COMPUTER-ASSISTED 2-D AGAROSE ELECTROPHORESIS OF SEMI-SYNTHETIC VACCINES CONTAINING PROTEIN-POLYSACCHARIDE PARTICLES WITH A CONTINUOUS SIZE DISTRIBUTION IN THE RANGE OF VIRUSES *

Dietmar Tietz **

Protein-conjugated meningitis vaccines have been developed by John Robbins, Rachel Schneerson and co-workers [1,2] for the immunization of small children, the main target group of bacterial meningitis. The physical characterization of these immunogens has been difficult, since their surface net charge is high and since the particles have a relatively large size which is in the range of intact viruses. Moreover, the size distribution of vaccine particles varies continuously over a wide range (polydisperse distribution) due to the randomizing steps in their method of preparation. When such samples are subjected to one-dimensional agarose electrophoresis, they exhibit an uninterpretable smear rather than a pattern of distinct zones (Fig. 1). However, when a second dimension is added to the separation (apparatus shown in Fig. 2), the results become interpretable: Distribution patterns are obtained which are characteristic for each vaccine. The patterns are digitized by scanning and then stored as computer images. Using the computer programs ELPHOFIT [3] and GELFIT [4], the size and charge distributions of these patterns as well as a number of other parameters are determined [5]. In particular, program ELPHOFIT is used to evaluate the gel electrophoretic data and to standardize the gel on the basis of the extended Ogston model [6,7], as shown in Fig. 3. Output of ELPHOFIT is then transferred to computer program GELFIT which transforms the original digital images (Fig. 4) to a rectangular coordinate system of particle radius and free mobility as it is exemplified in Fig. 5. GELFIT also computes frequency distributions of size and free mobility classes depicted by pseudocolors (Fig. 3 of [5]). Another application is the stripping of 2-D Gaussian surfaces demonstrated in Fig. 6.

The computerized gel electrophoretic technique appears generally applicable to the characterization of complex mixtures of small cell organelles, large DNA fragments and other subcellular-sized particles. An overview of methods is given in [12 - 15].

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* Lecture to honor Andreas Chrambach for his 40 years of achievement in science.

** Email: djt@his.com

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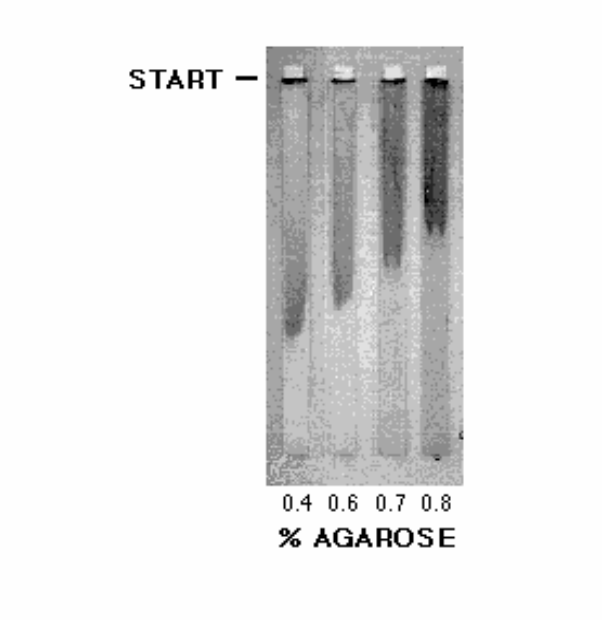

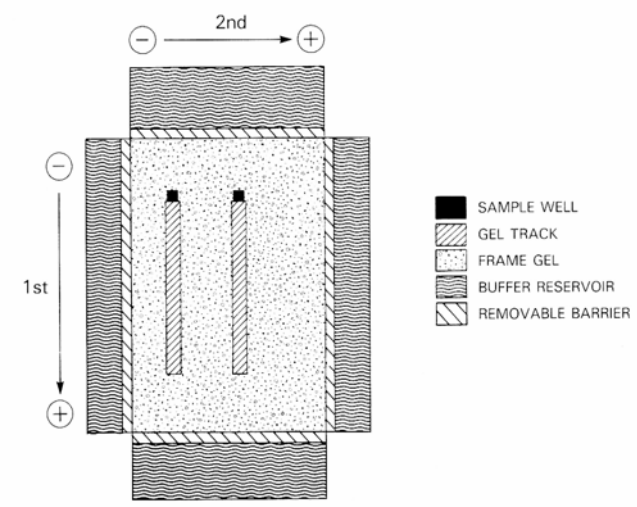

Fig. 1 (left): Gel patterns of meningitis vaccines at different agarose concentrations, using a 1-D submarine electrophoresis apparatus [8]. The samples yield an uninterpretable smear, although electrophoretic conditions are appropriate.

Fig. 2 (right): Schematic view of the 2-D submarine apparatus reported by Serwer [10]. Serwer's method, similar to the technique of O'Farrell, allows for a separation of particles predominantly according to charge in the first dimension, and according to size in the second dimension. However, the technique of Serwer rests on a different principle: Samples are first electrophoresed in a gel track of low concentration, then the field is switched perpendicularly and the samples are run into a relatively more concentrated frame gel which surrounds the first dimensional track. Gels need not be touched during the procedure; this makes it possible to handle the fragile gels suitable for the separation of *nondenatured* particles in the size range of viruses (> 3000 kDa). O'Farrell gels, by comparison, are typically used for much smaller proteins or protein fragments (10 - 500 kDa) which are *denatured* by sodium dodecyl sulfate (SDS). Adapted from Fig. 1 of [3].

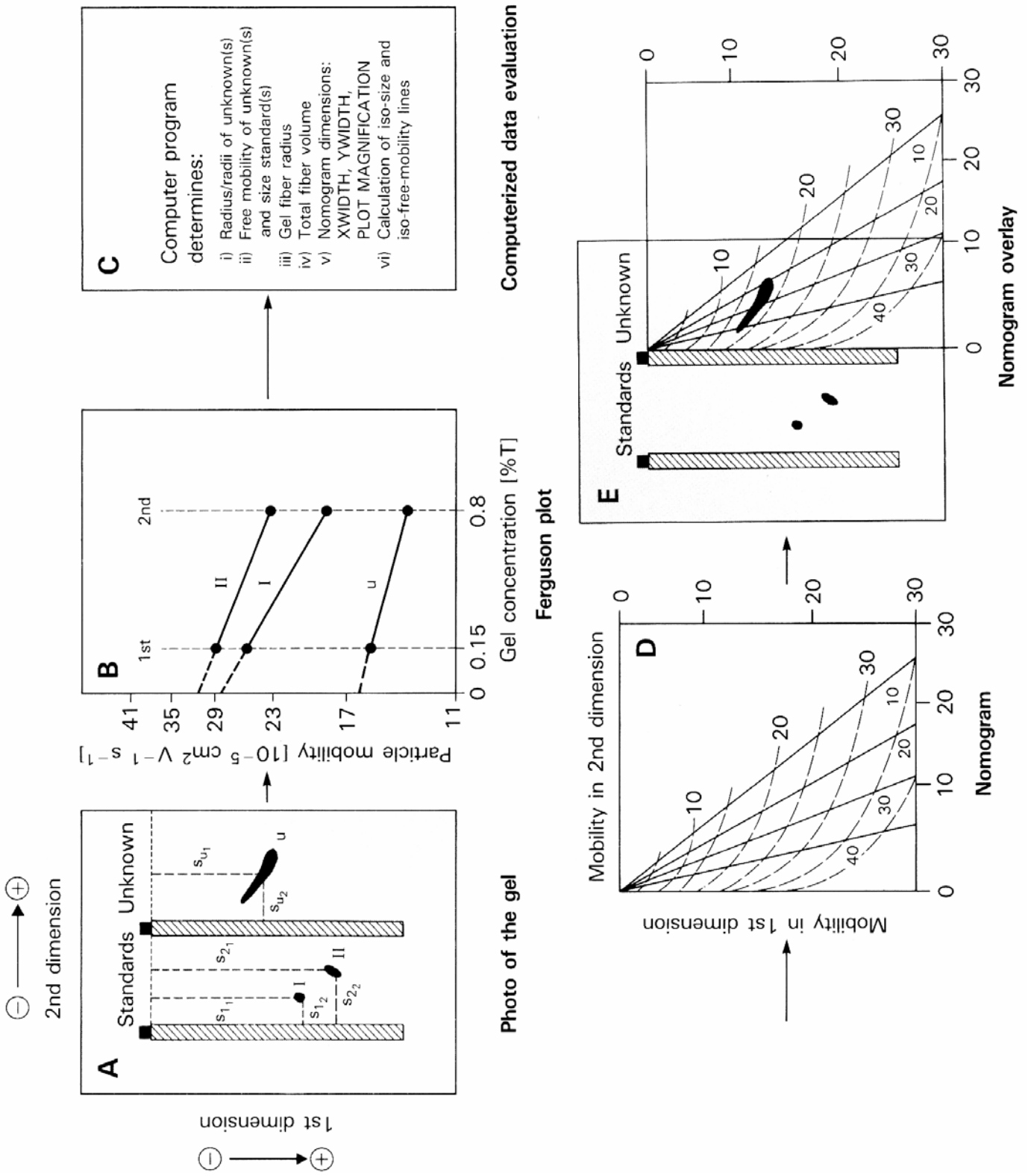

Fig. 3, please see legend on the following page.

Fig. 3: Procedure (schematic) for the construction of an iso-size/iso-free-mobility nomogram [9].
(A) A stained 2-D agarose gel is evaluated by determining the migration distances of standards and unknown in the first (s_1 , s_{u1}) and second dimensions (s_2 , s_{u2}).
(B) Particle mobility values are calculated from the migration distances, where mobility is defined as migration velocity (cm/s) divided by field strength (V/cm). The mobility values (two are available for each particle) are used to construct a linear Ferguson plot for each standard (I, II) and the unknown (U). The linear extrapolation of the Ferguson plots to 0 %T (absence of a gel) yields the free mobility for each particle, μ_0. The retardation coefficient, K_R, can be calculated as the absolute value of the slope of the linear Ferguson plot.
(C) Fitting Eqs. (1) and (2) of [3] to the data using a simultaneous curve-fitting algorithm (Newton-Gauss Marquardt-Levenberg), the gel is standardized by determining the gel fiber radius, r, and total fiber volume, V_F. Further output parameters of curve fitting are: radius and free mobility for specified centroid of the unknown, values of free mobility for the size standards. XWIDTH and YWIDTH determine the width and height of the box of the nomogram, which is scaled by a factor called PLOT MAGNIFICATION to fit the magnified photo of the stained gel pattern. Goodness of fit, standard errors and dependency values of fitted parameters are determined. High dependency values near 1.0 indicate that the mathematical model used for curve fitting is ill-defined. Such a condition may occur if insufficient experimental data are available in an important range, too many parameters are fitted or the values of fitted parameters degenerated during the curve-fitting procedure.
(D) After standardization, the nomogram can be constructed. Particles with similar radius are located on the straight diagonal lines (iso-size lines), particles with similar mobility are located on the curved dotted lines (iso-free-mobility lines). Iso-size lines, SL, are given by Eq. 3 of [3], Iso-free-mobility lines, FML, are defined by Eq. 4 of [3]. Numbers at the iso-size lines indicate effective particle radii (nm), labels at iso-free-mobility lines specify effective values of free mobility ($10^{-5}cm^2V^{-1}s^{-1}$). Serwer et al. [11] demonstrated the existence of iso-size lines experimentally.
(E) The nomogram is superimposed on a photograph of the stained gel pattern of the sample to be analyzed. This allows one to characterize each particle position in terms of particle size and free mobility.
Procedures of (B) to (D) are part of the program ELPHOFIT. Program GELFIT allows one to automate the nomogram overlay (E) and offers a number of additional features, as exemplified in Figs. 5 and 6. Adapted from Fig. 2 of [9]. ELPHOFIT and GELFIT are designed for the Macintosh platform.

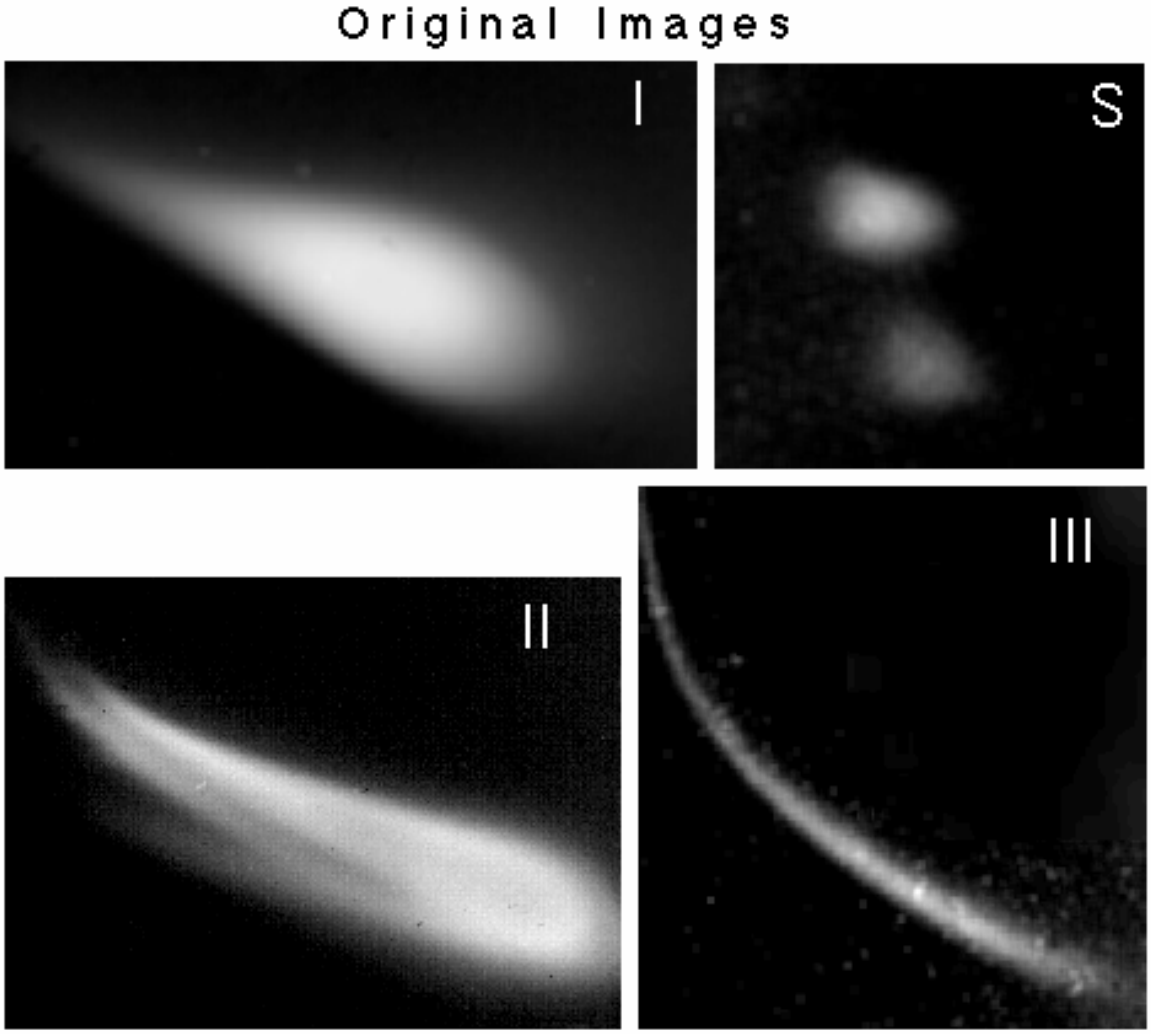

Fig. 4: Two-dimensional gel patterns of meningitis vaccines (**I** to **III**) and of hydrophilic polystyrene size standards (**S**) with 45 and 46.5 nm radius. The origin of electrophoresis lies outside the pictures. First dimension (top to bottom): 0.15 % agarose (SeaPlaque), 3 V/cm, second dimension (left to right): 0.8 % agarose, 1.5 V/cm. Electrophoresis in phosphate buffer pH 7.2 using a modified [9] 2-D submarine electrophoresis apparatus [10] shown in Fig.2, staining: Coomassie Blue R 250. Scanning of the stained patterns employed a Perkin-Elmer 1010MG microdensitometer interfaced with a PDP 11/34 (Digital). The vaccines produce characteristic gel patterns which depend on the nature of the sample. The samples consist of *Haemophilus influenzae*, type b, capsular polysaccharide crosslinked to tetanus toxoid (Panels **I** and **II**) or P2 protein (Panel **III**). The pattern of **II** can be interpreted as the composite of three subpopulations as has been shown in Fig .6 and may derive from combining different vaccine batches.

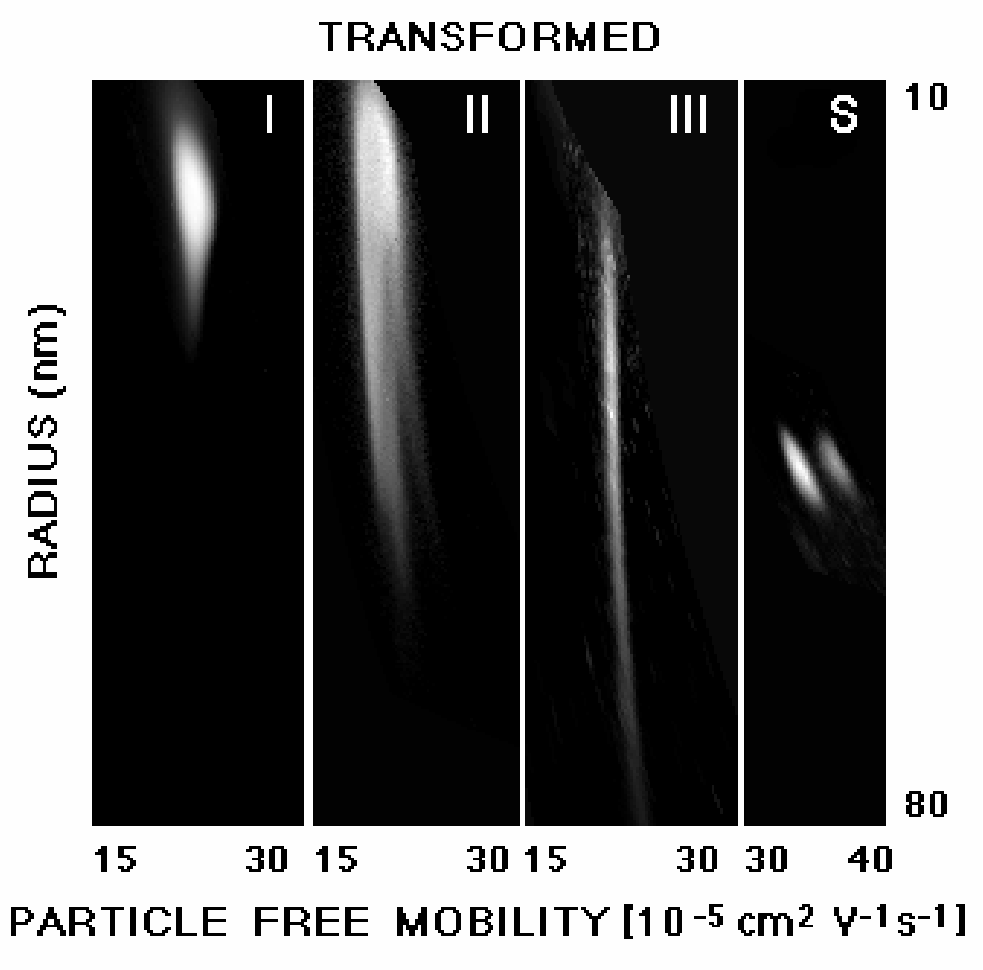

Fig. 5: Patterns of Fig. 4 which have been transformed from the original curvilinear to a rectangular coordinate system of particle size and free mobility (related to surface net charge density). Vaccines II and III which were effective immunogens have a considerably larger size distribution than sample I (not effective). The vaccines I and II have a much larger variation in free mobility than III, since protein P2 is well defined, whereas tetanus toxoid is a mixture of many components. It should be noted that the 2-D electrophoresis used here achieves results similar to O'Farrell's technique, but relies on a different principle: Predominant charge- (1st dimension) and predominant size-separations (2nd dimension) are achieved by using gels with low and relatively high agarose concentrations under *non-denaturing* conditions. By applying a mathematical approach [9,3] based on the extended Ogston model [6,7], one can distinguish the separation effects due to particle size and charge. The investigated particles are in the size range of viruses (10,000 to 2,000,000 kDa).

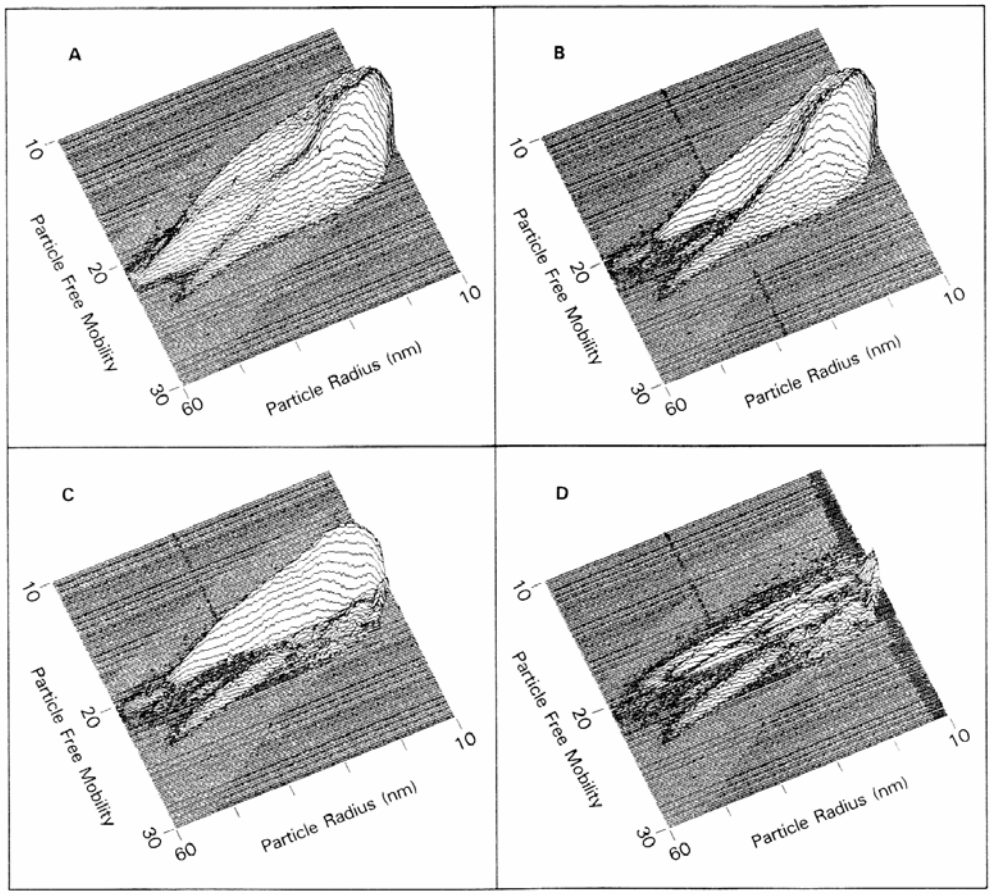

Fig. 6: Progressive stripping of 2-D Gaussian surfaces from the transformed image of vaccine II in Fig. 5. The distribution of signal energy (relative density) as a function of effective particle radius and free mobility is shown. **(A)** 3-D view of the original transformed image. **(B), (C)** and **(D)** show successive stages of stripping surfaces. These results suggest that the analyzed vaccine sample may consist of three major populations. Signal processing by GELFIT [4]. Plots were created by IMAGE (Wayne Rasband, NIH). Adapted from Fig. 7 of [5].

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