Lysozymes, Proteinase K, Bacteriophage E Lysis Proteins, and some Chemical Compounds for Microbial Ghosts Preparation: a Review and Food for Thought

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Abstract

Microbial Ghosts (MGs) is a new term that describes evacuated and dead microbes. Apparently, MGs will be able (soon) to substitute another term the "bacterial ghosts (BGs)". A new protocol for preparing MGs was introduced using the critical concentration or amounts of some chemical compounds and enzymes. In principle any compound such as SDS and NaOH or enzyme such as lysozyme and Proteinase K that could induce a pore(s) in the microbial cell wall could be used. "Evacology" might be a name for a new science that deals with living cells and viruses' evacuation. In addition, the bio-critical concentration of H_2O_2 enables Virus Ghosts (VGs) preparation. The bacteriophage *E* lysis gene based protocol is restricted only to the gram-negative bacteria. The Sponge-like Protocol (SL) has opened the window to nearly all microbes and all biological cells and viruses to be prepared as ghosts. In this shift point this review aims to cover the most important information about such a topic. SL protocol is based on determining the critical concentration of compounds that can kill, make pore(s), evacuate the cells, but did not deform or affect the cell wall or their antigens (under such concentration). Lysozyme has been used in the original protocol to complement any deficiency result in survive of any of the *E. coli* cells. Lysozymes and Proteinase K can stand-alone or can be combined with the other possible chemical compounds. The SL protocol for ghosts preparation is simple, inexpensive, in house, reliable, safe and cause pores starting from outside the cells to their inside. The future will show rising interest with such simple protocol, which could allow us to prepare our vaccine and drug delivery different ghosts' related formula in kitchen. In this, review most of the experiences gained from practicing experiments in ghosts' preparation and some idea about such subject were summarized and discussed.

Keywords: Bacterial Ghosts; Microbial Ghosts; Biological Ghosts; Sponge-Like protocol; Bacteriophage *E* lysis gene

Abbreviations

BGs: Bacterial Ghosts; MGs: Microbial Ghosts; MGC: Minimum Growth Concentration; MIC: Minimum Inhibition Concentration; SL: Sponge-Like; VCG: *Vibrio cholerae* ghost

Introduction

MGs in principle are empty microbial cells but with correct 3D structure. The cytoplasmic constituents of the MGs were come out, because of a pore was made due to the activity of the expression of the *E* lysis gene or by using the critical concentration of some chemical compounds and enzymes. The main idea is to remove the cytoplasm and its constituents without damaging the microbes' 3D structure or deforming their cells surface antigen. After being evacuated, the microbes are empty and dead. They are safe while they are unable to replicate. For that, they are the best candidates that can be used as vaccines. On the other hand, because they have each an empty internal space, they can be used in the drug delivery applications. Many microbial species were prepared as ghosts using both of the *E* lysis gene and the SL (Sponge-Like) protocol. Nevertheless, the bacteriophage *E* lysis gene is restricted only to the gram-negative bacteria. Alternatively, the SL protocol enables the preparation of MGs from both of the gram-negative and positive bacteria, yeasts, and even viruses. Amara(2015) has been suggested that such protocol (SL) and their modification using both of chemical compound at critical concentrations and enzymes at critical activity will emerge a new science might be carrying the name "Evacology"[1]. This review is a collection of the tools, idea, success, improvement, advantage, disadvantage, future, applications, and many facts and idea about the BGs, MGs, and biological cell ghosts. Such protocol might be able to establish a new science, which might be given the name "Evacology".

The first evacuation process

It is important to track the first root of evacuating a biological structure. The old civilizations somehow knew how to evacuate a complete mammalian organism or the human body (corpse). They know also that water is an essential factor for any living or enzymatic process. For that, they remove water correctly without

damaging the cells. Then they prevent water or moisture from their ability to re-enter to the dried organisms. They know also about many fine structures which were existing around them such as, fungi particularly species which are visible to the naked eye. Therefore, they had added some potent antimicrobial/water prevention agents such as resins. Additionally, they know how to use yeast for producing beer, wine, and bread. They know that boiled, smoked; salted etc food could survive (for different periods). In addition, they gained knowledge, experiences, and practice to avoid the bad effect of such spoiled food. In general, they should have the knowledge to control the microbial deterioration to design such perfect protocol for mummification.

Egyptian mummy

The ancient Egyptian might be the first to evacuate the corpse carefully from their organs. Then the evacuated corpse was subjected to careful treatment to remove its water content(s). Some reports have been described early that they have first treated the corpse with wines. Ethanol as one of the major constituent of the wine is a dehydrating agent. Other, bio-natural and chemical compounds were used for extending the protection and to avoid any physical, chemical or microbial degradation. Resins also were used. The Egyptian have used to wrap, embalmed corpses [2]. Then the corpse coated with cotton or any suitable Bandages and isolated by painting it with resins and putting it in well-isolated container. The process has given the name Mummification, which identified as: the process for embalming or artificially preserving lifeless bodies [1].

The microscope and the first empty observed microbe

Perhaps the first microbe, which was seen as a ghost was in the first sample, which was shown, using, lenses by the first microscopic examination to a water sample by Antonie van Leeuwenhoek in 1670s. Many microbiologists especially those, who were using the light microscope on a regular basis to show microbial specimens, should saw dead, evacuated, or partially damaged MGs. Even during the use of simple stain (crystal violet) one should remark that some microbes do not stain at all and some are deeply stained and some are only their wall edge were stained. Those, which are not stained (or get faint stain), are probably still life and resist the staining process that is clear in case of yeast when stained by crystal violet either when dead or viable. Such simple staining (using dilute crystal violet) could distinguish between the live and the dead microbes. Some researchers prefer to use special dye, like Trypan blue.

Ghost different names

Living cells after being evacuated can be given the name ghosts. For that, the term MGs should not be given to the dead microbes or cells but only to the dead and evacuated ones. There are different definitions and terms were used with "ghost" to give special meaning in the English language and literature. For examples, RBCGs ghosts for the RBCs cytoplasm free cells; BGs for bacterial ghosts and MGs for microbial ghosts; Phage ghosts were given for the first time to the genomic free bacteriophage and might be apparently the first name to be given for empty

virus. Virus ghosts, recently introduced after evacuating the Newcastle virus "Newcastle virus ghosts" from its genomic RNA [3]. For small parasite or a stage of them such as the trophozoite, it can be suggested to put the name followed by ghosts such as "trophozoite ghosts".

Did the mish (Mesh) cheese have been used for immunization?

Like any creature, each microbe has a life cycle, which end with death. The microbes' cell walls after their death could stay longer and resist decaying [4-7]. However, due to the process of the natural decay, environmental effect, enzymatic activity of other microbes, or any other expected mechanism for a pore or pores formation, natural loss of the cytoplasm or in better words, natural microbial ghosting will be happened. As being parts of the nature, microbes after being dead they are affected by their surrounding ecological environment. For that, cell ghosts and microbial ghosts are produced daily in our bodies (inside, or outside). They are produced in the lung, in the stomach, in the surface of our skin, in our aged food and so on. Therefore, dead and ghost microbes are natural phenomena.

In addition, they play different roles in immunization. Natural Ghosting phenomenon in our bodies plays important roles in immunization. Aged foods used by old civilization might have been used for the aim of immunization. The ancient Egyptian invented some type of cheese which named old cheese or Mish (Mesh) which combine between being so old so if any harm microbe is existed as a contaminate it is either being attenuated or dead. Such old cheese or Mish/Mesh cheese might be invented not as a type of cheese only but to protect the Egyptian from many diseases.

BGs and the mummy

When something (Physical, chemical or biological) cause microbes or cells to loss their cytoplasm contents, this will let their membrane or cell walls to stay longer (Figure 1 and 2). Ghost cells contain less or no cytoplasm constituents (protein and carbohydrates, salts etc.). The existence of the cytoplasm in the dead cells either enhance their degradation by the activity of the internal enzymes or by supporting the growth of other microbes which can secrete enzymes could degrade the cell wall or the membranes of the dead ones to be able to utilize the nutrients in such cytoplasm. Being dead, they become as food for other microbes. Additionally, as the mummy MGs have no water content. Some microbial cells which contain less protein resist the degradation process. For example, yeast contains only three percent of its cell wall as protein. For that Baker yeast can be preserved at room temperature. However, most of its cytoplasm content are protein in their nature. Being empty cells MGs show great resistance to the degradation process that was proved by their long shelf life.

Pore formation by the ϕ X174 bacteriophage E lysis protein

The mechanism of causing pore(s) in the bacterial cells infected by bacteriophage become clear by the power of the

Figure 1: Chemical, biological and physical values interact during the MGs preparation.

Figure 2: The most important values involved in MGs preparation.

electron microscope [8-12]. After the pore formation the bacterial cells wall is opened and the bacterial cells lose their cytoplasm content [13-23].

The ϕ X174 E lysis protein cause E specific transmembrane tunnel structure built through the cell envelope complex [24-27]. It form a fusion through the inner and outer cell membranes, forming a specific transmembrane tunnel structure. Through such tunnel or pore, the bacterial cytoplasmic content is passing out. Genetic engineering and molecular biology tools enables better control for the bacteriophage E lysis gene based protocol.

The produced lysis enzyme enable pores in the bacterial cell wall. There is still a need for degrading the residue of the

DNA, plasmid, lysozymes, proteinase K and so on, which are still contaminating the opened bacterial cells.

E lysis gene, its activity is restricted only to the gram-negative bacteria. The gene E was cloned and expressed in different gram-negative hosts. Such expression has been controlled by a heat sensitive promotor, which allows the expression of the E lysis gene. Using the heat sensitive promotor and regulator were used for better control. Nevertheless, its main weak point that it is restricted only to the gram-negative bacteria [4, 28].

Gene E codes for 91 amino acids

Gene E: The amino acids content of the E lysis protein sequence of the Enterobacteria phage ϕ X174 and its nucleotides content were well identified (as below). One can obtain uncut phage DNA from the market and clone the E lysis gene. Alternatively, one can isolate the phage and made DNA isolation then clone the E lysis gene or cut it with suitable enzyme and made gene library.

E lysis gene nucleotides sequence: atggtacgctggactttgtgggataccctcgctttcctgctcctgtgtgagttattgtcgccgtcattgcttattatgttcattcccgtaacattcaaacggcctgtctcatcatggaaggcgctgaattacggaaaacattattaatggcgtcgagcgtccagttaaagccgctgaattgttcgcgttacttgcgtgtacgcgcaggaaacactgacgttctgtgacgcagaagaaacgtgcgtcaaaaattgcgtgcagaaggagtga

Amino acids sequence: MVRWTLWDTLAFLLLSLLLPSL-LIMFIPSTFKRPVSSWKALNLRKTLMASSVQLKPLNCSRLPCVY-AQETLTLFLTQKKTCKVKNVCVQKE

One pore is enough!: A pore formation in the microbes' cell walls will lead to the removal of the cytoplasm which come out due to the cell wall imbalanced pressure force, osmosis differences, mechanical pressure, etc. The external medium can diffuse through the lysis tunnel filling the inner cell space of the still rigid BGs [27]. Apparently, and after observing many of the electron microscope images only one pore is usually existed. That might be due to the force happened as the result of the existence of the first pore. When first pore is being opened; it imbalance the internal pressure lead to getting rid of the cytoplasm contents and the rest of the E lysis protein so there is no chance to form another pore [29].

Foreign surface antigen and drug delivery: Ghost cells can be performed after the expression of foreign antigen, loading drugs, DNA, plasmid etc to the cells. However, in case of using MGs in the drug delivery, after such loading to the drug, the existed pore must be closed or in better word must be sealed [30]. Recently, another tactic have been introduced where *Saccharomyces cerevisiae* cell ghosts were loaded with dissolved ghosts gossypol acetic acid, then the dissolved ghosts gossypol acetic was allowed to crystallize inside the yeast cells. That will give the chance to load drugs without sealing the cells.

SL and the idea of the critical chemical concentrations

SL protocol introduce the idea of using the critical concentration of chemical compounds and recently enzymes for the ghosts preparation. The used steps were selected preciously to do in

sequence steps enable full evacuation for the treated microbes or cells from their cytoplasmic contents. The future might show a more perfect chemical compounds or a strong modification in the protocol but the Acknowledge should be given to the original six used chemical compounds. They are, NaOH, SDS, CaCO_3 , H_2O_2 , NaCl and Ethanol. Recently NaHCO_3 was used instead of CaCO_3 to produce yeast ghosts. H_2O_2 has stood alone to degrade Newcastle RNA using bio-critical concentration. Enzymes, which can affect on the microbial cell wall such as lysozyme and proteinase k are under optimization to give the equal results of the SL protocol [data not shown] but chemical compounds are more cost effective and omit the risk of incorrect immunization due to the use of enzymes which are protein in their nature [31].

The main idea of the SL protocol: The SL protocol main idea is simple and applicable; it depend on determining the (MIC) Minimum Inhibition Concentration and the (MGC) Minimum Growth Concentration of the used compounds or enzymes. The minimum killing effect in case of using MIC of the used compounds or enzymes they should cause minimum effect on the dead cells. In case of MGC the cells still alive. However, by using another MIC/MGC for the another compound(s) plus the physical effect of the repeated centrifugation steps, that all will lead to empty dead cells but with correct 3D structure and correct surface antigens which enables correct immunization upon the use of the experimental animals. SL protocol gives the chance to prepare ghosts from gram-positive and gram-negative bacteria, yeast, and virus and so on. Such a concept could be pass the microbes to any other biological cell forms. Many other forms from the biological system will join the ghost family after their preparation by this protocol (critical chemical concentration) soon.

Using critical chemical concentration for microbial killing is a natural phenomenon: *P. aeruginosa* is a dominant microbe in the hospitals while it is – in one word - hydrocarbon biodegradable microbes. H_2O_2 could turn any of the *P. aeruginosa* to mucoid strain hence to alginate producer. *P. aeruginosa* is able to be mutated and to produce a huge amount of exopolysaccharide, mainly the alginate if exposed to the H_2O_2 . That also could be happened in the patients' lungs. As a defense mechanism against the *P. aeruginosa* infection the lung produces H_2O_2 . How could the lung cells adjust the amount of the H_2O_2 to kill the *P. aeruginosa* but not to kill their own cells? In general, the lung cell must produce (somehow) H_2O_2 in critical concentration to kill such microbes and not to damage or to kill its own cells [32]. For one or another purpose, some of the *P. aeruginosa* cells were not killed by the killing dosage of the H_2O_2 , but were exposed instead to less amounts of the H_2O_2 by one or another mechanism. Less amount of H_2O_2 will induce mucoid mutant. Such mutants are able to produce a huge amount of exopolysaccharid. From such natural phenomena one can understand some facts about our "biological system" and how it could use the concept of "the critical chemical concentration" intelligently. That includes:

1. Our biological system knows how to produce chemical compounds in critical concentrations.
2. Concentrations less than the MIC could cause mutation, where such concentrations could keep the pathogens affected but alive.

3. Compounds produced by our biological system such as the oxidants and free radicals particularly those which produce to control pathogens should be given more concerns.
4. Misused of antioxidants could deteriorate our endogenous oxidant defense mechanism. For that, antioxidants should be taken wisely.
5. The biological system still proves that it is designed in perfect and intelligent way, so mechanisms used by such system should be given priority.
6. Lysozymes, proteases, DNases and other natural enzymes which are part of our defense system should be given more concerns to find more intelligent mechanism to control such pathogens.
7. Potant compound could be used upon dilution to do minimum side effect.

El-Baky and Amara(2014) combine between such phenomena and the idea of the SL protocol to *in vitro* degrade the Newcastle virus RNA to turn it to ghosts, which might be optimized to be *in vivo* protocol for controlling some pathogens without harming our biological system [3].

MGs omit the most important virulence factor: When foreign microbe enters to our body; our immune system starts to react with it and the battle will base on their number, type and our immune system quality. However, some microbes have extra virulence factors, which could be collectively stronger than our immune system. So, even, we are strong enough, the microbe can be fatal and exceed the speed of our immune system. Such conditions cause death, or severe illness or disorder. For that, scientists prepare killed microbes or attenuated ones to be sure that the immune system will manage the situation and control the invasion. Such in / less-active microbe(s) give our immune system the chance and the time to react with it. However, there are many reports prove that attenuated microbes can be turned to virulence ones in some conditions.

For that scientists spent time to reduce the virulence factors of the pathogenic strains or to find alternative solutions. Such alternative solutions might be summarized as follow:

1. Repeated cultivation, in media did not maintain the microbe's virulence factors.
2. Cell aging.
3. Viruses can be grown in an unspecific host, which induce safer mutants.
4. The use of recombinant strains enable expressing antigen(s) of some pathogenic microbes on their cell wall surfaces.
5. Genetically modified pathogens with less or completely deactivated virulence factors can be used.
6. Safe strains could induce antibodies which can protect against some virulence pathogens.

7. Close species are used which are able to induce immunization against certain targeted pathogens.
8. Using MGs (Figure 3).
9. Totally killed microbes but with with a suitable of effective surface antigens (Some killing process affect severely on the microbes surface antigen).

After that, our immune system produces the suitable antibodies. And, become ready for any pathogenic invader.

MGs are truly dead cells and they could not replicate. For that, if they are pathogens they will lose the chance to win the battle against our immune system (by increasing their numbers). For that, they are perfect candidates for activating safely our immune system. Old civilizations have aware by the knowledge and the tools of the vaccination. They know how to vaccine against the smallpox, the disease which kill millions of peoples. They use unsuccessful virus legion of smallpox to vaccine-uninfected patients. The original name of the vaccination is variolation or inoculation. Even such reports prove that the main idea was transferred to the new world, only after doing some improvement in a method from the Middle East and Africa, which was known as ventilation. We still -until nowadays- use the same old technique. Such simple tools saved the life of unknown number of peoples. It is an African practice is transferred to America nearly in 1706-1721 by a Sudanese slave [33]. However, it might be a shift in the concept when cowpox was used instead of the smallpox to give the full immunization against the later.

Some concerns about our immune system: There are some points that should be considered for those who are involved in the battle of the pathogens control. Some of such important concerns can be summarized as follows:

Figure 3: The unique criteria for M.

1. Our immune system is active in certain ages and less active in another. For that, we should be vaccinated in the correct time.
2. However, in case of using dead cells like BGs the equation is different. The vaccine types and the rout of administration is an important issue. Weak immune system, should give a correct time, formula (dead/attenuated microbes), site of administration and so on. MGs even an empty dead cells but it still have the correct 3D structure, so the immune system react with it with nearly the same power when it attack living cells. But one should consider the correct used number of cells. For that better and safer immunization were reported in case of using the MGs particularly for immunization.
3. One could take the maximum dosage from MGs safely while they are dead cells.
4. The genetic engineering and the molecular biology tools enable introducing or expressing the protein of the fatal microbes in the surface of recombinant safe microbe(s). After that the microbes turned to ghosts and become dead and safe.
5. BGs can be given safely in newly root of administration such nasal or on the surface of the wounded skin.
6. As a perfect biological package targeted by different immunological cells particularly the macrophage, they become the best choice for the gene therapy. BGS have increased our understanding to some biological factors such as our understanding to the resistance where intake cells (but not dead) might acquire new genetic material made them survive. Alternatively, before they loses their content under certain condition they might hybrid with other MGs.
7. Exopolysaccharide microbes could give false MIC where some microbes are survived due to biofilm formation. For that, microbes, which can be affected or mutated by the used chemical compounds, are recommended to be killed by NaOH using its MIC concentration first H_2O_2 , SDS can induce exopolysacchaide production. As the other empty similar containers, MGs have more respective size and stable envelope and can be used in the field of the drug delivery.
8. BGs can be used in the diagnostic Kit as a reference antigen, where it should give positive reaction with serum containing the proper antibodies [34].
9. BGs will be in its better form after removing their cytoplasmic contents including the DNA, RNA, and the protein and so on.

The elements of the evacuation process

The protocol can use either the introduced chemical compounds or enzymes in critical amounts or the combination between both. The original SL protocol and its reduced and modified forms were succeeding to prepare BGs from gram-negative,

Gram-positive, yeast and viruses till nowadays. In fact, the concept of the protocol enables preparing ghosts from any microbe or even from any biological cells. That because it based on some chemical compounds or enzymes were selected based on their ability to kill the microbes and induce pores in their cell walls, degrade/or remove their DNA, RNA and the protein. The compounds were used in concentrations that enable induction of minimal effect on the cell wall and the protocol itself use physical parameters to get rid of the cytoplasm such as the shaking and the cell pressing using centrifugation. For that, it is given the name SL protocol. The lysozyme and proteins K which are able to lysis most types of different microbes will give another chances for improving a commercial protocol for ghosts preparation. In fact, most of the molecular biologist have used such enzymes to prepare the DNA from various microbes. Only, they are in need to be used in critical activities enables ghosts preparation rather than the lysis of the microbes.

The first used chemical compounds: The selected compounds, which were used to prepare BGs using SL protocol, are NaOH, SDS, CaCO_3 , and H_2O_2 and both of NaCl and Ethanol are included. Lysozyme and proteinase K upon their use in a critical amount also give the same results. Some physical factors are involved (Centrifugation, shaking and temperature). Biological parameters are playing the central role in the success of the protocol such as, the type of the used cell or the microbe and their age. The ghosts' preparation condition(s) are effective factor(s). Such as cells density during the preparation. The cells quality should be monitored either by light or electron microscopes. The ability of the prepared ghosts' cells to induce correct immunization should be investigated. The sequence of the treatment is also an important issue. For example, exopolysaccharide-producing microbes must be treated with NaOH firstly. After preparing MGs, the cells must be investigated for the existence of any viable colonies. In addition, the viable cells should be deactivated, converted again to ghosts or the overall batches should be sterilized (in case of fatal microbes). It is might be interesting to highlight that the vaccine and the immunological technologies are fine technology and in some cases grams from correctly prepared MGs is in need to satisfy the demand.

Why NaOH and Why SDS? : NaOH and SDS are two well-known compounds used for plasmid isolation from gram-negative bacteria. The protocol is given the name "alkaline lysis protocol for plasmid isolation". The lysis buffer made from ten percentage NaOH (not autoclave) and SDS (need to be autoclaved) as stock solutions (400 μl of the ten percentage SDS on 3400 μl water and then add 80 μl of ten percent NaOH). Both of NaOH and SDS are used to prepare the lysis buffer by mixing them with water. SDS introduces pores in the bacterial cells. For that, it is added to most of the toothpaste, used in lysis buffer for the plasmid isolation and for some of the DNA isolation protocol. If one use SDS in its MIC that might give minimum effect and introduce pores in the bacterial cells, such concept was one of the successful keys to isolate the DNA from any gram-negative microbes without a need for buying expensive kits. Increasing the temperature and the exposure time were additional option in case

of gram-positive bacteria [35]. NaOH prove to be effective on the cell wall and for that, it is better to use its MGC. However, it is a potent bacterial killer. For that, it is recommended to use it at the first to deactivate strains able to be mutate and to resist the ghost preparation steps.

Why MIC and MGC?: MIC is a critical point where the used chemical, drug, enzymes or the antimicrobial agents are able to kill the microbe under the investigation condition with minimum side effect on their cells. In the serial dilution experiment, the tube after the MIC, which shows the first growth and which, given the name MGC such concentration should still effective on the microbe somehow. Using the concept that chemical compounds and enzymes were used in their MIC in a time or a combination between the MIC and MGC of other compounds this will support the concept of generating the minimum effect on the cell wall of the microbial strains. In another word, the used combinations collaborate to do relaxed job by complementing each other.

Experimental Design-Plackett-Burman, Box-Behnken and the excel solver for optimization: Experimental design has been used to optimize many of the biological processes. It has been also used to optimize the production of the BGs. Experimental design needs two levels for each variable, one is low and the other is high. And, it is preferable to use from four to ten variables. In the original protocol twelve experiments are conducted. Each experiment contains either the high level or the low level of each of the used microbe. All of the used experiments must be different and followed the Plackett-Burman design. Therefore, concerning the used chemical compounds concentration; the MIC is the higher one +1 and the MGC is the lowest one -1 [36].

CaCO_3 or NaHCO_3 : CaCl_2 is used in the competent cells preparation. CaCl_2 is able to facilitate the movement of the plasmid from outside the cells toward its inside. It can do the same with the SDS and other compounds.

Using another stronger "Ca" based compound will give better results with some microbes. Some could have dual activities as a membrane transfer and as an alkali such as CaCO_3 and NaHCO_3 . CaCO_3 was selected because it has another unique property that it is poorly water dissolved even after its autoclavation and it can be used as a suspension.

NaHCO_3 was used with the first Eukaryotic prepared as ghosts using the idea of the critical chemical concentration [37,38].

Ethanol: Ethanol can be used to precipitate the DNA and protein if used as 90 percentage. If it is used as 70-percent concentration that enables both of precipitation and salts elimination, (The 30 percent water content enables that). However, if it is used in concentration less than 70 percentage it could eliminate the salt, DNA, RNA and the soluble protein as well. In the SL protocol, 60 percent of the ethanol was used to evacuate the bacterial cells content from their DNA, RNA and the salts.

Lysozyme: Lysozyme is an enzyme found in some tissues and secretions and is considered as a part of the defense mechanism against pathogens while it is able to lyse some microbes. The mechanism of its activity is mainly by its targeting to the bacte-

rial exopolysaccharide existed in the cell wall causing osmotic shock or lysis. The most famous source is the hen egg, saliva, milk and bacteriophage T4. Its substrate is consisting of alternate residues of (1-4)-linked β -N-acetylmuramic acid (MurNAc) and β -N-acetylglucosamine (GlcNAc). Lysozyme hydrolysis the bond between C-1 of β -N-acetylmuramic acid and C-4 of GlcNAc. Chitin (β -1-4-linked GlcNAc) is also a substrate [31].

Proteinase K: One of the endopeptidases able to digest keratin and it is a broad-spectrum serine proteinase. It is activated by calcium. It is used to remove protein contaminating nucleic acid. The enzyme's activity is stimulated by denaturants such as SDS. It is always used side by side with lysozymes. Proteinase K can be used to improve the MGs preparation [39].

Centrifugation: What can be happened if the pore in the microbial cell wall is so small and the bacterial cells still able to maintain their content (even the microbe have such a small pore). For solving such a problem, centrifugation will be the best process for pressing the microbial cells.

After introducing single pore (small or big) or more than single pore the microbe cells might still be able to maintain. Their cytoplasmic content due to the preplasmic membrane, the centrifugation will be able to press the cells to get rid of their contents. One can imagine the cells coming down to the test tube bottom due to the centrifugation force. After being settled and aggregated. The cells pressed like the sponge and the cells continue to get rid of their contents. Therefore, the protocol has been given the name SL protocol. So one should use a suitable centrifugation speed. One should not exceed 4000 rpm/min speed during the ghosts preparation. Speed from 2000 to 3500 rpm/min will give better cell quality.

Washing: Washing is an important step that because after the cultivation and during the centrifugation step; the microbe surface and biomass trap debris and fatty acids. Such cells contaminating elements should be eliminated. In most cases, such cytoplasmic constituent or the rest of the growth condition could neutralize the effect of the used chemical compounds or enzymes. Also, washing enable getting rid from the elaborated cytoplasmic contents. So several washing steps using saline solution could improve the MGs quality. In fact after each centrifugation step samples from each supernatant should be investigated spectrophotometrically or by using gel electrophoresis to monitor the remove of the DNA and the protein. After the success of the protocol, its steps can be reduced and optimized.

The BGs Quality: The above step is concerned with monitoring the remove of cytoplasm which is not an indication about that the microbial cell walls are safe and did not deteriorate or damage. One question is immersed, how to evaluate the quality of the MGs cell walls during the ghosts preparation steps? The quality of the prepared MGs is an important issue. Light microscope should give correct judgment. However, electron microscope will give sharp evaluation for the bacterial 3D structure. To evaluate the quality of the BGs one should count randomly in a certain area ten bacteria and count the number of the cells which have correct 3D structure and use them as a percentage. Hemocytometer

can be used for more precise results. Both of the scanning and transmission electron microscopes can be used to evaluate the quality of the MGs.

Electron microscope is absolutely proof that the prepared BGs or MGs are excellent, very good, good, or damaged cells. In case of using the electron microscopes, simply one can spread one drop of distilled water on the surface of the microscope slide. After that the diluted MGs have been added, spread gently and left for air dry. After being well dried one should rewash the microbial smear to remove any of the salt crystals (to get better electron microscope result) [40]. The microbial smear then dried again. In case of transmission microscope the standard protocol is used.

Stocks and Microbial Cells Preparation

Stocks

10 percentage NaOH: Ten percentage of NaOH is sterile by itself and did not need to be autoclaved.

10 percentage SDS: Ten percentage of SDS need to be autoclaved.

30 percentage H_2O_2 : Thirty percentage of H_2O_2 can be purchased from any local pharmacy and did not need to be autoclaved. But one should be sure that it does not contain any other ingredients.

10 percentage of $NaHCO_3$: Ten percentage of $NaHCO_3$ need to be autoclaved. $NaHCO_3$ has been used in the preparation of the yeast ghosts instead of $CaCO_3$.

In case of $CaCO_3$, it did not cause any apparent toxicity to the bacterial cells in high concentrations, so it is used either 1.05 μ g/mL or 0.35 μ g/mL.

The First Prepared Bacterial Strain Using The SL Protocol

Microbial cells preparation

1. Prepare a pure and an identified microbial strain, simply by doing streak method for spreading the cells in the proper medium or on the selective medium plate. You should be care that the used medium does not mutate your strain.
2. Pick up single colony and re-spreads it on the surface of suitable medium and remark its phenotype.
3. Test the bacterial cells using light microscope for being sure of its type and purity. Both of simple stain and gram stain should be used.
4. Pick up a single pure colony either by sterile needle or by tooth pick and inoculate it to 25 ml flask containing 10 ml of the proper broth medium. And incubate for overnight. Fresh cells will give better MIC and MGC result.

Determination of the MIC and MGC for NaOH, SDS, and H_2O_2 , enzymes etc.

1. Prepare several test tubes containing 5 ml or 4.5 ml of the proper medium aiming to conduct the serial dilution experiment.

2. Add 0.5 ml from the above solutions each in the first test tube of a set of tubes (7 tubes) and transfer 0.5 ml from one to second tube as in the standard protocol of the serial dilution. Remove 20 μ l of each tube and add 20 μ l of about 10^8 of the overnight cell culture in each tube (for each of the above chemical compound related experiment). Any change in the dilution should be considered. Incubate the tubes at 37°C for overnight (or at the growth temperature, which is recommended for the microbe under investigation). Calculate the MIC and the MGC for each of the used chemical compounds.

3. In case of CaCO_3 it was used either as 1.05 $\mu\text{g}/\text{mL}$ or 0.35 $\mu\text{g}/\text{mL}$ was used.

MGs Evacuation Protocol

Microbial aging

The culture of the used microbe was cultivated in one liter flask contains 500 mL NB or any other suitable medium under static condition at 37°C for 72 hr. Some microbes need adding blood to support their growth. Long time cultivation was used aiming to give old culture and thick cell wall. That could be better for ghost preparation. Further more old culture reduced the virulence of the used microbe (in most cases).

The five used strategies for MGs preparation

After collecting, the aged cells by centrifugation and washing them with saline several times; upon the type of the used microbe one can follow one of the below strategies.

Strategy No. 1 (The original SL protocol with twelve Plackett-Burman experiments protocol) (Figure 4).

The original used method is based on using 5x concentration strategy. NaOH, SDS and CaCO_3 were used in one step. Then the washed cells treated with H_2O_2 in the second step. After enough washing and centrifugation steps, 60 percent ethanol is used in the third step. Each of the twelve used experiments is following the Plackett-Burman design as in the original protocol [41].

Strategy No. 2 (The Plackett-Burman reduced protocol) (Figure 5) same as in the strategy no-1., exactly but only the best two experiments were selected. Such reduced protocol was designed to be used only for the similar strains such as *E. coli* BL21 and JM109. However, it has been used for other microbes and proved to be effective [42].

Strategy No. 3 (The 2x strategy) (Figure 6), one can use 2x concentration of the used chemical compounds each in separate step instead of 5 x. For example; add one ml of the suspended bacterial cells to one ml of 2x of the NaOH (NaOH 10 percentage is sterile by itself but if diluted one should avoid any contamination). The 2x stock (prepared according to the results of the MIC and the MGC as recommended, in Plackett-Burman reduced protocol) are prepared and added to the bacteria. That depends on the experiment one or two in the reduced protocol. After finishing the treatment, centrifuging the cells and washing, the treatment with the 2x of each compound of MIC/MGC is started. That

Figure 4: *E. coli* Ghosts.

Figure 5: SL reduced protocol reduced-5x strategy (Strategy No 2).

Figure 6: SL reduced protocol 2x strategy (Strategy No 3).

enables us to use NaOH alone with the expolysaccharide producing microbe to kill it and to take from the cells the chance to produce the expolysaccharide.

Strategy No. 4, (The special protocol for mutated strains such as expolysaccharide producing strain (for example *P. aeruginosa*). After working with an expolysaccharide producing strains, or eukaryotic and viruses strains. It becomes clear that H₂O₂ might induce resistance. In such case, NaOH must be used in the first treatment to kill the microbial cells and to prevent it from producing the expolysaccharide. And in the same case the MIC must be test correctly while expolysaccharide will give wrong result. In case of obtaining wrong MIC one can use the MIC which is calculated the *E. coli* or use lesser concentration while expolysaccharide will give high MIC value [43].

Strategy No. 5 Using enzymes after determining their MIC and MGC side by side with the used chemical compounds particularly with some gram-positive and halophilic strains [under optimization].

General Advices for a Better BGs Preparation

The SL protocol is a simple, inexpensive and in-house protocol. However, one aim of this review is to give the reader the most tested tactic, problems, solutions and image during his experiment design. This review should be read in detailed steps to enable the reader to implement or design his own protocol for a particular microbial strain. The protocol can be used to prepare ghosts from nearly all the bacterial strains, yeasts, and viruses. One can use any of the strategies explained in this protocol and included in this review. It is important to determine the MIC of the used chemical compounds and enzymes to start the protocol. One should also know some important information about the used microbes either gram-negative or positive, expolysaccharide producer, the strains ability to be mutagenized and so on.

The following advices will give the reader some image during his experiment design. This review should be read carefully to gain all the idea within.

1. You should do all your experiment under aseptic condition and well-planned microbiological experiment in a microbiological lab containing all the needed facility. For example any contamination with a spore former microbe such as *Bacillus sp* will give wrong MIC and MGC.
2. For fatal pathogenic microbes, special care should be taken. One should refer to the technical advices concerning the handling of the microbe(s) under investigation.
3. You should know suitable information about the microbial strain, which you are going to evacuate its cytoplasm (gram-positive or negative, spore former, expolysaccharide producer, yeast, fungi, trophozoite and so on).
4. You should use pure strain. Better to recheck the strain purity.
5. You should know its morphology on plats and under the light microscope.

6. Some microbes are sensitive to the chemical compounds used in the protocol such as *P. aeruginosa*, which is sensitive to the H_2O_2 and can be turned to be mucoid (exopolysaccharide producer). For that, it is recommended to use NaOH at the first step.
 7. You can change the protocol by using each compound in separate steps (2x strategy). For that, you can simply prepare 2x stock from both of the MIC and the MGC you are going to use.
 8. In some special cases where MIC and MGC could not be calculated correctly one can use either the *E. coli* MIC and MGC or that of another related strains.
 9. In the serial dilution step, you should use glass tube to get correct MIC and MGC result. Plastic tubes might give wrong judgment.
 10. You should avoid aggregation or clamping, so suitable volume should be used to allow correct contact between the microbial cells and the used chemical compounds.
 11. Prepare double (or more) concentration of the MIC and the MGC. Therefore, you can reach the correct concentration after adding the solution which contains the microbe under investigation. In such case it is recommended that one might use the 5x strategy as in the original protocol. However, for the beginner it is recommended to apply NaOH, SDS, H_2O_2 and $CaCO_3$ using 2x protocol separately.
 12. Better to age the microbe to get stronger cell wall and in some cases to reduce the microbe's virulence factors. Alternatively, some chemical compounds can induce cell rigidity.
 13. One should monitor the release of the DNA and the protein, spectrophotometrically and by using gel electrophoresis.
 14. Use gentle centrifugation not to exceed 3500 rpm.
 15. Wash several times after each step to get rid of the residue of the chemical compounds by using saline (0.5-0.9 percentage) as well as to remove more DNA and protein (etc.).
 16. Investigate for the exopolysaccharide producing strain and start the protocol with NaOH to kill them first.
 17. You can keep the test tube of the serial dilution for more than one day and you can reevaluate the concentration you use. That because some microbes grow slowly.
 18. Calculate the concentration correctly. Any wrong calculation may damage the cells. Light microscope is a suitable tool to monitor the quality of the cells during the adjustment of the MIC and the MGC effect.
 19. You can also change the exposing time of the microbe to the used chemical compounds or enzymes. However, regular control for the cell quality by using light microscope should be followed to prevent cell-lysis.
 20. You can extend the microbial exposure to H_2O_2 for overnight.
 21. Do not forget that NaOH in the original protocol was used in both experiments (1 and 2) as -1, which mean the MGC. For a certain microbe, you can increase the concentration of the NaOH until the MIC but under the control of the light microscope. And in such case one can reduce the exposure time. It is recommended to conduct both of the experiment number ones and two in the SL reduced protocol. For the design of experiment one and two refer to Amara et al. (2013) [42].
 22. After completing the MGs preparation steps, you should examine the cell viability. In case of the existence of viable cells in one or both of the conducted experiments, you should repeat the ghost preparation steps but you still be able to use your incomplete prepared MGs again. There is no need to prepare new cells if the cells are in good quality (investigate the cell quality using the light microscope).
 23. In complicated microbes, experimental design should be used. Plackett-Burman, Box Behnken and Excel solver can be used in sequence to get the best-expected optimization. In fact, experimental design could map the critical points involved in the cell ghosts preparation and could be able to optimize perfectly any complicated process. For that MIC for the used compounds in the SL protocol might be used in strains differentiation. Similar strains such as in case of *E. coli* BL21 and JM109 prove that they have different MIC and MGC.
- Optimizing the gap between MIC and the MGC that can be achieved using Plackett-Burman, Box-Behnken and the Excel solver.
24. Spectrophotometer and electrophoresis can be used to monitor the evacuation of the cytoplasm content.
 25. The best MGs are ones which are dead, empty from their cytoplasmic content, have correct 3D structure and are able to induce the immune system upon their treatment.
 26. Polyacrylamide gel electrophoresis can be used to show the differences between the viable cells and the ghosts. In original protocol viable cells, which existed after running this protocol (did not turn to dead ghosts and still viable), were subjected to lysis by inducing the lysozyme gene carried on pLysS plasmid.

MGs Definition

After the introducing of the critical chemical concentration and activity method for preparing the MGs, many facts were changed and wide range of microbial and mammalian cells as well as other biological containers could be evacuated using such protocol. Therefore, new definition of the MGs can be introduced as follows:

"MGs are empty and dead microbial cells or viruses (envelopes) devoid of cytoplasmic contents or any internal fluidized or any ge-

Figure 7: The most important applications for MGs .

Figure 8: Five steps for produce high quality MGs : Microbe feature; Visual control; MIC and MGC; Ghosts preparation; immunological studies.

Figure 9: Disadvantages of MGs.

netic element. That were caused by one or more than one pore happened in their cell walls. Or direct remove of the genetic elements. They have correct 3D structure, morphology and native surface antigens structure able to induce the immune system of the delivered host to produce specific antibodies that could react correctly with the mother viable cell or viruses. Additionally as being empty cells they can be used as drug delivery system for various drugs, genes and antigen or surface antigen expressed protein from another potent pathogenic bacteria. The critical chemical concentration and enzymes activity methods have extended the spectrum of the BGs from the gram-negative bacteria to all types of bacterial strains including gram-positive, Archaea as well as Eukaryotic such as yeast ghosts. Viruses were achieved and parasites also are expected to join the Ghosts Family. The future of the MGs is bright"

One should observe that the previously introduced E lysis protocol was restricted only to the gram-negative bacteria. However, the critical chemical concentration and the critical enzymes activities enables ghosts preparation from any microbes.

The advantage of MGs (Figure 7,8)

After introducing the SL protocol most microbes become target to be prepared as ghosts. Microbial or Biological Ghosts have some advantages which could be concluded in the following points:

1. After purification, MGs could be subjected to long-term storage at ambient room temperature as lyophilized products.

2. Genetically engineered or surface expressed antigens can be targeted to the inner or outer membrane.
3. Before being prepared as MGs, they were alive active cells, so they can be manipulated through the molecular and genetic engineering tools such as loading surface protein antigen of a potent microbial pathogenic strain. That can be happened on the surface of a suitable recombinant strain such as *E. coli*. Such ghosts could be used as vaccine against such protein or such strain. As carriers, there is no limitation in the size of foreign antigens that can be inserted in the membrane and any spaces in the cells.
4. Being dead cells make them safe during their handling, transfer, transport, processing and so on.
5. Being dead cells enable many new root of administration including oral, inhalation, superficial, ocular, wound, skin ulcer, beside the classical ones such as subcutaneous, aerosol, intradermal, intramuscular, intravenous, intraperitoneal, intragastric, rectal and intravaginal and so on.
6. MGs can be fractionated further to smaller fragment for certain applications, particularly if foreign surface antigen was introduced on the surface of recombinant strain (for example *E. coli*), hence such antigen can be re-purified and reused for research or immunization purposes. BGs can be produced inexpensively in large quantities.

7. They are dead cells, so they cannot replicate or do any metabolic activity.
8. They proved to be recognized by the immune system in manner similar to the living cells, so they can be used for application aim to target certain cells particularly the macrophage. Drugs of different types, compounds and proteins can be loaded to the internal lumen or periplasmic space of the BGs.
9. High potential to target different cell types; such as dendritic cells, macrophages, tumor cells, endothelial cells and epithelial cells.
10. MGs can be an alternative to the adjuvant, so if they are used as a drug delivery they can do more than one function.
11. Initial aerosol vaccinations showed that this route of administration can establish protective immunity and are able to target the mucosal immune system.
12. Ghosts introduced to the experimental animals are able to induce specific humoral and cellular immune responses against microbial components.
13. Large microbial ghost cells can be used for immobilizing small one, in certain industrial applications.
14. Multiple antigenic determinants can be presented simultaneously.
15. They can be suspended after being prepared as ghost in solvent such as ethanol which may contain an active ingredient such as gossypol acetic acid and then the ethanol is evaporated and the gossypol acetic acid is crystallized inside the cells. Water which did not dissolve the gossypol acetic acid can then be used to wash the outer cells crystal of the gossypol acetic acid. This strategy can be used to prepare some types of drug delivery products [38].
16. Multiple antigens of the native BG envelope and recombinant protein or DNA can be combined in a single type of MG.
17. No cytotoxic and genotoxic impacts on the viability and metabolic activity of cells recognizing BGs.
18. Simple and high-dose manufacturing process of MGs can be easily and quickly performed either in disposable fermenters, small laboratory steel fermenters or in large-scale fermenters.
19. They can be used in small or large quantities.
20. They have broad spectrum of possible safe applications.
21. They can induce several potent immune regulatory cytokines.
22. Their quality can be validated, using both the light and electron microscope for validating their 3D structure. Regular electrophoresis and spectrophotometer (260-280 nm) methods can be used to validate their content (as contaminant) of DNA and protein. But the most important point is to prove that they are still able to induce correct immunization. The research on eukaryotic cells and viruses has been started using the critical chemical concentration method and proved some advantages over the old used method [3,37,38].

Figure 10: *Saccharomyces cerevisiae* Ghosts.

23. After adding the critical activity of the enzymes more relaxed protocol are expected to cover a wide range of microbes.

Disadvantage of the BGs (Figure 9)

By screening the literature published so far about the BGs, apparently, there is no mentioned disadvantage; however, MGs still have some disadvantages such as:

1. They have one open pore (at least), for that; they need to be sealed in case of their use as drug delivery system.
2. Even, their preparation become clear and straightforward, but aggregation should be avoided to avoid any survival cells.
3. Exopolysaccharide producing strains can give some wrong MIC result.
4. H_2O_2 could induce mutation if not calculated correctly. For that, some microbes such as *P. aeruginosa*, which mutated upon their exposure to H_2O_2 can produce mucoid strains. Such microbes should be treated with NaOH firstly.
5. Being dead cells, they should be manually counted preferably by using the hemocytometer.
6. Being dead, they should be given with higher dosage to equalize the effect of the active cells
7. Being empty cells, they can return some of the last solution, they suspended on, so they should be carefully washed and dried.
8. They should be gently subjected to the drying process to maintain the feature of their 3D structure.
9. After being empty cells, they can lose their 3D structure not by deforming the cell wall but by shrinking or getting rid of the empty space [43].
10. In case of preparing MGs using enzymes, which are protein in nature, enzymes should be removed after finishing the process. Any contamination with protein will induce the host immune system upon treatment.

Bacterial Cells Prepared As Ghosts Or Their Surface Protein Expressed In Ghosts

By revising the literatures there are many microbial cells that were prepared as ghosts as well as yeasts, viruses and cells.

E gene mediated lysis was achieved in a variety of gram-negative bacteria while SL protocol has extended the spectra to the eukaryotic and viruses. That includes *Acholeplasma laidlawii* [E lysis][44] ; *Actinobacillus pleuropneumoniae* [E lysis] [45] [46] [47]; *Aeromonas hydrophila* [E lysis] [48]; *Bacillus megaterium* [E lysis][49]; *Chlamydia trachomatis* [E lysis] [50] [51]; *Clostridium botulinum* [E lysis](Ellison, Mattern, et al. 1971); *E. coli* (EHEC) O157:H7 [E lysis] (Mayr, Haller, et al. 2005) [52] ; *E. coli* (EPEC) E2348 /69 [E lysis] [53]; *E. coli* BL21 (DE3) pLysS (Promega) [SL] (Figure 3) [54, 55]; *E. coli* JM109 [SL][36]; *E. coli* K12 [E lysis][56] ; *E. coli* Nissle 1917 [E lysis][57]; *E. coli* NK9373 [E lysis][58]; *E. coli* NM522 [E lysis][30, 59]; *E. coli* O157:H7 [E lysis][52]; *E. coli* O26:B6 [E lysis][60]; Enteropathogenic *E. coli* (EPEC) E2348/69 [E lysis] [53]; *Edwardsiella tarda* [E lysis] [61]; *Flavobacterium columnare* [E lysis] [62]; *Haemophilus influenzae* [E lysis][63]; *Haemophilus parasuis* serovar five reference strain Nagasaki [E lysis][64]; *Helicobacter pylori* [E lysis] [65]; *Mannheimia haemolytica* [E lysis] [66] [67] [68]; *Mycobacterium phlei* [E lysis] [69] [70]; *Neisseria sicca* [E lysis] [71]; *Pasteurella multocida* [E lysis] [72] [72]; *Pseudomonas aeruginosa* [E lysis and SL] [73]; *Salmonella* Enteritidis (and *Yersinia enterocolitica*) [E lysis][26, 74]; *Salmonella gallinarum* [E lysis][75]; *Salmonella typhi* Ty21a [E lysis][76]; *Salmonella typhimurium* [E lysis][60, 77]; *Salmonella typhimurium* ATCC 14028 [SL][34]; *Salmonella typhimurium* C5 [E lysis][60]; *Streptococcus fecalis* [E lysis][78]; *Trichosurus vulpecula* (pest) [E lysis] [59]; *Vibrio cholerae* [E lysis][79]; *V. cholerae* O1 or O139 [E lysis] [80]; *Vibrio anguillarum* [E lysis][81]; *Yersinia enterocolitica* [E lysis][82] [83]; Yeast [84]; *Saccharomyces cerevisiae* [SL] [37, 38] (Figure 9) and Viruses such as bacteriophage [85] and Newcastle virus [3].

Ghosts Future

The protocols introduced in this review show that there are big opportunities to prepare different types of microbial and viruses ghosts. The most interesting one is that which based on using the critical concentration of some chemical compounds or the critical activity of some enzymes. Additionally, the future will show extending the ghosts preparation to nearly all types of cells. For that such protocols as well as the different types of ghosts will flourish the science of the immunology and pharmacology. The problem of any vaccine will be solved and new intelligent drug delivery systems will be introduced. There will be no need to go through the risk of using alive or attenuated vaccines. MGs will make a break through in many scientific fields and will be extended to the other forms of cells. This will enable many industrial, biotechnological, pharmaceutical, medicinal etc applications. In addition, it will be a great chance to study dead microbes and cells but still have their correct 3D structures.

Conflict Of Interest Statement

The author declares that there is no conflict of interest in this review article.

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