J Appl Biotechnol Rep. 2020 Mar;7(1):7-15

doi 10.30491/JABR.2020.105916

Journal of
Applied Biotechnology
Reports

Review Article

A Review of the Methods for Concentrating M13 Phage

Faezeh Fouladvand[1], Peyman Bemani[2], Mozafar Mohammadi[3*], Razieh Amini[1*], Farid Azizi Jalilian[4]

[1]Department of Molecular Medicine and Genetic, Faculty of Medicine, Hamedan University of Medical Sciences, Hamedan, Iran
[2]Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[4]Department of Virology, Faculty of Medicine, Hamedan University of Medical Sciences, Hamedan, Iran

Corresponding Authors: 1. Mozafar Mohammadi, PhD, Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: +98-9167379116, Email: mohammadi83@live.com
2. Razieh Amini, PhD, Associate Professor, Department of Molecular Medicine, Faculty of Medicine, Hamedan University of Medical Sciences, Hamedan, Iran. Tel: +98-9184441405, Email: Aminra14@gmail.com

Received July 22, 2019; **Accepted** December 19, 2019; **Online Published** March 11, 2020

Abstract
Bacteriophages, viruses which infect the bacteria are the most abundant organisms on the earth. Among them, the best studied and the most-exploited group is the filamentous phages especially M13 phage. They have shown a lot of interesting applications because of their unique features. Therefore, to get maximum performance of bacteriophage like M13, techniques need to be engaged for proper concentrations. In this review, most of these methods were explored in PubMed, Scopus and Google Scholar, using keywords including M13 bacteriophage, phage concentration, phage purification, phage display. Accordingly, the most important research papers about this subject have been collected, categorized and discussed. As a conclusion, to select an appropriate method for the concentration of M13 bacteriophages different criteria should be considered, including cost, equipment, yield and purity of the product. In general, subsequent applications of M13 phage is the most important factor for the selection of the concentration method.
Keywords: M13 Bacteriophage, Phage Concentration, Phage Purification, Phage Display, Phage Precipitation
Citation: Fouladvand F, Bemani P, Mohammadi M, Amini R, Azizi Jalilian F. A review of the methods for concentrating M13 phage . J Appl Biotechnol Rep. 2020;7(1):7-15. doi:10.30491/JABR.2020.105916.

Introduction

Bacteriophages or phages which have a bacterial host which infect them are the most abundant organisms in the biosphere.[1] In terms of bacteriophages capsid, they are classified into 3 types: icosahedral, filamentous, and head-tail in shape.[2,3] Among filamentous phage, the best studied and most-exploited group are the F pilus-specific phage or Ff, known as f1, M13 and fd.[4] Filamentous bacteriophages (particularly M13) are normally used for phage display to screen and select recombinant antibodies, therapeutic peptides, the new ligand to target proteins and drug discovery, etc.[5-9] The M13 bacteriophage has been used as a template to align inorganic, organic, and biological nanomaterials to generate different nanostructures, such as nanowires and nanofilms.[10-15] Furthermore, the most amazing applications are high-power phage batteries, metal nanowire catalysts, biological, cell-targeting agents, gene transfer vectors, and targeted cancer therapies.[16-21] Thus to get maximum performance of bacteriophages like M13, methods for high-quality separation and purification of these microorganisms need to be engaged. In this review, extensive investigations in PubMed, Scopus and Google Scholar have been performed using keywords including M13 bacteriophage, phage concentration, phage purification, and phage display. Accordingly, the most important research papers about this subject based on quality and level of evidences have been collected, categorized and discussed.

M13 Phage Structure

The M13 is a cylindrical bacteriophage with 880 nm length and 6 nm diameters. The body of M13 phage is composed of pVIII protein, as the major coat protein with 2700 copies. One end of the particle is capped by 3- 5 copies of minor coat proteins pIII and pVI, and the other end is capped by 5 copies of pVII and pIX proteins. (Figure 1).[22-24]

Density-Based Methods

Density Gradient Ultracentrifugation

Density gradient ultracentrifugation (DGU) is a highly versatile method that has been widely employed in different fields of biology including separating protein complexes,[25] subcellular organelles[26] and extracellular vesicles[27] such as exosomes.[28] Cesium chloride (CsCl) DGU as a type of DGU has been used for the concentration and purification of

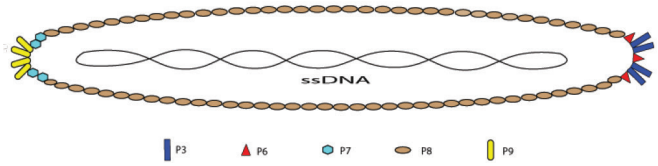

Figure 1. Schematic illustration of M13 bacteriophage.

biological molecules including RNA, DNA, and proteins.[29] This method has also been used for purification of a range of viruses[30-35] including bacteriophage[36-38] as well as M13 phage.[39-42] This approach allows the separation of substance on the basis of buoyant density differences, independently of their size and shape.[43] In this method, the sample is usually precipitated through a steep density gradient that contains a very high concentration of CsCl and high speed centrifuge. The target of interest begins to move down the gradient, but it eventually reaches a position where the density of the solution is equal to its own density. At this point, the phage floats and can move no farther. After CsCl ultracentrifugation of phage-containing bacterial culture supernatant, two bands are visible; the top one corresponds to bacterial cell debris, especially membranes, endotoxins (LPS) as well as empty phage capsids (with density lower than 1.3) and the other is the lower band which contains the desired phage (with density higher than 1.3).[38,44,45] Thus, the phage forms a band at a specific place in the CsCl gradient that corresponds to its density which can be simply collected. Finally, to remove CsCl from phage suspensions, dialysis is performed for two to three times.[38,44,45]

Extremely high pure phage is one of the most important advantages of the CsCl-based purification method.[39,44,46] Another advantage of the CsCl method compared to the other methods is that the method could distinct phages containing genome from genome-free phage capsids. This relies on the difference in the buoyant density between DNA/RNA-filled and empty particles. This is important especially about M13 phage library displaying diverse binder moiety (such as peptide, receptor, antibody, etc), as the presence of empty-genome capsids can prevent the promotion of panning process for screening and selecting best binder moiety. This is also important for other purposes such as gene therapy studies. While CsCl DGU as a conventional method of phage purification is yielded high purity,[39,44] some drawbacks are associated with this method; the purification of phages by CsCl DGU usually requires ultracentrifugation at more than 100 000 ×g force[44,46,47] which cannot be achieved by ordinary centrifuges and requires an expensive set of ultracentrifuge, rotor, and special tubes. Hence, all laboratories cannot access this powerful method. In addition, the purification efficiency of DGU in CsCl differs depending on the phage; some phages cannot withstand long periods of centrifugation and in most cases, this method exhibits a relatively low yield, yet for some phages, it does not work.[48,49] Phages purified by this method also could be destructed by the centrifugal forces, because of osmotic shock or interaction with CsCl which lead to loss of their infectivity.[50] Recently Nasukawa et al introduced a modified method of CsCl to reduce the imposed cost related to the need to high-speed centrifuge and the risk of damage to the phage by reducing centrifuge speed from 100 000×g (1 hour) to 40 000×g (2 hours). It demonstrated almost the same concentration efficiency as conventional CsCl DGU.[51]

Precipitation-Based Methods

PEG-Based Precipitation

Polyethylene glycols (PEG) is a condensation polymer of ethylene oxide and water which has several chemical properties that make it useful for chemical, biological, and pharmaceutical applications.[52,53] Precipitation of viruses is generally achieved using PEG in the presence of a high concentration of a monovalent salt as co-precipitant. The PEG is usually used in combination with NaCl salt as co-precipitant.[54] The mode of the action of phage precipitation with PEG is very similar to the salting out of proteins in protein precipitation.[55,56] As bacteriophage particles are made up mostly of coat proteins around their genetic material, solutions containing PEG and high salt content separate water molecules forcing the bacteriophages to aggregate into clusters. Indeed, by adding salt such as NaCl to the PEG, the interaction of some water molecules with the charged coat protein of the phage could be replaced by the salt and thus decreases the number of available interacting water molecules with the phage, hence help to better aggregation and precipitation of the phage (Figure 2). Also, NaCl helps membrane-bound phage to separate from the bacterial membrane.[48] Thus, the precipitated phages can easily be collected by centrifugation.

Different factors affect the efficiency of PEG phage precipitation such as the concentration of co-precipitant (salt), the concentration of PEG and the molecular weight of the PEG.[45] At a final concentration of 2.5M, NaCl is usually used and at the concentrations lower than 0.5 M, however, the efficiency of sedimentation or bacteriophage viability is reduced.[45] Regards to the concentration of PEG, and based on a study on a number of bacteriophages, an increasing fraction of each of the phages could be found in the pellet as the PEG concentration is increased, and concentrations of 10% or higher allow at least 90% of the infective titer of phage to be recovered in the pellet. However, some studies demonstrated that high level recovery rates of M13 phage can keep in the lower concentration of PEG (2%-5%).[57-59] The PEG molecular

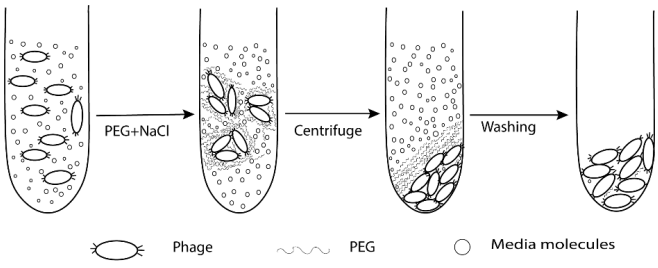

Figure 2. Schematic Illustration of the PEG Phage Precipitation.

weight (MW) is another factor that affects the yield of phage. The PEG molecules with a molecular weight lower than 6000 are less effective in precipitating bacteriophages, as the PEG molecular weight decreases, increasing concentrations of PEG are needed to reach a specified level of pelleted phage. While MWs equaling or above 6000 are more effective for concentrating bacteriophages and appear to behave identically.[45]

The PEG-based precipitation is the traditional approach for purifying bacteriophages like M13.[57-61] The advantage of this method is its low cost as it needs minimal equipment and doesn't require expensive ultracentrifuge, and the precipitation can be performed by general centrifuge which is commonly available in laboratories.[45] However, this method faces several disadvantages; the PEG precipitation method requires the addition of large amounts of PEG and 2.5 M NaCl, some of which remain as a residue in the phage pellet and are difficult to be eliminated.[62,63] Other certain contaminants such as *E. coli* lipopolysaccharide (LPS) could remain in the PEG-concentrated phage preparation.[64,65] Certain enzymes from the *E. coli* host could also be other contaminating agents in PEG-purified phage. They could be co-precipitated with phages that hamper the selection of phage displaying the same catalytic activity. For example, contaminating RNAses from *E. coli* interferes with RNAse-displaing phages.[40] For these reasons, depending on the purpose, the PEG-precipitated phage can be subjected to the next method of purification in further steps (usually CsCl gradient centrifugation) to remove impurities.[44,66]

Isoelectric-Based Precipitation

Bacteriophages, as well as other (bio)-colloids, show a pH-dependent surface charge in polar media.[67] The pH value at which a particular molecule or surface carries no net electrical charge is referred to as the isoelectric point (pI).[63,67] The pI is a parameter which characterizes the (bio)-colloids in an equilibrium state with its environmental water chemistry, which is due to a superposition of protonated and un-protonated states of functional groups.[67] The pI of bacteriophages are in pH range from 1.9 to 8.4; most frequently, between 3.5 and 7.[67] The M13 phage is covered by 2700 copies of the pVIII major coat protein, representing the largest fraction of the viral mass.[68] The surface amino acid sequence of the pVIII protein is AEGDDPAK which is rich in acidic amino acid residues. These residues give the M13 phage a low pI of 4.2 and a net negative charge at a neutral pH. Thus, it could be easily precipitated in acidic pH around its pI.[63,68] For isoelectric precipitation of phage, after removing bacterial cells by centrifugation, the pH of the resultant phage-containing supernatant is adjusted to pI of the phage (for example 4.2 for M13) by adding an acid (HCl). After brief mixing, the precipitated phage is simply pelleted by centrifugation.[63]

As the isoelectric precipitation separates phage particles on the basis of pI, this method has the advantage of requiring only acid (or base) addition to induce precipitation.[57] This eliminates additive contamination such as PEG residue. The only potential contaminating source may be the components of media and/or secreted proteins by *E. coli* during culturing which have a similar pI to the target (for example 4.2 for M13).[57,63] However, some studies have shown that the amount of contaminating proteins is minimal to non-existent.[63] In addition, studies have demonstrated that the phage recovery rate of this method is very high (98.4 %) and PFU numbers of the M13 phage has not any significant variation before and after pH adjustment with no significant loss of viability.[57,63] In addition, a dynamic light scattering analysis showed that the phage structure was not damaged by the pH adjusting. Consistently, PFU counts confirmed that there is no considering decreasing in phage infectivity during 8h interval following pH adjustment from 4.2 to 7.0[63] which indicates phage resistance to acidic pH. Thus, M13 appears resistant to denaturation by isoelectric precipitation, its oft-cited and the greatest drawback as a general method of protein precipitation.[68,69] Therefore, simplicity, high efficiency, low cost, and high speed could be considered as the advantages of this method. It should be noted that studies have shown that electro-kinetic features of phages (including the isoelectric point) have not only been affected by their outer protein components but also have been effected by internal genomic content.[70,71] Thus, by considering the difference in the pI of a bacteriophage and its empty capsid particle, it may be assumable that the isoelectric precipitation may theoretically be able to separate virus from their corresponding genome-free virus-like particles.

Purification of M13 phage by isoelectric precipitation has advantages compared to using traditional PEG/NaCl-based precipitation. The PEG-based precipitation is based on the "salting out" mechanism of PEG and NaCl which needs the addition of large amounts of PEG and NaCl, some of which remain as a residue in the phage pellet[62] and consequently interfere with further application of M13 phage. This is while for isoelectric precipitation, only a small amount of acid or base is added, and no residue is left in the phage pellet, thus obtaining a purer phage.[67] The PEG-based precipitation requires one week of dialysis to remove culture media, the residual PEG and NaCl.[72] Whereas in the isoelectric precipitation, the phage pellet is only required to be rinsed with water and collected via centrifugation to remove the residue of the culture media which can be performed in 15 minutes. For isoelectric precipitation, the NaOH used to adjust the pH can simply be substituted by any other bases. Isoelectric precipitation doesn't need expensive ultracentrifuge and the precipitation can be performed by general centrifuge which is commonly available in laboratories.[63] Also, precipitation can be performed faster than other methods. As mentioned above, the main disadvantage of this method is the risk of contaminant proteins with the pI similar to that of the phage of interested.

Spermidine-Based Precipitation

Spermidine is a natural polycationic aliphatic amine that plays different roles in all living cells.[73] It notably can precipitate M13 bacteriophage at a low concentration of 1.5 mM. In this regard, M13 phage behaves like DNA and other polyvalent anions molecules. Branston et al. used this method

and achieved about 95% M13 bacteriophage recovery.[57] It seems that the concentration of the monovalent ion such as NaCl in solution is a key factor which determines the effectiveness of the phage precipitation. Based on Branston et al's experiment, while monovalent ions are increased, the spermidine concentration must be increased subsequently, to achieve the desired phage precipitation rate. For example, M13 phage precipitation by spermidine is prevented at 50 mM NaCl addition.[57] Therefore, in this approach, the control of equilibration between spermidine and monovalent salts concentration, determines the rate of M13 bacteriophage recovery.

Chromatography-Based Methods

Chromatography is a powerful and diverse technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.[74] This separation technique is based on the interaction of the target of interest with the mobile phase or stationary phase. The target of interest (protein or virus) can be purified based on their interaction types as well as their characteristics such as shape, size and, total charge, hydrophobic groups on their surface, and binding capacity with the stationary phase. Accordingly, the separation functionality can be broadly categorized into 1) size, 2) charge, 3) hydrophobic and 4) affinity, etc. Although based on the stationary bed, several types of chromatography techniques are developed,[74] column chromatography is typically used for virus purification. Bellow the most frequent chromatography methods which have been applied for bacteriophages purification are discussed.

Size-Exclusion Chromatography

Size exclusion chromatography (SEC), also known as gel filtration chromatography, is a separation technique using a tightly packed stationery matrix of beads that contain pores of a particular size.[75] The beads of gel filtration columns consist of cross-linked polyacrylamide, Sephadex G, agarose gel, dextran, silica or a combination of any of these.[64-75] The basic principle of this method is separating macromolecules based on their differences in molecular sizes.[74] Given the large size of phages relative to the most bacterial components as well as other small compounds such as ions and buffer salts, SEC can be applied as a sole or complementary step for phage purification.

In a study conducted by Zakharova et al,[64] SEC was applied to purify a filamentous M13 phage under mild conditions and to differ its recovery, purity and infectivity rate compared to PEG precipitation by using a Sephacryl resin-filled SEC column. The electrophoretic analysis showed that all contaminants and impurities found in the PEG-purified preparations were removed after SEC. Also, the SEC did not significantly decrease phage viability as the loss of the phage viability after SEC was about 10%. In addition, SEC did not reduce the panning efficiency in the three analyzed display formats (pIII-based peptide display, pVIII-based peptide display, and pIII-protein display).[64]

Phage purification by SEC has some other advantages; the main advantages of SEC are the low costs of resins and simplicity in operation as samples are eluted isocratically, so there is no need to use different buffers during the separation. However, this technique lacks selectivity and suffers from low productivity. Macromolecules, which stay adsorbed within the active SEC column packing may strongly reduce the effective volume of the separation pores.[76] Thus, the scaling up process using SEC is restricted because the column can get saturated with host cell proteins consequently preventing the separation of large bacteriophages from host cell proteins. Another drawback is the limitation in increasing pressure to increase the flow rate and decrease purification time; increasing the pressure leads to an increase in temperature which may cause some changes in the protein structure which leaves the risk of on-column protein denaturation and aggregation.[77] This causes an additional risk of decreasing in the column lifetime. Hence, the necessity of periodically re-calibration of the SEC columns as well as removing macromolecules adsorbed within packing in the course of analyses are other problems with this technique.[76]

Ion Exchange Chromatography

Ion exchange chromatography (IEC) is one of the most efficient methods for separating charged particles. The IEC is based on electrostatic interactions between charged groups of a particle (in this case virus) and solid support matrix.[74] There are two types of IEC; anion- and cation-exchange chromatography (AEC and CEC, respectively). Positively charged ion-exchange matrices are known as anion-exchange matrices that interact with negatively charged virus. While matrices loaded with groups of negatively charged are called cation-exchange matrices, and viruses with positive charge are adsorbed.[74] Charge-based virus binding to the solid matrix is dependent on the difference between the pI of the virus and the charge on the surface matrix. To create electrostatic interaction, the pH of the adsorption buffer is selected in such a way that the virus is negatively charged and the matrix is positively charged or vice versa. After binding the virus to the solid surface, it can be separated from the column by changing pH, ionic strength or the combination of both pH and ionic strength of the elution buffer.[39,78] Anion-exchange chromatography has been used to purify several bacteriophages.[39,78,79] The purification of M13 with anion exchange chromatography has also been reported thrice.[39,79,80] For the anion-exchange chromatography purification of M13 phage, the adsorption buffer has a pH higher than the pI of the viral particle, thus the M13 phage particles have a net negative charge and thus, bind onto the positively charged adsorbent. On the other hand, the elution buffer has a pH lower than pI of the phage, thus it has a net positive charge and can be eluted out from a positively charged adsorbent.[39,79]

pH optimization of the elution buffer and ionic strength is essential to reach the highest recovery and infectivity rate. In a study conducted by Monjezi et al,[39] the effect of pH and ionic strength was evaluated on the recovery percentage and infectivity of the eluted M13 phage particles. To evaluate the effect of pH, citrate buffer containing 1.5 M NaCl at different pH levels of 3.5, 4, and 5 were used. The highest recovery percentage was achieved when pH 4 was applied (about

69%). At the pH levels higher than the pI of M13 (pH 5), the lowest recovery was observed (18%); this is logical as at this pH, the particles have a net negative charge and bind onto the positively charged adsorbent and could not elute. The lower recovery percentage of elution buffer at pH 3.5 was also observed (19%). It should be noted that studies have shown that at pH levels lower than 3 the phage structure folds to a looser arrangement.[81] Henceforth, the lower recovery percentage of elution buffer at pH 3.5 was most likely due to the destructive effect of low pH on the phage structure. Thus, the selected pH for elution of phage should be lower than its pI, but not very low to cause damage to the structure of the phage.

Another factor that is applied to elute phage from the ion exchanger is changing the ionic strength of elution buffer. Elution from the ion exchanger is usually accomplished by increasing the ionic strength of the eluting buffer; as a result, this reduces the forces between bound phage and the adsorbent. In the same study, the effect of different ionic strength of citrate buffer with pH 4 on recovery percentage was compared. Results showed that the NaCl concentration of 1 M eluted the lowest amount of M13 (37%) compared to those containing 1.5 M (78.6%).[39] When the salt concentration was increased from 1.5 M to 2 M, an increase in recovery percentage was expected; nonetheless, the recovery in 2 M NaCl (44.3%) was lower than that of 1.5 M NaCl (78.6%). The authors hypothesize that this was most likely due to highly viscous elution buffer containing 2 M NaCl which may pose a problem in elution operation. This emphasizes the necessity of optimizing elution buffer salt concentration to achieve the highest recovery percentage. It is worth noting that the complete elution process is only achieved when optimization in the combination of both pH and ionic strength is applied. Studies have shown that using a low pH elution buffer to switch the phage charge from negative to positive, without adding salt solution, was not completely effective (48.6 ± 18.1% of elution efficiency).

Furthermore, the increase of Cl anion concentration without decreasing the pH was not enough to fully disrupt the ionic interactions between negative-charged M13 particles and the positive-charged adsorbent (36.4 ± 10.6% elution efficiency). In contrast, elution buffer with pH 4 in the presence of a high salt concentration was most effective for M13 phage elution (86.4 ± 14.9% elution efficiency).[79] Other factors that affect the yield of phage include the chemistry type of separation matrices, buffer type, and the column capacity. The protocol of purification should be individually optimized for each bacteriophage. For each phage, taking into account the required end product titer, the appropriate volume and column type are needed to be chosen. For large-scaling, optimization of the protocol on an analytical scale or a laboratory scale column can be done, and then for large-scale applications, a larger volume column with an industrial scale can be used.[49]

Some studies have compared AEC and traditional PEG/NaCl or CsCl DGU as a function of yield, purity and time consumption. In a study conducted by Ling et al.[80] the performance of the two methods, conventional multiple steps PEG/NaCl and expanded bed anion exchange chromatography were evaluated and compared for purifying M13 bacteriophage. By using expanded bed AEC, the purification of the M13 bacteriophage, yielded a higher recovery percentage (82.86%) compared to the PEG/NaCl method (36.07%).[80] In another study, comparing the performance of AEC and conventional CsCl gradient density ultracentrifugation method showed that an average yield of 74% was achieved from AEC. The purification process was substantially shortened from 18h in the ultracentrifugation method to less than 2h in ACE. The SDS-PAGE evaluation demonstrated that the purity of phages was comparable to that of the CsCl DGU method. In addition, plaque forming assay revealed that the AEC-purified phages were still infectious.[39] The anion-exchange columns have other advantages. They can be exploited several times, although, it should be considered that after multiple usages the capacity may sometimes be reduced.[49] Additionally, given the fact that the pI of a bacteriophage and its corresponding genome-free particle is different, IEC confers the advantage of the capability of purifying virion from its genome-free particles.[71] Moreover, due to the centrifugation step in the traditional methods, the volume of phage suspension which is used in each sample is restricted, while in the chromatography method by using appropriate HPLC or FPLC pump and loading system, on each column the volume of bacteriophages which can be loaded are unlimited. This offers an extra advantage especially for phages that are not properly amplified in the previous stage of a plate or liquid amplification.[49]

In summary, the AEC chromatography method offers a valid and effective substitution for conventional benchtop purification and concentration methods, particularly for bacteriophages which are not sufficiently stable in these traditional approaches.[49] The main drawbacks are the need to connect to the chromatography column to costly FPLC[39] or HPLC[78,82] systems that are not available in all laboratories,[39] as well as a potentially long optimization process. Nevertheless, after optimization, the scalability of each column is achieved without any additional optimization.[49]

CIMs Monolith Anion-Exchange Chromatography

Recently, convective interactive medias (CIMs) monoliths based anion-exchange chromatography demonstrated the effective purification and concentration potential for several bacteriophages, including Staphylococcus phage VDX-10, Escherichia phages T4, lambda and M13.[78,82] The method has shown that it could be a useful alternative approach to CsCl gradient concentration of bacteriophages as a standard method. Taking into account the phage yield, per sample, CsCl purification can generally give a higher amount of phage particles than the 0.34 ml and 8 ml columns employed in CIMs monoliths based on anion-exchange chromatography. This is while the centrifugation step in the CsCl method is a limiting factor due to the restricted phage suspension used in each sample. Meanwhile, the CIMs monoliths chromatography method doesn't encounter with the mentioned problem

because of an unlimited volume of phages which can be loaded on each column. The bacteriophages which did not have enough opportunity for a proper amplification in the previous step will be able to amplify themselves. Also, the CIMs monoliths' can be optimized for larger columns to use in the industrial scales purification of phages.[49]

Conclusions

To select an appropriate method for the concentration of M13 bacteriophages, different criteria should be considered including cost, equipment, yield and purity of the product. However, application of M13 phage is the most important factor for the selection of the concentration method. Regarding to the routine phage applications, isoelectric precipitation technique is suggest because of its low cost, fast performance, high recovery rate and also minimal equipment. On the other hand, if very high purification is considered, especially about the M13 phage library displaying system, CsCl-based concentration could be exploited. Also, for a large scale production of concentrated M13, chromatography approaches is recommended. All methods regarding concentration of M13 bacteriophage are categorized in Table 1, and scientists who intend to work with M13 can employ each of them based on the advantages and disadvantages of the methods.

Authors' Contributions

FF, PB and MM have searched and contributed to the article writing. RA and FJ edited the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

Acknowledgments

The authors would like to thank Dr. Seyed Nooreddin Faraji for his valuable comments.

References

1. Clokie MR, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage. 2011;1(1):31-45. doi:10.4161/bact.1.1.14942.
2. Salmond GP, Fineran PC. A century of the phage: past, present and future. Nat Rev Microbiol. 2015;13(12):777-786. doi:10.1038/nrmicro3564.
3. Dimmock NJ, Easton AJ, Leppard KN. Introduction to Modern Virology. Hoboken, NJ, USA: John Wiley & Sons; 2016.
4. Skerra A, Pluckthun A. Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. Science. 1988;240(4855):1038-1041. doi:10.1126/science.3285470.
5. Mohammadi M, Bemani P, Zarei N. Applying the bioinformatics methods to design and evaluate the SapM-M13 pIX fusion protein and its theoretical role in phage ELISA system. J Appl Biotechnol Rep. 2016;3(2):419-424.

Table 1. Brief Comparison of the M13 Phage Concentration Methods

Method	Advantages	Disadvantage	References
Cesium chloride (CsCl) density gradient ultracentrifugation	(1) Very high pure phage; (2) Separation of phages containing genome from genome-free phage	(1) Requiring costly ultracentrifugation at more than 100 000 ×g which is not available in all laboratories; (2) Need to performing time consuming two or three rounds of dialysis to remove CsCl as interaction of phage with CsCl lead to loss of phage infectivity; (3) Some phages cannot withstand long period and high speed conditions of centrifugation. This leads to phage damage, which is associated with lower yield and infectivity rate	38,39,44-50
PEG-based precipitation	(1) Low cost; (2) Needs minimal equipment and does not require expensive ultracentrifuge	(1) Residual PEG, NaCl and LPS and protein contaminants after PEG-purification. These contaminants are cytotoxic and hinders phage panning utilizing living eukaryotic cells, interferes with downstream assays such as ELISA, prevent selection of phage displaying some moieties; (2) Need to subject PEG-precipitated phage to the next method of purification	40,44,45,62-66
Isoelectric precipitation	(1) Low cost; (2) Lack of PEG/NaCl contaminants in PEG precipitations; (3) Needs minimal material and equipment and does not require expensive ultracentrifuge; (4) Fast; (5) High phage recovery rate; Separation of the virus from their corresponding genome-free virus-like particles (weaker than AEC method)	(1) Risk of contaminants protein with isoelectric point similar to the phage	57,63,70,71
Spermidine-based precipitation	(1) Lack of PEG/NaCl contaminants in PEG precipitations	-Its effectiveness depends on the NaCl concentration	57
Size-exclusion chromatography (SEC)	(1) No need to using harsh buffers or conditions; (2) High purity; (3) High recovery rate; Low cost	(1) Lack of selectivity; (2) Low productivity; (3) Restriction in scaling up as the column can get saturated with host proteins after a few times; (4) Limitation in increasing pressure to increase the flow rate and decrease purification time. Increasing pressure can lead to phage protein denaturation and aggregation; (5) The necessity of periodically re-calibration of the columns as well as removing adsorbed macromolecules. (6) Does not district and separate virus from their corresponding genome-free particles	64,76,77
Ion exchange chromatography	(1) High yield; (2) High purity; (3) Simple and fast after optimization; (4) Columns can be used more than once; (4) The capability of purifying virion from its genome-free particles; (5) Suitable for phages which do not amplify well on a plate or liquid growth media	(1) Time-consuming and laborious optimization pH and ionic strength of buffers; (2) Purification protocol needs to be optimized for each phage individually; (3) Need costly FPLC or HPLC systems which are not available in all laboratories	39,49,71,78, 80,82

6. Bazan J, Całkosiński I, Gamian A. Phage display--a powerful technique for immunotherapy: 1. Introduction and potential of therapeutic applications. Hum Vaccin Immunother. 2012;8(12):1817-1828. doi:10.4161/hv.21703.
7. Ehsaei B, Nejatollahi F, Mohammadi M. Specific single chain antibodies against a neuronal growth inhibitor receptor, nogo receptor 1: promising new antibodies for the immunotherapy of multiple sclerosis. Shiraz E-Med J. 2017;18(1):e45358. doi:10.17795/semj45358.
8. Bemani P, Mohammadi M, Hakakian A. Anti-ROR1 scFv-EndoG as a novel anti-cancer therapeutic drug. Asian Pac J Cancer Prev. 2018;19(1):97-102. doi:10.22034/apjcp.2018.19.1.97.
9. Lunder M, Bratkovič T, Doljak B, et al. Comparison of bacterial and phage display peptide libraries in search of target-binding motif. Appl Biochem Biotechnol. 2005;127(2):125-131. doi:10.1385/ABAB:127:2:125.
10. Yang LM, Diaz JE, McIntire TM, Weiss GA, Penner RM. Covalent virus layer for mass-based biosensing. Anal Chem. 2008;80(4):933-943. doi:10.1021/ac071470f.
11. Yang LM, Diaz JE, McIntire TM, Weiss GA, Penner RM. Direct electrical transduction of antibody binding to a covalent virus layer using electrochemical impedance. Anal Chem. 2008;80(15):5695-5705. doi:10.1021/ac8008109.
12. Yang LM, Tam PY, Murray BJ, et al. Virus electrodes for universal biodetection. Anal Chem. 2006;78(10):3265-3270. doi:10.1021/ac052287u.
13. Souza GR, Christianson DR, Staquicini FI, et al. Networks of gold nanoparticles and bacteriophage as biological sensors and cell-targeting agents. Proc Natl Acad Sci U S A. 2006;103(5):1215-1220. doi:10.1073/pnas.0509739103.
14. Kassner PD, Burg MA, Baird A, Larocca D. Genetic selection of phage engineered for receptor-mediated gene transfer to mammalian cells. Biochem Biophys Res Commun. 1999;264(3):921-928. doi:10.1006/bbrc.1999.1603.
15. Larocca D, Baird A. Receptor-mediated gene transfer by phage-display vectors: applications in functional genomics and gene therapy. Drug Discov Today. 2001;6(15):793-801. doi:10.1016/s1359-6446(01)01837-2.
16. Nam KT, Kim DW, Yoo PJ, et al. Virus-enabled synthesis and assembly of nanowires for lithium ion battery electrodes. Science. 2006;312(5775):885-888. doi:10.1126/science.1122716.
17. Nam KT, Wartena R, Yoo PJ, et al. Stamped microbattery electrodes based on self-assembled M13 viruses. Proc Natl Acad Sci U S A. 2008;105(45):17227-17231. doi:10.1073/pnas.0711620105.
18. Smith GP, Petrenko VA. Phage Display. Chem Rev. 1997;97(2):391-410. doi:10.1021/cr960065d.
19. Lee YJ, Yi H, Kim WJ, et al. Fabricating genetically engineered high-power lithium-ion batteries using multiple virus genes. Science. 2009;324(5930):1051-1055. doi:10.1126/science.1171541.
20. Merzlyak A, Indrakanti S, Lee SW. Genetically engineered nanofiber-like viruses for tissue regenerating materials. Nano Lett. 2009;9(2):846-852. doi:10.1021/nl8036728.
21. Zarei N, Fazeli M, Mohammadi M, Nejatollahi F. Cell growth inhibition and apoptosis in breast cancer cells induced by anti-FZD7 scFvs: involvement of bioinformatics-based design of novel epitopes. Breast Cancer Res Treat. 2018;169(3):427-436. doi:10.1007/s10549-017-4641-6.
22. Hess GT, Cragnolini JJ, Popp MW, et al. M13 bacteriophage display framework that allows sortase-mediated modification of surface-accessible phage proteins. Bioconjug Chem. 2012;23(7):1478-1487. doi:10.1021/bc300130z.
23. Sidhu SS. Engineering M13 for phage display. Biomol Eng. 2001;18(2):57-63. doi:10.1016/s1389-0344(01)00087-9.
24. Bratkovic T. Progress in phage display: evolution of the technique and its application. Cell Mol Life Sci. 2010;67(5):749-767. doi:10.1007/s00018-009-0192-2.
25. Shinzawa-Itoh K, Shimomura H, Yanagisawa S, et al. Purification of active respiratory supercomplex from bovine heart mitochondria enables functional studies. J Biol Chem. 2016;291(8):4178-4184. doi:10.1074/jbc.M115.680553.
26. Williamson CD, Wong DS, Bozidis P, Zhang A, Colberg-Poley AM. Isolation of endoplasmic reticulum, mitochondria, and mitochondria-associated membrane and detergent resistant membrane fractions from transfected cells and from human cytomegalovirus-infected primary fibroblasts. Curr Protoc Cell Biol. 2015;68:3.27.21-23.27.33. doi:10.1002/0471143030.cb0327s68.
27. Onódi Z, Pelyhe C, Terézia Nagy C, et al. Isolation of high-purity extracellular vesicles by the combination of iodixanol density gradient ultracentrifugation and bind-elute chromatography from blood plasma. Front Physiol. 2018;9:1479. doi:10.3389/fphys.2018.01479.
28. Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. Methods Mol Biol. 2015;1295:179-209. doi:10.1007/978-1-4939-2550-6_15.
29. Zhang H, Chen HT, Glisin V. Isolation of DNA-free RNA, DNA, and proteins by cesium trifluoroacetate centrifugation. Biochem Biophys Res Commun. 2003;312(1):131-137. doi:10.1016/j.bbrc.2003.09.246.
30. Takemoto M, Koike M, Mori Y, et al. Human herpesvirus 6 open reading frame U14 protein and cellular p53 interact with each other and are contained in the virion. J Virol. 2005;79(20):13037-13046. doi:10.1128/jvi.79.20.13037-13046.2005.
31. Farkas T, Sestak K, Wei C, Jiang X. Characterization of a rhesus monkey calicivirus representing a new genus of Caliciviridae. J Virol. 2008;82(11):5408-5416. doi:10.1128/jvi.00070-08.
32. Kreider JW, Howett MK, Leure-Dupree AE, Zaino RJ, Weber JA. Laboratory production in vivo of infectious human papillomavirus type 11. J Virol. 1987;61(2):590-593. doi:10.1128/JVI.61.2.590-593.1987.
33. Strobel B, Miller FD, Rist W, Lamla T. Comparative analysis of cesium chloride- and iodixanol-based purification of recombinant adeno-associated viral vectors for preclinical applications. Hum Gene Ther Methods. 2015;26(4):147-157. doi:10.1089/hgtb.2015.051.
34. Mueller C, Ratner D, Zhong L, Esteves-Sena M, Gao G. Production and discovery of novel recombinant adeno-associated viral vectors. Curr Protoc Microbiol. 2012;Chapter 14:Unit14D.11. doi:10.1002/9780471729259.mc14d01s26.
35. Tan J, Lancaster M, Hyatt A, van Driel R, Wong F, Warner S. Purification of a herpes-like virus from abalone (Haliotis spp.) with ganglioneuritis and detection by transmission electron microscopy. J Virol Methods. 2008;149(2):338-341. doi:10.1016/j.jviromet.2007.12.019.
36. Bachrach U, Friedmann A. Practical procedures for the purification of bacterial viruses. Appl Microbiol. 1971;22(4):706-715. doi:10.1128/aem.22.4.706-715.1971.
37. Brown-Jaque M, Rodriguez Oyarzun L, Cornejo-Sánchez T, et al. Detection of bacteriophage particles containing antibiotic resistance genes in the sputum of cystic fibrosis patients. Front Microbiol. 2018;9:856. doi:10.3389/fmicb.2018.00856.
38. Zeng L, Golding I. Following cell-fate in E. coli after infection by phage lambda. J Vis Exp. 2011(56):e3363. doi:10.3791/3363.
39. Monjezi R, Tey BT, Sieo CC, Tan WS. Purification of bacteriophage M13 by anion exchange chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878(21):1855-1859. doi:10.1016/j.jchromb.2010.05.028.
40. Korn K, Foerster HH, Hahn U. Phage display of RNase A and an

improved method for purification of phages displaying RNases. Biol Chem. 2000;381(2):179-181. doi:10.1515/bc.2000.024.
41. Imai S, Mukai Y, Takeda T, et al. Effect of protein properties on display efficiency using the M13 phage display system. Pharmazie. 2008;63(10):760-764.
42. Henry TJ, Pratt D. The proteins of bacteriophage M13. Proc Natl Acad Sci U S A. 1969;62(3):800-807. doi:10.1073/pnas.62.3.800.
43. Yu H, Lu JJ, Rao W, Liu S. Capitalizing resolving power of density gradient ultracentrifugation by freezing and precisely slicing centrifuged solution: enabling identification of complex proteins from mitochondria by matrix assisted laser desorption/ionization time-of-flight mass spectrometry. J Anal Methods Chem. 2016;2016:8183656. doi:10.1155/2016/8183656.
44. Boulanger P. Purification of bacteriophages and SDS-PAGE analysis of phage structural proteins from ghost particles. Methods Mol Biol. 2009;502:227-238. doi:10.1007/978-1-60327-565-1_13.
45. Yamamoto KR, Alberts BM, Benzinger R, Lawhorne L, Treiber G. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology. 1970;40(3):734-744. doi:10.1016/0042-6822(70)90218-7.
46. James KT, Cooney B, Agopsowicz K, et al. Novel high-throughput approach for purification of infectious virions. Sci Rep. 2016;6:36826. doi:10.1038/srep36826.
47. Szelechowski M, Bergeron C, Gonzalez-Dunia D, Klonjkowski B. Production and purification of non replicative canine adenovirus type 2 derived vectors. J Vis Exp. 2013(82):50833. doi:10.3791/50833.
48. Cox CS. Bacterial survival in suspension in polyethylene glycol solutions. J Gen Microbiol. 1966;45(2):275-281. doi:10.1099/00221287-45-2-275.
49. Adriaenssens EM, Lehman SM, Vandersteegen K, et al. CIM((R)) monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. Virology. 2012;434(2):265-270. doi:10.1016/j.virol.2012.09.018.
50. Carlson K. Appendix: working with bacteriophages: common techniques and methodological approaches. In: Kutter E, Sulakvelidze A, ed. Bacteriophages: Biology and Applications. Boca Raton, FL: CRC Press; 2005:437-494.
51. Nasukawa T, Uchiyama J, Taharaguchi S, et al. Virus purification by CsCl density gradient using general centrifugation. Arch Virol. 2017;162(11):3523-3528. doi:10.1007/s00705-017-3513-z.
52. Veronese FM, Mero A. The impact of PEGylation on biological therapies. BioDrugs. 2008;22(5):315-329. doi:10.2165/00063030-200822050-00004.
53. Jang HJ, Shin CY, Kim KB. Safety evaluation of polyethylene glycol (PEG) compounds for cosmetic use. Toxicol Res. 2015;31(2):105-136. doi:10.5487/tr.2015.31.2.105.
54. Colombet J, Sime-Ngando T. Use of PEG, polyethylene glycol, to characterize the diversity of environmental viruses. Microb Ecol. 2012;58:728-36.
55. Atha DH, Ingham KC. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. J Biol Chem. 1981;256(23):12108-12117.
56. Arakawa T, Timasheff SN. Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding. Biochemistry. 1984;23(25):5912-5923. doi:10.1021/bi00320a004.
57. Branston S, Stanley E, Keshavarz-Moore E, Ward J. Precipitation of filamentous bacteriophages for their selective recovery in primary purification. Biotechnol Prog. 2012;28(1):129-136. doi:10.1002/btpr.705.
58. Sattar S, Bennett NJ, Wen WX, et al. Ff-nano, short functionalized nanorods derived from Ff (f1, fd, or M13) filamentous bacteriophage. Front Microbiol. 2015;6:316. doi:10.3389/fmicb.2015.00316.
59. Lin TC, Webster RE, Konigsberg W. Isolation and characterization of the C and D proteins coded by gene IX and gene VI in the filamentous bacteriophage fl and fd. J Biol Chem. 1980;255(21):10331-10337.
60. Høydahl LS, Nilssen NR, Gunnarsen KS, et al. Multivalent pIX phage display selects for distinct and improved antibody properties. Sci Rep. 2016;6:39066. doi:10.1038/srep39066.
61. Lee JH, Warner CM, Jin HE, et al. Production of tunable nanomaterials using hierarchically assembled bacteriophages. Nat Protoc. 2017;12(9):1999-2013. doi:10.1038/nprot.2017.085.
62. Reitinger S, Petriv OI, Mehr K, Hansen CL, Withers SG. Purification and quantitation of bacteriophage M13 using desalting spin columns and digital PCR. J Virol Methods. 2012;185(1):171-174. doi:10.1016/j.jviromet.2012.06.021.
63. Dong D, Sutaria S, Hwangbo JY, Chen P. A simple and rapid method to isolate purer M13 phage by isoelectric precipitation. Appl Microbiol Biotechnol. 2013;97(18):8023-8029. doi:10.1007/s00253-013-5049-9.
64. Zakharova MY, Kozyr AV, Ignatova AN, Vinnikov IA, Shemyakin IG, Kolesnikov AV. Purification of filamentous bacteriophage for phage display using size-exclusion chromatography. Biotechniques. 2005;38(2):194, 196, 198. doi:10.2144/05382bm04.
65. Gao C, Lin CH, Lo CH, et al. Making chemistry selectable by linking it to infectivity. Proc Natl Acad Sci U S A. 1997;94(22):11777-11782. doi:10.1073/pnas.94.22.11777.
66. Castro-Mejía JL, Muhammed MK, Kot W, et al. Optimizing protocols for extraction of bacteriophages prior to metagenomic analyses of phage communities in the human gut. Microbiome. 2015;3:64. doi:10.1186/s40168-015-0131-4.
67. Michen B, Graule T. Isoelectric points of viruses. J Appl Microbiol. 2010;109(2):388-397. doi:10.1111/j.1365-2672.2010.04663.x.
68. González-Mora A, Ruiz-Ruiz F, Benavides J, Willson RC, Rito-Palomares M. Recovery and primary purification of bacteriophage M13 using aqueous two-phase systems. J Chem Technol Biotechnol. 2017;92(11):2808-2816. doi:10.1002/jctb.5359.
69. Novák P, Havlíček V. Protein extraction and precipitation. In: Ciborowski P, Silberring J, eds. Proteomic Profiling and Analytical Chemistry. 2nd ed. Boston: Elsevier; 2016:51-62. doi:10.1016/B978-0-444-63688-1.00004-5.
70. Dika C, Gantzer C, Perrin A, Duval JF. Impact of the virus purification protocol on aggregation and electrokinetics of MS2 phages and corresponding virus-like particles. Phys Chem Chem Phys. 2013;15(15):5691-5700. doi:10.1039/c3cp44128h.
71. Urabe M, Xin KQ, Obara Y, et al. Removal of empty capsids from type 1 adeno-associated virus vector stocks by anion-exchange chromatography potentiates transgene expression. Mol Ther. 2006;13(4):823-828. doi:10.1016/j.ymthe.2005.11.024.
72. Yu L, Yu PS, Yee Yen Mui E, et al. Phage display screening against a set of targets to establish peptide-based sugar mimetics and molecular docking to predict binding site. Bioorg Med Chem. 2009;17(13):4825-4832. doi:10.1016/j.bmc.2009.03.054.
73. Madeo F, Bauer MA, Carmona-Gutierrez D, Kroemer G. Spermidine: a physiological autophagy inducer acting as an anti-aging vitamin in humans? Autophagy. 2019;15(1):165-168. doi:10.1080/15548627.2018.1530929.
74. Coskun O. Separation techniques: chromatography. North Clin Istanb. 2016;3(2):156-160. doi:10.14744/nci.2016.32757.
75. Duong-Ly KC, Gabelli SB. Gel filtration chromatography (size exclusion chromatography) of proteins. Methods Enzymol. 2014;541:105-114. doi:10.1016/b978-0-12-420119-4.00009-4.
76. Simeková M, Berek D. Studies on high-performance size-exclusion chromatography of synthetic polymers. I. Volume of silica gel column packing pores reduced by retained macromolecules.

J Chromatogr A. 2005;1084(1-2):167-172. doi:10.1016/j.chroma.2005.01.068.
77. Fekete S, Beck A, Veuthey JL, Guillarme D. Theory and practice of size exclusion chromatography for the analysis of protein aggregates. J Pharm Biomed Anal. 2014;101:161-173. doi:10.1016/j.jpba.2014.04.011.
78. Kramberger P, Honour RC, Herman RE, Smrekar F, Peterka M. Purification of the Staphylococcus aureus bacteriophages VDX-10 on methacrylate monoliths. J Virol Methods. 2010;166(1-2):60-64. doi:10.1016/j.jviromet.2010.02.020.
79. Jacinto MJ, Patinha DJS, Marrucho IM, et al. M13 bacteriophage purification using poly(ionic liquids) as alternative separation matrices. J Chromatogr A. 2018;1532:246-250. doi:10.1016/j.chroma.2017.12.005.
80. Ling TC, Loong CK, Tan WS, Tey BT, Abdullah WM, Ariff A. Purification of filamentous bacteriophage M13 by expanded bed anion exchange chromatography. J Microbiol. 2004;42(3):228-232.
81. Marvin DA, Wiseman RL, Wachtel EJ. Filamentous bacterial viruses. XI. Molecular architecture of the class II (Pf1, Xf) virion. J Mol Biol. 1974;82(2):121-138.doi:10.1016/0022-2836(74)90336-2.
82. Smrekar F, Ciringer M, Strancar A, Podgornik A. Characterisation of methacrylate monoliths for bacteriophage purification. J Chromatogr A. 2011;1218(17):2438-2444. doi:10.1016/j.chroma.2010.12.083.