rapidly-attaching contaminating hemocyte cells [50]. Then, fragments can be mechanically homogenized and transferred to culture dishes with appropriate growth medium [50]. Both chemically defined and serum-supplemented formulations have been used to support fat body cell culture [50]. In a *P. americana* experiment, continuous cell lines were established from fat body cultures, exhibiting doubling times of 5-8 days and observed to store glycogen and lipids for over six months [50]. Lipid-containing cells have also been observed in *M. sexta* embryonic cell cultures, although they were not detected to proliferate [22]. Fat body cell culture has historically been utilized to study insect hormone (e.g., 20-hydroxyecdysone) and protein (e.g., vitellogenin) synthesis [47]. For novel applications, fat body cell cultivation may prove useful due to unique functionality including antimicrobial peptide synthesis, energy storage and mobilization and nutrition profiles [51].

Bioactuation devices

Insect muscles offer potential to engineer new types of actuators for soft robotic and medical device applications (Figure 1B). The majority of commercial actuators (e.g. electric motors, solenoids) are rigid, and current soft actuators, typically based on inflation and deflation of soft materials, rely on rigid components and heavy off-board power sources making them incompatible for biological and soft robotics applications [52]. In contrast, muscles are intrinsically soft and allow for complex movements in both soft- and rigid-bodied animals.

There are several intrinsic properties that present muscle as an attractive choice for actuation in devices. Muscles are often able to produce higher forces than soft actuators of similar size and they can be powered by simple sugars, amino acids and lipids, producing only biodegradable waste products [53, 54]. These biofuels have an energy density comparable to gasoline and kerosene but they are safe and converted into mechanical energy at low temperature in an aqueous environment. Fat body co-cultures could be implemented to provide an energy source for muscle cells by mobilization of glycogen stores [22, 48]. One advantage of this approach is that muscle actuators can be controlled by electrical or optogenetic stimulation. In both cases this activates the intrinsic contractile mechanism using an exceedingly small amount of external energy thereby removing the need to carry heavy batteries. Muscles also provide better mechanical impedance matching for locomotion in complex environments [55]. Insect muscles provide key advantages over vertebrate muscles for bioactuator applications; more robust in that they can survive a relatively wider range of temperatures, do not require vasculature, do not atrophy when deprived of innervation, and can withstand greater levels of oxidative stress, anoxia and radiation [56-58]. The molecular basis of insect muscle development and fate specification is known in detail, which provides powerful tools for controlling the physiological characteristics of engineered bio-actuators. Additionally, many insects are holometabolous, going through metamorphosis to change from a soft larval form to a rigid adult body, so insect muscle can be sourced from different life stages depending on the intended application.

In recent years, several examples of insect muscle-based actuators have been developed [59, 60]. These actuators have been used to drive small rigid and soft devices in action such as inching, pinching, swimming and pumping [61-64]. For example, excised dorsal vessel muscle from *M. sexta* was used to power a micropillar inching robot [61]. This insect muscle actuator functioned for 90 days without media changes, in a temperature range of 5-40 °C and was controllable both chemically and electrically [61]. While most existing bioactuators are composed of excised muscle, the ability to grow insect bio-actuators *in vitro* would provide additional benefits such as the ability to customize the actuator for size and scale towards specific applications. Embryonic cells from *M. sexta* have been successfully used to

grow contractile muscle cells that are capable of actuation in *in vitro* culture [22]. These muscle cells autonomously assemble into a functional contractile unit and stayed viable for up to 14 days [22, 23]. Myogenic cells originating from *D. melanogaster* embryonic cultures are also able to form contractile units and survive for multiple weeks without medium refreshment [15, 24, 37].

The commercial use of insect muscle actuators in devices is currently limited by scalability and culture methods. Excised muscles are robust but cannot be customized to specific applications; conversely, cultured insect muscle actuators can be designed and matched to specific applications but are not yet as robust as their excised counterparts [22]. Future studies in molecular mechanisms including gene editing and molecular cues, as well as improvements in scaffolding technology to support muscle tissues grown from insect cells and tissues, will allow for the development of robust insect-based actuators for a range of devices.

Cultured meat

Driven by the negative externalities (e.g., environmental impact, public health interests, animal welfare concerns, food safety) of conventional animal agriculture, the alternative protein industry is expanding rapidly. Focus in this area is divided between plant-based meat alternatives, cultured meat and edible insects. Cultured meat is meat grown from *in vitro* cell cultivation as opposed to flesh harvested from an animal. In brief, the production process entails isolating adipose and skeletal muscle progenitor cells from a donor animal, proliferating cells in serum-free growth medium and differentiating tissue within a scaffold system [65]. There are multiple prototypes of cultured beef, pork, chicken and seafood mentioned over the past decade but products have yet to be commercialized. Impediments to bring cultured meat to the market include both technical and regulatory hurdles [66, 67]. Key technical hurdles include formulation of low-cost, xeno-free growth medium, designing scalable production systems for adherent cell types and addressing unknowns surrounding food safety, nutrition and organoleptic properties [68].

While the majority of cultured meat research is based on cultivating cells from familiar food species (e.g., livestock animals, seafood), insect cells, while unconventional, may be uniquely suited for this purpose (Figure 1C). The unique properties of invertebrate cell culture enable more cost-effective and scalable manufacture when compared to cells of more conventional animal species [51]. Specifically, insect cells can grow under a wider range of external conditions (e.g., osmolarity, pH, temperature), do not require serum or recombinant growth factors and may present superior nutritional value [24, 69]. Furthermore, many insect cells can grow in single-cell suspension, consume nutrients at a lower rate and produce lower levels of toxic byproducts (e.g., ammonia, lactic acid) [1, 24]. Another advantage of insect cells is ease of immortalization, through both spontaneous and genetic strategies [2, 15]. These characteristics enable insect muscle cells to be potentially scaled and produced at lower-cost than mammalian muscle cells, similar to how insect cells are often preferable to mammalian cell types for recombinant protein production [1]. Cell immortalization is important with regard to quality control at industrial scale production. For the fat component of meat, adipose tissue can be recapitulated by cultivating cells from insect fat body tissue [49, 51]. Insect fat body cells can also synthesize and secrete antimicrobial peptides which may reduce or eliminate the need for supplementation of external antibiotics during culture [12, 70].

Meat produced from insect cell cultures will be markedly different than food products based on edible insects. In the United States and Canada, entomophagy most commonly takes the form of baked goods or protein bars produced with flour made from ground crickets or mealworms or as novelty foods (e.g., chocolate coated insects) and as such, edible insects are currently considered protein alternatives but not meat alternatives [71, 72]. By generating structured tissue from insect skeletal muscle and fat body cells, cultured insect meat will likely look, taste and feel more similar to conventional meat than to edible insects, which also include significant fractions of exoskeleton and other organs [51]. Tissue engineering

techniques (e.g., genetic modification, growth medium formulation, scaffold integration) can facilitate control over fat and muscle composition, as well as tissue density, organization and structure to produce meat products with specific properties [65]. Cultured insect meat may more closely emulate seafood as sensory evaluations demonstrate edible insects are often described as tasting like fish or shrimp [72, 73].

Ingestible vaccines

Vaccination provides a strong defense against infectious diseases, both directly protecting immunized individuals and propagating herd immunity [74]. Vaccines are composed of live attenuated microbes, killed whole cells or subunits (e.g., conjugates, polysaccharides, proteins, toxoids) [75]. Protein subunit vaccines are manufactured by genetically engineering cells to synthesize antigens associated with the target pathogen. Antigens are purified and often co-delivered with an adjuvant to stimulate immunity. The baculovirus-insect cell expression system is capable of producing complex proteins at industrial scale and is thus an option for vaccine manufacture [76]. Multiple protein subunit vaccines produced in insect cells have been approved for human immunization against cervical cancer (Cervarix®), prostate cancer (Provenge®) and the seasonal influenza (FluBlok®) [77]. FluBlok® offers a number of advantages over conventional egg-based vaccines including more rapid production, as the cloning to production process only takes approximately two months, is lower cost due to less stringent facility requirements and has the benefit of the absence of egg allergens [13]. The versatility and relatively low cost barriers of baculovirus-insect cell expression present the platform as a strong candidate for further vaccine development [76].

While the majority of vaccines are designed to be administered as intramuscular or subcutaneous injections, oral vaccines against adenovirus, cholera, rotavirus and typhoid have been developed and approved by the FDA [78]. Oral administration has numerous benefits over injection routes including distribution ease, patient compliance, self-administration and stimulation of mucosal as well as systemic immunity [75]. Challenges involved with oral administration include delivering active antigens through the gastrointestinal tract, transport of antigens across the mucosal barrier and activating antigen-presenting cells [79]. Oral vaccines can take the form of lyophilized powders that are reconstituted in liquids or capsules and edible vaccines have already been developed in the form of transgenic plants. Vaccines against hepatitis B, Norwalk virus, rabies and the human immunodeficiency virus have been expressed in tobacco plants, potatoes, lettuce, tomatoes and maize [80]. Edible vaccines could circumvent the complex and costly purification requirements with cell culture, provide an option for patients who have difficulty swallowing pills and present new options for broad distribution in the developing world [80, 81].

Advances in biofabricated food, recombinant protein production and edible plant-based vaccines set the stage for innovation: ingestible cell-based vaccines (Figure 1D). Ingestible cell-based vaccines retain the benefits of edible plant-based vaccines and have the potential to address some of the limitations, namely antigen yield and dosage consistency [80]. In a clinical trial investigating heat labile enterotoxin subunit proteins expressed in transgenic potatoes, three doses of 100 g of raw potatoes were required to elicit a sufficient antibody response in human volunteers and the antigen concentration fluctuated between 3.7-15.7 μg/g [82]. Engineering edible cells, such as those used in cultured meat production, to express antigens against infectious disease should enable better control over antigen levels and consistency relative to agricultural methods. Insect cells are promising candidates due to prior establishment in the vaccine production space and the aforementioned benefits relative to mammalian cells with regard to biofabricated food. The production process could look similar to recombinant subunit vaccine manufacture with select discrepancies. For insect cell platforms, the baculovirus expression vector system may not be feasible as cells lyse in late phases of infection; thus it may be preferable for cells to remain viable for further processing into food products [83]. In addition, it would be important to ensure the expressed antigens remain stable during food preparation (e.g., cooking) and

through the gastrointestinal tract which may be feasible through virus-like particle formation [75, 84].

CONCLUSIONS

Advances in biotechnology and biofabrication continually give rise to new possibilities for materials design, future food systems and human medicine. To date, most efforts in these realms have focused on mammalian cells due to established protocols and clinical relevance to humans. Insect cells have been widely employed in certain industries, most notably for recombinant protein production and basic research and are lauded for their ease of maintenance, diversity in cell sources, and unique functionalities. Because cultivation of insect cells is often more cost-effective than that of mammalian cells, insect cells may be a promising platform for future technologies that require large-scale production and are constrained by low price points. Insect muscle cells, in particular, have many favorable traits including: (1) growth in ambient conditions, (2) continued survival and functionality (i.e., contractions) throughout months-long cultures without fresh nutrients, (3) high contraction force and (4) nutrient density. These properties can be exploited to generate the next-generation bioactuator devices, cultured meat products and therapeutics. To advance research and development within these areas, next steps could include the design of control systems for contraction regulation, adaptation of tissue engineering techniques for invertebrate cells for optimization and scale, and evaluation of food nutrition and safety.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

ABBREVIATION

FDA : Food and Drug Administration

REFERENCES

1. Ikonomou, L., Schneider, Y.-J. and Agathos, S. N. 2003, Appl. Microbiol. Biotechnol., 62, 1-20.
2. Lynn, D. E. 2002, J. Insect Sci., 2, 9.
3. Ejiofor, A. O. 2016, Insect Biotechnology, Springer, Cham. doi:10.1007/978-3-319-24244-6_8.
4. Harnden, D. G. 1977, Cell Biology and Cell Culture Methods - A Review, R. A. Harkness and F. Cockburn (Eds.), Springer, Dordrecht. doi:10.1007/978-94-011-6627-0_1.
5. Harrison, R. G. 1907, Anat. Rec., 1, 116-128.
6. Arunkarthick, S., Asokan, R., Aravintharaj, R., Niveditha, M. and Kumar, N. K. K. 2017, J. Entomol. Sci., 52, 261-273.
7. Glaser, R. W. and Chapman, J. W. 1912, Science, 36, 219-224.
8. Grace, T. D. C. 1962, Nature, 195, 788-789.
9. Smagghe, G., Goodman, C. L. and Stanley, D. 2009, Vitr. Cell. Dev. Biol. - Anim., 45, 93-105.
10. Claydon, K. 2009, James Cook Uni., 156.
11. Bairoch, A. 2018, J. Biomol. Tech., 29, 25-38.
12. Wu, Q., Patočka, J. and Kuča, K. 2018, Toxins, 10, 11.
13. Cox, M. M. J. and Hollister, J. R. 2009, Biologicals, 37, 182-189.
14. Nolletti, C. 2016, Biologics License Applications Clinical Review Memorandum, FDA.
15. Dequéant, M.-L., Fagegaltier, D., Hu, Y., Spirohn, K., Simcox, A., Hannon, G. J. and Perrimon, N. 2015, Proc. Natl. Acad. Sci., 112, 12974-12979.
16. Lynn, D. E. and Hung, A. C. F. 1991, Arch. Insect Biochem. Physiol., 18, 99-104.
17. Inoue, H., Kobayashi, J., Kawakita, H., Miyazaki, J. and Hirabayashi, T. 1991, Vitr. Cell. Dev. Biol., 27, 837-840.
18. Akiyama, Y., Iwabuchi, K., Furukawa, Y. and Morishima, K. 2008, Int. Conf. Proc. Miniaturized Syst. Chem. Life Sci., 1669-1671.
19. Luedeman, R. and Levine, R. B. 1996, Dev. Biol., 173, 51-68.
20. Soya, S. and Can, H. 2019, J. Anim. Plant Sci., 29, 325-332.

21. Akiyama, Y., Iwabuchi, K., Furukawa, Y. and Morishima, K. 2009, Lab Chip, 9, 140-144.
22. Baryshyan, A. L., Woods, W., Trimmer, B. A. and Kaplan, D. L. 2012, PLoS One, 7, e31598.
23. Baryshyan, A. L., Domigan, L. J., Hunt, B., Trimmer, B. A. and Kaplan, D. A. 2014, R. Soc. Chem. Adv., 75, 39962-39968.
24. Rubio, N. R., Fish, K. D., Trimmer, B. A. and Kaplan, D. L. 2019, ACS Biomater. Sci. Eng., 5, 1071-1082.
25. Gunage, R. D., Dhanyasi, N., Reichert, H. and VijayRaghavan, K. 2017, Semin. Cell Dev. Biol., 72, 56-66.
26. Carmena, A., Bate, M. and Jimenez, F. 1995, Genes Dev., 9, 2373-2383.
27. Dobi, K. C., Metzge, T. and Baylies, M. K. 2011, Fly, 5, 68-75.
28. Richardson, B. E., Nowak, S. J. and Baylies, M. K. 2008, Traffic, 9, 1050-1059.
29. Weitkunat, M. and Schnorrer, F. 2014, Methods, 68, 2-14.
30. Roy, S. and VijayRaghavan, K. 1998, J. Cell Biol., 141, 1135-1145.
31. Duch, C., Bayline R.J. and Levine, R. B. 2000, J. Comp. Neurol., 422, 1-17.
32. Dutta, D., Anant, S., Ruiz-Gomez, M., Bate, M. and VijayRaghavan, K. 2004, Development, 131, 3761-3772.
33. Wigglesworth, V. B. 2012, Muscular System & Locomotion. Springer Science & Business Media, 146-178.
34. Hooper, S. L., Hobbs, K. H. and Thuma, J. B. 2008, Prog. Neurobiol., 86, 72-127.
35. Baryshyan, A. 2013, Tufts University, http://hdl.handle.net/10427/012133
36. Kurtti, T. J. and Brooks, M. A. 1970, Exp. Cell Res., 61, 407-412.
37. Seecof, R. L., Alleaume, N., Tepliz, R. L. and Gerson, I. 1971, Exp. Cell Res., 69, 161-173.
38. Shimizu, K., Takayuki, H., Akiyama, Y. Y., Iwabuchi, K., Akiyama, Y. Y., Yamato, M., Okano, T., Morishima, K., Hoshino, T., Akiyama, Y. Y., Iwabuchi, K., Yamato, M., Okano, T. and Morishima, K. 2010, 3rd IEEE RAS EMBS Int. Conf. Biomed. Robot. Biomechatronics, BioRob 2010, 425-430. doi:10.1109/BIOROB.2010.5626762.
39. Akiyama, Y., Iwabuchi, K., Furukawa, Y. and Morishima, K. 2008, J. Biotechnol., 133, 261-266.
40. Seecof, R. L. and Dewhurst, S. 1974, Cell Differ., 3, 63-70.
41. Storti, R. V., Horovitch, S. J., Scott, M. P., Rich, A. and Pardue, M. L. 1978, Cell, 13, 589-598.
42. Hossain, M. S., Akimitsu, N., Kurokawa, K. and Sekimizu, K. 2003, Differentiation, 71, 271-280.
43. Seecof, R. L. and Donady, J. J. 1972, Mech. Ageing Dev., 1, 165-174.
44. Seecof, R. L., Gerson, I., Donady, J. J. and Teplitz, R. L. 1973, Dev. Biol., 35, 250-261.
45. Bermudez, I., Lees, G., Botham, R. P. and Beadle, D. J. 1986, Dev. Biol., 116, 467-476.
46. Uesugi, K., Sakuma, Y., Akiyama, Y., Akiyama, Y., Iwabuchi, K., Okano, T. and Morishima, K. 2019, Adv. Robot., 33, 219-231.
47. Raikhel, A. S., Deitsch, K. W. and Sappington, T. W. 1997, Culture and analysis of the insect fat body. Springer, Dordrecht. doi:10.1007/978-94-009-1535-0_41.
48. Hoshizaki, D. 2013, Fat body. Cambridge University Press.
49. Arrese, E. L. and Soulages, J. L. 2010, Annu. Rev. Entomol., 55, 207-225.
50. Philippe, C. 1982, J. Insect Physiol., 28, 257-265.
51. Rubio, N. R., Fish, K. D., Trimmer, B. A. and Kaplan, D. L. 2019, Front. Sustain. Food Syst., 3, 24.
52. Bahramzadeh, Y. and Shahinpoor, M. 2014, Soft Robot., 1, 38-52.
53. Marden, J. H. and Allen, L. R. 2002, Proc. Natl. Acad. Sci. USA, 99, 4161-4166.
54. Dennis, R. G. and Herr, H. 2005, Engineered Muscle Actuators: Cells and Tissues, CRC Press, New York.
55. Delcomyn, F. 2004, Annu. Rev. Entomol., 49, 51-70.
56. Koval, T. M. 1983, Proc. Natl. Acad. Sci., 80, 4752-4755.
57. Hegstrom, C. D. and Truman, J. W. 1996, J. Neurobiol., 29, 535-550.
58. Koval, T. M. 1996, BioEssays, 18, 149-156.

59. Kim, S., Laschi, C. and Trimmer, B. 2013, Trends Biotechnol., 31, 287-294.
60. Ricotti, L., Trimmer, B., Feinberg, A. W., Raman, R., Parker, K. K., Bashir, R., Sitti, M., Martel, S., Dario, P. and Menciassi, A. 2017, Sci. Robot, 2, 29.
61. Akiyama, Y., Hoshino, T., Iwabuchi, K. and Morishima, K. 2012, PLoS One, 7, e38274.
62. Akiyama, Y., Sakuma, T., Funakoshi, K., Hoshino, T., Iwabuchi, K. and Morishima, K. 2013, Lab Chip, 13, 4870.
63. Yalikun, Y., Uesugi, K., Hiroki, M., Shen, Y., Tanaka, Y., Akiyama, Y. and Morishima, K. 2019, Actuators, 8, 30.
64. Yamatsuta, E., Ping Beh, S., Uesugi, K., Tsujimura, H. and Morishima, K. 2019, Engineering, 5, 580-585.
65. Datar, I. and Betti, M. 2010, Innov. Food Sci. Emerg. Technol., 11, 13-22.
66. Post, M. J. 2012, Meat Sci., 92, 297-301.
67. USDA FSIS. 2019, Formal Agreement to Regulate Cell-Cultured Food Products, Release No. 0027-19.
68. Specht, L. 2020, Good Food Institute.
69. Verkerk, M. C. C., Tramper, J., van Trijp, J. C. M. C. M. and Martens, D. E. E. 2006, Biotechnol. Adv., 25, 198-202.
70. Yakovlev, A. Y., Nesin, A. P., Simonenko, N. P., Gordya, N. A., Tulin, D. V, Kruglikova, A. A. and Chernysh, S. I. 2017, In Vitro Cell Dev. Biol. Anim., 53, 1, 33-42.
71. Huis, A. van, Itterbeeck, J. Van, Klunder, H., Mertens, E., Halloran, A., Muir, G., Vantomme, P., van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G. and Vantomme, P. 2013, Food and Agriculture Organization of the United Nations, Rome. doi:10.1017/CBO978110 7415324.004.
72. Mishyna, M., Chen, J. and Benjamin, O. 2020, Trends Food Sci. Technol., 95, 141-148.
73. Kouřimská, L. and Adámková, A. 2016, NFS J., 4, 22-26.
74. Doherty, M., Buchy, P., Standaert, B., Giaquinto, C. and Prado-Cohrs, D. 2016, Vaccine, 34, 6707-6714.
75. Vela Ramirez, J. E., Sharpe, L. A. and Peppas, N. A. 2017, Adv. Drug Deliv. Rev., 114, 116-131.
76. Cox, M. M. J. 2012, Vaccine, 30, 1759-1766.
77. Yang, L. P. H. 2013, Drugs, 73, 1357-1366.
78. FDA. 2020, Food and Drug Administration Vaccines Licensed for Use in the United States.
79. Mitragotri, S., Burke, P. A. and Langer, R. 2014, Nat. Rev. Drug Discov., 13, 655-672.
80. Mishra, N., Gupta, N., Khatri, K., Goyal, A. K. and Vyas, S. P. 2008, Indian J. Biotechnol., 7, 283-294.
81. Schiele, J. T., Quinzler, R., Klimm, H. D., Pruszydlo, M. G. and Haefeli, W. E. 2013, Eur. J. Clin. Pharmacol., 69, 937-948.
82. Tacket, C. O., Mason, H. S., Losonsky, G., Clements, J. D., Levine, M. M. and Arntzen, C. J. 1998, Nat. Med., 4, 607-609.
83. Levin, D. M. 2007, Kansas State Univ., 125.
84. Tacket, C. O., Sztein, M. B., Losonsky, G. A., Wasserman, S. S. and Estes, M. K. 2003, Clin. Immunol., 108, 241-247.