ISSN 2075-1729
www.mdpi.com/journal/life

Peer-Review Record:

Protocells: At the Interface of Life and Non-Life

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***Life* 2015, *5*, 447-458, doi:10.3390/life5010447**

Reviewer 1: Peter Strazewski
Reviewer 2: Anonymous
Reviewer 3: Anonymous
Editor: Pasquale Stano and Fabio Mavelli (Guest editor of Special Issue "Protocells - Designs for Life")

Received: 25 October 2014 / Accepted: 2 February 2015 / Published: 9 February 2015

First Round of Evaluation

Round 1: Reviewer 1 Report and Author Response

A very useful and enjoyable minireview on the misconception of a "primordial RNA World". The authors reflect very carefully all major arguments and concepts that are used by those researchers that steadfastly hold to the assumed existence of a primordial RNA World and to the main (and original) argument in favor of it: some kind of "Natural Parsimony" postulated to exist and to reign over reality from the beginning of time. This theory continues to ignore the (early) importance of a genetic code, of translation, of the huge size, complexity and ancient origin of the ribosome (which, yes, is a ribozyme but so what), of the fact that amino acids and peptides appear spontaneously and are chemically more persistent than the nucleosides and nucleic acids, and therefore must have been offered by Nature very early to take an essential part in life, not only because of their persistence, but because proteins are so incredibly useful and adaptable. In the opinion of the reviewer, the idea of a primordial RNA World is born of a fundamentally purist (or puritan) attitude towards life which has not much bearing on real life. It is as if you'd ignore the importance, even existence of African Americans and Asian Americans in the US society, although they did all the hands-on work to make it function – and bequeathed the world the music and food to enjoy the work. On what grounds should we assume that Nature is inherently parsimonious? Isn't parsimony a goal or ideal rather than an origin? If so, then cooperation is not something that needs to be avoided at first, not something that becomes inevitable when all walls are crashing. Cooperation is an early spontaneous, utterly Darwinian achievement, parsimony is not.

Response: The main purpose of this paper is to explain why cellular form should have been already significant near the very beginning of the origin of life, and how the primitive cellular form, protocells, may have evolved. If, as the reviewer points out, the explanation is based on the scenario of a "primordial RNA World", it is because the hypothesis is to date the only idea that can "mediate" the conceptual discussion here. As mentioned in the text, "the notion of the RNA-based protocell serves well as a 'working concept' for us to examine some fundamental issues concerning the primitive cellular form".

Then why is this scenario of a primordial RNA World the only idea that can serve as the base of the discussion? The ultimate reason should be that to date only this idea states explicitly how Darwinian evolution could have got start, given that the genetic feature and the functional feature are both indispensable for the occurrence of Darwinian evolution. And without Darwinian evolution, any process would be purely chemical—that is, the starting point of Darwinian evolution was associated with the interface of life and non-life, which is the focus of this discussion.

Although the main purpose of this paper is not to defend the idea about a primordial RNA World, as a paradigm concerning the notion of a parsimonious origin, we do think that a parsimonious origin is in some sense reasonable, and the logic should be as follows. Any thing's emergence in the evolution of life should not simply mean its "chemical" existence, but that it is incorporated into the "living system" and thus could be duplicated and delivered to the "offspring". For example, even if proteins (with random sequence) may have appeared spontaneously and were chemically more persistent than RNA in those prebiotic environments, they had not "emerged in life" at that moment, because their formation was not in an inheritable way—they are in some degree no more than environmental factors, rather than the living system's components. Lacking a mechanism for their own replication, to be synthesized in an inheritable way, they should be useful to certain living system already existing which harbors the genetic feature (e.g., some "RNA-based organism") and is capable of synthesizing them—and then they could be "adopted" by this system on account of nature selection. And proteins are "incredibly useful and adaptable" only when they carry special sequences that could fold into special structures to perform special functions. Thus, they would "emerge in life" only when they could be coded by nucleic acids and thereby passed on generation after generation—owing to their special functions. In other words, a parsimonious origin does not mean the circumstance at that time was pure in chemistry, but that for a start of Darwinian evolution, the most primordial system could not have been complex—because all the components of the system, each has its mechanism and its justification (i.e., some "beneficial" function) to recur across generations, must "happen to" appear through purely chemical events (since they cannot be inherited from a former generation) in the same time and in the same place. In this sense, cooperation is indeed something that needs to be avoided at the outset and should have arisen only in the subsequent evolution, when "efficiency" could cover the cost of becoming complex.

Corrections and suggestions not to be published in an open review report (but to be taken into account by the authors in a revision):

Either in Refs. [17–22] or, even more importantly, in Refs. [37–39] the work of Holliger and coll. is missing, especially Attwater, Wochner, Holliger, *Nature Chemistry* 2013, *5*, 1011. They succeeded to design a polymerase ribozyme capable of catalysing the accurate synthesis of an RNA sequence longer than itself (adding up to 206 nucleotides).

Response: Yes, thanks. We have added three papers of theirs into "References". (i.e., [23] - cited in Lines 85 and 87; [49,50] – cited in Line 127 in the revised version), and have modified our relevant descriptions accordingly (Lines 125–127 in the revised version).

Line137: "The main 'trouble' is concerning the growth". This is technically not true. To make lipid vesicles grow in size is not so difficult. The main trouble is to make them divide into several smaller vesicles.

Response: This should be a misunderstanding. What we mean here is the coupled growth of membrane and core, not purely the enlargement of the vesicle through the growth of membrane. We have explained this point in a clearer way in the revised version (Lines 148–151).

The English language should be corrected/modified in a number of instances.

Response: Many thanks. The English language has been corrected/modified in these instances. Please see the revised version for details. Additionally, we have edited the language throughout the manuscript in light of the opinions from a professional English editing service.

- Line 39: "are based upon" (not are base upon)-- ***Corrected***
- Lines 41–42: "more detailed analysis is deserved for" is bad English -- ***Modified***
- Line 46: "it is also not quite doubtful" ?? Doubtful or not? Bad English -- ***Modified***
- Line 47: "which render natural selection [...] possible" Not good English -- ***Modified***
- Line 57: "it makes the point that" and "might be able to be avoided" is bad English -- ***Modified***
- Lines 59–62: better replace "sort" by "kind" -- ***Modified***
- Line 163: "(note that the membrane would grow by naturally ... " (not growth) -- ***Corrected***
- Line 192: [c517] ?? -- ***Corrected***
- Lines 222–224: "given that single molecules were 'futureless' to act as Darwinian entities" ?! Single molecules never were "deemed" (see also line 130) a Darwinian entity. Or else you could dissolve a piece of RNA at it would begin to live. The step between a single replicator molecule (if existing at all) and a "unitary-protocell" is so huge (jumping over "pseudo" and "true" in one big leap) does not help the argument. Rather: Given that "pseudo-protocells" and "true-protocells" were not sufficiently robust against changes in the environment ... or something like that. -- ***Modified***.
- Line 245: "the most 'parsimonious' and ..." (not parsimony) -- ***Corrected***
- Line 254: "became sufficiently sophisticated" (not sufficient) -- ***Corrected***

Round 1: Reviewer 2 Report and Author Response

In this manuscript, the authors intend to delineate how the first protocell emerged at the origin of life in a hypothetical RNA world. Although this is a really interesting topic for a special issue devoted to protocells and their importance in the fields of origin of life and synthetic biology, I think that the manuscript is not fulfilling the expectative, since it is not presenting any novel and significant results nor adding new insights into this matter. Additionally, I see a fundamental problem: the scenario put together by the authors is purely speculative. Even if it were possible to synthesize such an RNA-protocell, that will not prove that it happened this way in the origin of life.

Response: As mentioned in our response to reviewer 1's comments, the main purpose of this paper is to show why cellular form should have been already significant near the very beginning of the origin of life, and how the primitive cellular form, protocells, may have evolved. We rely on the hypothesis of the RNA world because the hypothesis is perhaps the only idea that can "mediate" the conceptual discussion here. To make this point clearer, we have added relevant explanations at the end of Section 2. Thanks for the reviewers' comments. Here we disagree with the reviewer on his attitude concerning the history and rules involved in the origin of life. On one hand, we do not intend to assert the scenario would indeed happen this way in the origin of life—who can assert with certainty a scenario with such great antiquity?; on the other hand, it should be nevertheless valuable to conceive the scenario according to those reasonable deductions—especially, if we could synthesize these protocells in lab in the light of the deductions, our understanding concerning the essence of life would improve a lot. That is what we should really care about, rather than what exactly occurred in the history. As to the relationship between the history and rules concerning the problem of the origin of life, please see a recent publication of ours [61] for a detailed discussion.

Dr. Ma and colleagues have been working on this subject for the last decade, and this work seems to be an attempt to summarize the models derived from their previous computer simulations about molecule cooperation and the emergence of membranes and chromosomes in RNA-based protocells. However, the summary is too succinct to be informative enough for a reader that could not be familiar with their previous work.

Response: To be sure, in the discussion of this paper, we rely heavily on our previous simulation studies using RNA-based systems as working models. However, we do not merely attempt to summarize our previous models. We talk much about these models because the scenario described in them is just concerning the starting point of Darwinian evolution, lying between the interface of life and non-life. In fact, the purpose of these previous modeling studies were just to shed light on this area, which is dim but significant for our understanding on the essence of life. Because here we focus on the conceptual discussion concerning the protocells, rather than these modeling studies, readers with deeper interests to the models could read the original papers.

As the authors already state, with our current knowledge about living systems and the physicochemical properties of the biological molecules, the hypothesis of the RNA world can be considered the most plausible explanation for the origin of self-sustaining and self-replicating systems able of Darwinian evolution. Furthermore, compartmentalization and molecular cooperation appear to be central for life. However, the straightforward scenario presented in this manuscript is just a plausible model about how life could have emerged, but it could have happened this way or not, since there are no evidences that support it. Therefore, it should be clearly stated from the beginning that this is just speculation about one possible scenario for the origin of life. The proposed protocells would be at the interface between living and non-living matter, but do not necessarily represent the origin of life. Sure, it would be interesting to try to implement a system able to gain in complexity to end up being a true-protocell, and this would be quite relevant in the field of synthetic biology, but this is not a new idea.

Response: This issue is associated with the first issue. The answer is that the main purpose of our discussion is not to talk about a special scenario. However, as the reviewer points out, "the straightforward scenario presented in this manuscript is just a plausible model about how life could have emerged", so, to avoid misunderstandings, we have stated (at the end of Section 2), according to the reviewers' opinions, that If the discussion is deemed to be delineating a detailed scenario, one should keep in mind that it just represents one speculation regarding the early stage in the origin of life.

Additionally, indeed, to implement a system as a true-protocell is not a new idea in the field of synthetic biology, but to implement a system as a unitary-protocell, which harbors a chromosome with linked gene, should be a new idea. That is, emphasizing the significance of a central genetic molecule to the protocell is a novel notion regarding the goal in the field of synthetic biology.

Additionally, the way the different ideas are connected throughout the text is not clear at all, starting from the abstract, where the first and the second sentence have no clear relationship between them.

On the first section, it is surprising that they consider viruses as "living things" and talk about "living activities" without defining them. Even though there is no consensus on a definition for life (although I wouldn't say there is a "violent controversy" regarding such definition), the authors should try to give an insight about what they call life in order to introduce the subject for the readers. Furthermore, to present "the genuine cellular form" they describe as "the most natural choice" does not seem to be a proper scientific argument.

Response: We think the logic of our discussion is clear. As to the points raised by the reviewer, first, concerning the relationship between the first and the second sentence, the logic is that because molecular cooperation is inevitable, a membrane-bounded vesicle is needed to keep those functional molecules together. To make it clearer, we have added a note "(comprising various functional molecules)" in the first sentence. Second, it is indeed disputable whether viruses are living things, and just because of this, we complement the wording with a clause: "which, though capable of existing 'physically' independent of cells, are only 'biologically' active within host cells". Third, we have adopted the wording "there is no consensus on a definition for life", as suggested by the reviewer, thanks. However, we believe that if we define life in an exact way, any way it may be, there would indeed be violent opposition. For example, if we define that life is anything capable of Darwinian evolution, the reviewer would object violently since he thinks it serious to consider viruses as "living things" without defining them. If we adopt that popular definition of NASA: "Life is a self-sustained chemical system capable of Darwinian evolution", things may also become troublesome: what is "self-sustained"? Who can state it clear, especially when we talk about the interface between life and non-life. And so we prefer to start with the assertion like: "there is no doubt that life is something capable of Darwinian evolution". Fourth, our assertion that the genuine cellular form (with genetic and functional molecules bounded by lipid membrane composed of amphiphilic molecules) should be the most natural choice for life represents our inference concerning why life is ultimately based on cellular form, instead of a scientific argument on firm sense.

It is also surprising the beginning of the second section. I don't see the point to introduce at this level how complicated modern cells are.

Response: The logic is that cells are complicated in modern life, but at the outset in the evolutionary history, they cannot be so complicated—otherwise it would be difficult to interpret how the cellular form could emerge in the beginning. And so there should have been some much simpler cellular form(s), i.e., "primitive cells" or "protocells".

But it is even more surprising to define Mycoplasma genitalium as "the simplest cell in nature known to date". And there are more recent attempts to approach the minimal gene set than those selected by the authors.

Response: What we mean is the simplest cell of free-living organisms in nature. Corresponding modifications have been made, including the number of essential genes (modified from 250 to 200~250, in light of the more recent ref. [37]), and the introduction of Nasuia deltocephalinicola as the simplest cell in nature known to date (if the living style is not constrained) [39]. Many thanks.

Finally, the manuscript needs some language corrections. Many of the sentences are not easy to understand and there are some awkward/incorrect word choices.

Response: Yes, we have edited the language in light of the opinions from a professional English editing service.

Round 1: Reviewer 3 Report and Author Response

This is a rather convincing defence for a protocellular origin of life. The theoretical survey is well documented although some references are missing such as the pioneer contribution of Harold Morowitz.

Harold Morowitz (H. Morowitz, "Beginnings of Cellular Life." Yale University Press, New Haven and London, 1992) already postulated that the first step toward the origin of life was the spontaneous condensation of amphiphilic molecules to form vesicules. Examples of auto-catalytic micelle growth have been published by the group of Luisi (P.A. Bachmann, P.L. Luisi, and J. Lang, Nature 357, 57, 1992). These autocatalytic systems alone do not store hereditary information and cannot therefore evolve by natural selection. However, Jack Szostak (M.M. Hanczyc, S.S. Mansy, and J.W. Szostak, Orig. Life Evol. Biosph. 37, 67, 2007) found that clay particles, such as montmorillonite, can help to assemble vesicles and, in the process, bring bound RNA into the interior of the vesicles, thus providing information to the vesicular system. Computer modeling by Doron Lancet (B. Shenhav, A. Bar-Even, R. Kafri, and D. Lancet, Origins Life Evol. Biosph. 35, 111, 2005) may also help support this vesicular life. Even more ambitious, the "minimal cell project" aims to synthesize a cell model having the minimal number of components to be defined as living. Liposomes are used as shell membranes and attempts are made to introduce a minimal genome (P.L. Luisi, Chem. Biodiv. 4, 603, 2007 and R.V. Solé, S. Rasmussen, and M. Bedau, Philos. Trans. Royal Soc. 362, 1486, 2007).

Response: These references, as well as some explanations relevant to them, have been added in the revised version ([27,28,32,33,68,69]). Many thanks.

Second Round of Evaluation

Round 2: Reviewer 2 Report and Author Response

The manuscript has been significantly improved, and has taken into account all my previous comments.

The improvement in English language makes the manuscript much easier to follow. However, Lines 267–270 are still unclear. Please review the last paragraph on Section 4.

Response: Yes, we have modified this paragraph further, please see the revised version. Thanks.

I totally agree on the fact that the scenario put together in this manuscript "just represents one speculation regarding the early stage in the origin of life" and, therefore, and that's why I thought this point should be clearly stated. The text added at the end of Section 2 makes this point clear to the reader now.

Response: Yes, thanks.

Regarding the topic about the simplest cell in nature, if the living status is not constrained, it is truth that Nasuia deltocephalinicola possess the shortest genome. However, the genome with the smaller number of genes (116 protein coding genes) corresponds to Tremblaya princeps PCVAL from the mealybug Planococcus citri (Lopez-Madrigal *et al.*, 2011; http://www.ncbi.nlm.nih.gov/genome/genomes/3208).

Response: Yes, we have taken Tremblaya princeps PCVAL into account and added the relevant reference (Lopez-Madrigal et al., 2011, i.e., [38]). Thanks.

Finally, I would like to point out that the lack of consensus does not imply violent attitudes at all. There are no sign of violence on my objections; they are just a sign of polite and critical discrepancy that (I hope) have helped to improve the manuscript.

Response: Yes, many thanks.