

Research Article – Histology and Cell Biology

From Kepler's conjecture and fcc lattice to modelling of crowding in living matter

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Summary

Up to now, sphere packing has been investigated without any reference to living matter. This study focuses on the void space (VS) of sphere packing to mimic the extracellular spaces of living tissues. It was inspired by the importance of the extracellular matrix, the vehicle of micro and macromolecules involved in cell metabolism, intercellular communication and drug delivery. The analysis of sphere packing evidenced that in uniform random packing VS is about 1.9 times greater than in the face centered cubic (fcc) lattice (thus being very close to the 1.9 volume ratio of the cube to the sphere). This datum is a good reference for cell packing in vivo. The disproportionate increase of VS per sphere in loose packing in vitro is analyzed having in mind the variability in volume and composition of the interstitial spaces in vivo and cell trafficking. Arrangements of lymphocytes mimicking a two-dimensional hexagonal pattern and dense packing of disks generated by numerical procedures, are described in 7 μm -thick haematoxylin and eosin-stained histological slices from a human lymph node. In narrow tubes simulating roundish cells arranged in limited compartments of the interstice, sphere packing is characterized by noticeable increases of VS. The VS of this packing in vitro is compatible with variability in volume and composition of the interstitial spaces and with cell trafficking in vivo. This paper stresses that in mammalian tissues and organs cells can be packed quite more densely than spheres in the fcc lattice. As to pathology, attention is focused: (i) on overcrowding of cell organelles in some diseases, (ii) on shrinking or swelling of high amplitude, whose opposite effects are to concentrate or dilute intracellular structures and crowding of macromolecules, and (iii) on neoplastic tissues.

Key words

Sphere packing; fcc lattice; void space; cell packing; extracellular matrix; interstitial space.

Glossary

Fcc lattice is the abbreviation indicating spheres most tightly packed according to the face centered cubic packing.

ECM is the extracellular matrix filling the interstice of tissues.

The term: "void space" (VS) (synonym: interstice) means: (i) the space inside sphere packings, including the space within the packing and the surrounding bound-

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aries; whereas, (ii) the "extracellular space" of a tissue is the space outside the cells, all-inclusive (ECM, fibers, vessels, nerves). However, (iii) VS referred to a cell is used here to indicate the cytoplasm free of organelles (mitochondria, lysosomes, etc.).

The term "wall effect" means a noticeable change, usually an increase, in VS referable to the space between the surface of the packing and the wall of the container.

1. Introduction

How densely objects can fill a volume is an old problem of mathematics, physics and biology. Four centuries ago, Johannes Kepler claimed that spheres such as cannonballs stacked according to the face-centered cubic packing (known as fcc lattice) occupy the available space with a density equal to 0.7405 (Conway and Sloane, 1993; Zellberger, 2003), that is, 74.05% of the total space. Such a configuration is used by grocers as the most efficient one to stack roundish fruits (Steward, 2003; Szpiro, 2003). Arrangements resembling the fcc lattice of nanoparticle catalysts noticeably variable in size have been depicted (Bell, 2003). The size tolerance of these catalysts helped us to envisage spreading of packing properties from nanoparticles to cells, that is, from inanimate to living matter.

This study investigates whether basic properties of an experimental model may adapt to the complexity of living matter. First, attention should be focused on two facts concerning the void space (VS) properties of sphere packing: (i) the VS of the fcc lattice is 25.95% of the total packing volume ($100 - 74.05 = 25.95$). (ii) the concept of "random packing" is made elusive by the many possible combinations. To simplify, here it is assumed that spheres uniformly packed at random occupy 60 % of the total space available (Torquato et al., 2000), taking into account that similar values have been reported also for the volume fraction of disordered sphere packing (Aste et al., 2005). Instead, in the living matter VS is represented by fluids and by intercellular substance. Moreover, it differs from one tissue to another in terms of volume per cent (and its chemical composition as well) and adapts during normal development, in aging and in various pathophysiological conditions. Furthermore, the extra- and intracellular spaces may dilate or shrink, depending on hydration and on several other variables, such as changes of cell shape deformed by external forces (Gladilin et al., 2007), and/or by cell volume and by the number of subcellular organelles and their metabolic activity. Finally, in the living matter spaces available for cell packings tend to be narrow because, in contrast with computational and inanimate models, VS is compartmentalized structurally (in tissues by interlacing bundles, in cells by membranes) and functionally (still ill-defined). Therefore, roundish-shaped cells may form clusters and the interstitial space (VS) available per cell can vary appreciably.

Starting from an evaluation in models of VS available per calibrated sphere packing of different densities, this paper is aimed at focusing on the density of cell packing in terms of VS available per roundish lymphoid cell in the nodes as a paradigm for other situations in living organisms. The analyses based on simulation models with calibrated spheres *in vitro*, combined with image analyses of lymph nodes, might help to better evaluate the effects of cell crowding in tissues, for instance in some conditions pertinent to pathology. Embryos at early developmental stages forming clusters of few cells, are out of the scopes of this paper.

2. Background

2.1. Theoretical and experimental background

Computer algorithms used to generate and study random packing of spheres or particles yield packing volumes in a range between 0.60 and 0.64, depending on the utilized protocol (Torquato et al., 2000). However, experimental simulations based on a configuration allowing the particles to be in contact with one another yielded packing volumes approximately equal to 0.60, a value that we assumed to correspond to uniform random packing. Preliminary simulation experiments carried out in this laboratory using lead shot pellets and 25 ml-packing volumes (see Materials and Methods) yielded an average VS = 0.3957 (mean of 4; range 0.39-0.40) and an average packing volume equal to 0.6043. These preliminary results were not surprising because in a model assuming a cylindrical container to be filled with spheres of diameter d , they will fit it with a basic configuration given by a right prism, with equilateral triangular base of length d , and height d .

The prism volume is given by:

$$V_{PR} = Area_{base} \cdot h = \frac{1}{2} \cdot d \cdot \frac{d\sqrt{3}}{2} \cdot d = \frac{\sqrt{3}}{4} \cdot d^3$$

The spheres fit the prism with only 1/6 of their volume each, thus the void volume is given by:

$$V_{VS} = V_{PR} - 3 \cdot \frac{1}{6} \cdot V_{SPH} = \frac{\sqrt{3}}{4} \cdot d^3 - \frac{1}{2} \cdot \frac{4}{3} \cdot \pi \cdot \left(\frac{d}{2}\right)^3 = \frac{\sqrt{3}}{2} \cdot d^3 \cdot \left(1 - \frac{\pi}{3\sqrt{3}}\right)$$

The resulting packing volume equals to 0.6, independently from d , and VS equals 0.4, a theoretical value closely approached in the above experiments.

2.2. Some problems inherent to living matter

The central point to be first examined theoretically and then to be investigated by simulations with sphere models and estimated with the aid of image analyses at the tissue level, is the space available per cell in those tissues which are mainly composed by roundish cells. Therefore, variations of amplitude of the extracellular space (VS) in one or other tissue deserve preliminary attention. Extracellular spaces (VS) in the living matter are compartmentalized. Therefore, roundish cells (as well as intracellular organelles) may assemble in limited spaces and form clusters. Again, the inverse relationship between the packing volume of a cluster and the extent of its surface (which determines the amount of void space between the surface of the packing and the wall) should be considered and quantified by experimental simulations.

Table 1 – Comparison of VS in rat and human tissues with VS of sphere packings.

| Material | VS (%) | References |
|----------------------------------|---------------|---|
| <i>Mammalian tissues</i> | | |
| Kidney (rat) | 26.9 | } McIver and MacKnight, 1974; Cieslar et al., 1998 |
| Liver (rat) | 16.4-17.9 | |
| Heart (rat) | 14.8 | |
| Skeletal muscle (rat) | 11.0 | |
| Adipose tissue (rat) | ~9 § | Levitt, 2003 |
| Diaphragm (rat): neonate - adult | ~47- ~18 | Gosselin et al., 1973 |
| Lymph node (human) | ~20-30 to >50 | This paper |
| <i>Sphere packing</i> | | |
| fcc lattice | 25.95 | Calculated from Conway and Sloane, 1993 |
| Uniform random packing | ~40 | Szpiro, 2003 (and references therein) |

VS are mean values and are expressed as % of the total space occupied by tissues or by sphere packings.

§ Assumed here to be half the value of total tissue water (Levitt, 2003, footnote to Table 4).

2.3. Background from data on VS in inanimate and living matter

The packing of ellipsoid objects, studied in detail with candies (Donev et al., 2004), is consistent with previous data for yeast cells showing that, due to a slight departure from spherical shape, mature yeast cells reach a packing density of about 0.78 (Arnold, 2000) and a VS value (22%) slightly lower than in the fcc lattice. However, a comparison of VS in mammalian tissues and in sphere packing (Table 1) shows similar values only for the kidney and the fcc lattice. In fact, likely because biological structures are designed so as to meet, but not exceed, the maximal demand (West et al., 1999), VS values are quite lower in liver, heart, muscle and adipose tissues. Data available for diaphragm muscle (Gosselin et al., 1993) are a striking example of age-related changes in VS and are of particular interest here, because they were obtained by image analysis techniques in two dimensions.

3. Materials and Methods

3.1. Simulation Experiments

Materials used for simulations were equilibrated, and experiments were performed, in a room where temperature was maintained at $20 \pm 1^\circ \text{C}$. Calibrated stainless-steel microspheres for ball bearings (diameter: 2.381 mm, weight 55 mg) or, when indicated, lead shot pellets (average diameter 1.653 mm, weight 23.8 mg, range: 27.8-22.1 mg), were poured into tubes or cylinders of neutral glass (internal diameters 22, 21.5, 18, 14, 11, 10 and 9.7 mm, respectively). Denatured alcohol or water was then added up to fill

the VS among the crowded spheres. The volume of trapped fluid was taken as a reliable measure of VS (checked by increased weights of the tubes containing the packing, combined, when required, with calculation). Noticeably, differences in the shape of the bottom of the tubes did not influence the results. Total packing volumes ranging from 22 to 7 ml were used. Reproducibility (differences within 3 per cent), checked by duplicate experiments carried out in parallel, was good enough for present purposes. Due to differences in surface tension between alcohol and water, in particular for small packing in narrow tubes, care was taken in standardizing the liquid surface.

3.2. Image analyses.

The images were taken both from internet (as specified in section 4.3) and from histological sections. Images of 7 μm -thick haematoxylin and eosin stained sections of paraffin-embedded human lymph nodes were taken with a Zeiss microscope equipped with a telecamera Ikegami (Tokyo, Japan), using an objective of 40 X. VS is evaluated as % of total cross-sectional areas by computing optically-free areas with the aid of the program Image-Pro Plus, version 4.5.1 (Media Cybernetics, Bethesda, Maryland, USA).

Image analyses of lymph nodes were first performed by measuring the optically void areas (extracellular space) and solid spaces (occupied by fibers and cells) as they appeared in coloured figures found in internet (Yahoo, "Lymph node structure", item 1, figure enlarged from the inset: Medulla T-lymphocytes). These measurements were done in two ways: (i) by hand, by cutting the areas in the figure with scissors and weighing the pooled fragments with an electronic balance; (ii) by computing the areas with the aid of the program Image-Pro Plus. Results obtained by the two methods by two independent operators were similar (difference of 3.7% for the void space, and less than 2% for the solid space). The computing method was used routinely.

4. Results

4.1. VS in sphere packing

Figure 1 shows that in the same space where 1,000 equal spheres are stacked according to the fcc lattice (VS = 25.95%) only 810 spheres fit according to the uniform random (and disordered) packing (VS assumed to be 40%). Therefore, in random packing VS is $\sim 55\%$ greater than in fcc lattice. Remarkably, the ratio of VS to sphere number turns out to be nearly 91% greater than in the fcc lattice. The approximated value of 1.9 shown in the figure depends on the value of 60% assigned to the space occupied by the random close packing (see Introduction, and Torquato et al., 2000) and to the corresponding 40% value of VS. Amazingly, the volumetric ratio of a cube to a sphere (for side = diameter) is 1.91 (density of sphere packing = 60.31 and VS = 39,685). The inverse relationship between sphere density and VS per sphere is depicted in Table 2.

As better shown in Figure 2, sphere density changes linearly whereas VS per sphere changes exponentially. Thus, the VS of sphere packing should not be disregarded when reasoning on the topic of VS available per roundish cells loosely assembled *in vivo*.

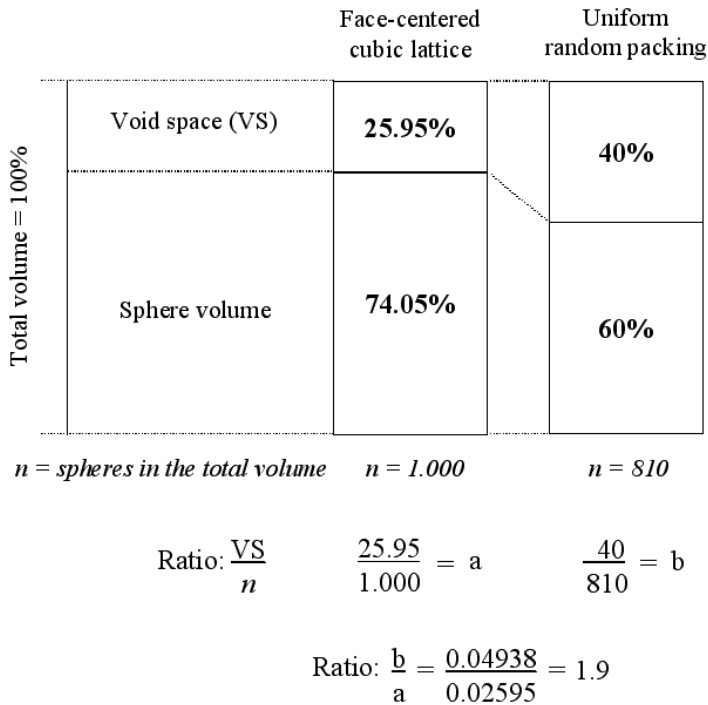


Figure 1 – Differences between the fcc lattice and random packing. Taking 1,000 spheres (*n*) + void space (VS) = 100% (total volume), the random packing compared with the fcc lattice is characterized by 19% lower sphere number (*n*), 54.14% larger VS and 90% greater VS/*n* ratio.

Table 2 – Changes of sphere density and VS per sphere in the space which accommodates 1,000 spheres packed according to the fcc lattice. Two possible conditions of loose packing are indicated.

| Conditions | Sphere number (<i>n</i>) | Sphere density (%) | VS (%) |
|---|-------------------------------|-----------------------|-----------|
| Face-centered cubic packing (fcc lattice) | 1,000 | 74.05 | 25.95 |
| Uniform random packing; | 810 | 60 | 40 |
| Disordered, loose packing | 675 | 50 | 50 |
| Disordered, loose packing | 540 | 40 | 60 |

4.2. Experimental simulation of VS and the wall effect

Figure 3 shows the relation between VS and the tube diameters used in experimental simulations. VS values around 41% were obtained with tube-shaped containers (test tubes or cylinders) ranging between 22 and 14 mm in diameter and filled

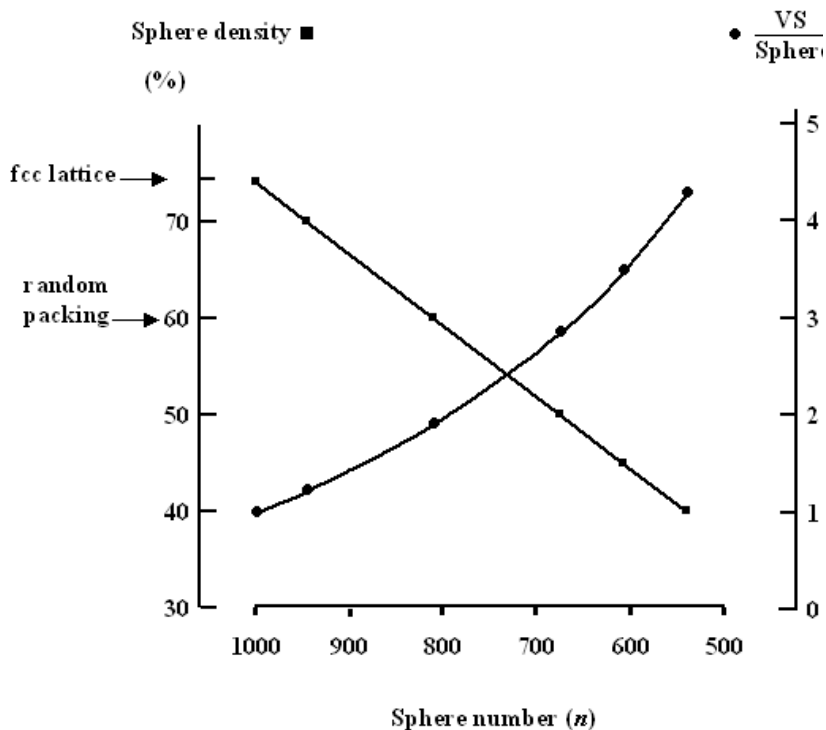


Figure 2 – Inverse relationship between sphere packing density and VS per sphere. The maximum possible density (74.05%) corresponds to 1,000 spheres packed according to the fcc lattice. The values of the ratio VS/sphere have been normalized taking as unit the value corresponding to 1,000 spheres.

with calibrated microspheres for ball bearings. These values are in good accordance with those of preliminary experiments carried out with lead shot pellets (See 2.1). Narrowing the tubes down to 9.7 mm diameter (four times greater than the sphere diameter) produced a noticeable increase of VS (+~10%). It seems therefore that, when round cells are densely packed in limited spaces of interstice, changes of this order of magnitude should affect VS *in vivo*.

4.3. Histology and image analyses

Giving for granted that packing of identical spheres in two or three dimensions is essentially a problem of mathematics (Wouterse et al., 2005), in living tissues cells are packed in three dimensions. However, their microscopic images in thin histological sections are substantially in two dimensions (Figure 4a and 4b). It is worth noting that arrangements of seven or eight lymphocytes in clusters (red-coloured cell packings in Figure 4b) are consistent with “dense packings of disks and spheres inside various geometrical shapes” generated by numerical procedures (Boll et al., 2000, and references therein). The VS in the images examined ranged between 20 and 40 per cent of

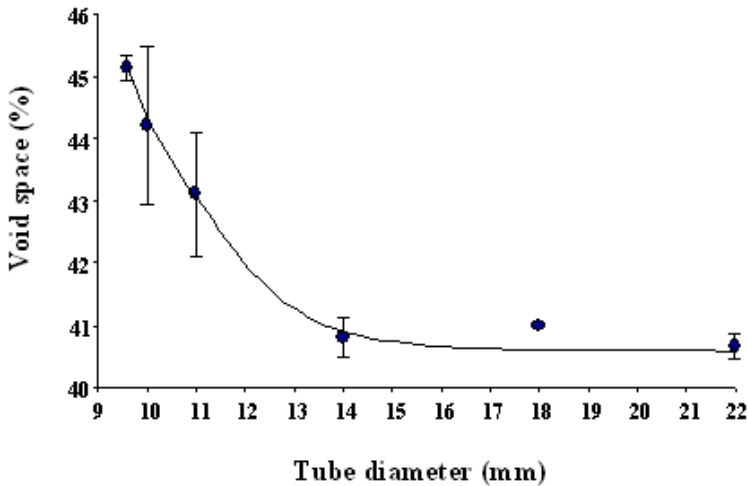


Figure 3 – Relationship of the void volume (VS) of sphere packings with the diameter of the cylindrical containers. The points in the figure are, from left to right, means of 4, 8, 6, 4, 1 and 3 determinations with calibrated spheres. SD is given by the bars. The value of the last tube on the right (diameter 22 mm) is the average of one point with calibrated spheres and two points with lead-shot pellets.

the microscopic fields analyzed. VS values below $\sim 26\%$ (the VS of the fcc lattice, three dimensions) found in some areas of the microscopic images (two dimensions) have been interpreted as due to cell overlapping and cells deformation in the images analyzed.

Further results can be summarized as follows. (a) Local differences of lymphocyte densities may have more than a single explanation. The content of fibers and the considerable amounts of space attributable to macrophages should be taken into account in any case. The space occupied by silver-stained fibrils in different fields of slices ranged from 3.23 to 11.98 % (average 6.76 %). It was not investigated further. (b) Cell density varied considerably in different fields of the same slice (analysed by hand as specified in the Methods). (c) Average diameter of lymphocytes worked out from electron microscope images taken from three different books was near $6 \mu\text{m}$ (mean: 5.9; range: 5.5-6.2), that is, very close to values found in the literature (Pollack et al., 1973). Anyhow, in EM images the lymphocyte diameter is quite similar to the thickness of the histologic samples analyzed in this study. The surfaces (μm^2) of the lymphocytes and their nuclei (calculated by the formula: πr^2) were as follows: lymphocytes 27.4 (range: 23.6-29.8); nuclei 20.1 (range: 18.8-20.7). Allowance should be made for possibly noticeable differences from true values of nuclear and cellular diameters due to the sources of EM images and to laboratory techniques. Anyhow, the arrangements (observed by optical microscopy in $7 \mu\text{m}$ -thick tissue slices) of seven or eight lymphocytes mimicking a two-dimensional hexagonal pattern (Figure 4b) are consistent with "dense packings of disks and spheres inside various geometrical shapes" generated by numerical procedures (Boll et al., 2000, and references therein). Images of lymphocyte clusters like those in Figure 4b are not rare in areas of high cell density. They recall a two-dimensional hexagonal lattice in which a disk is surrounded

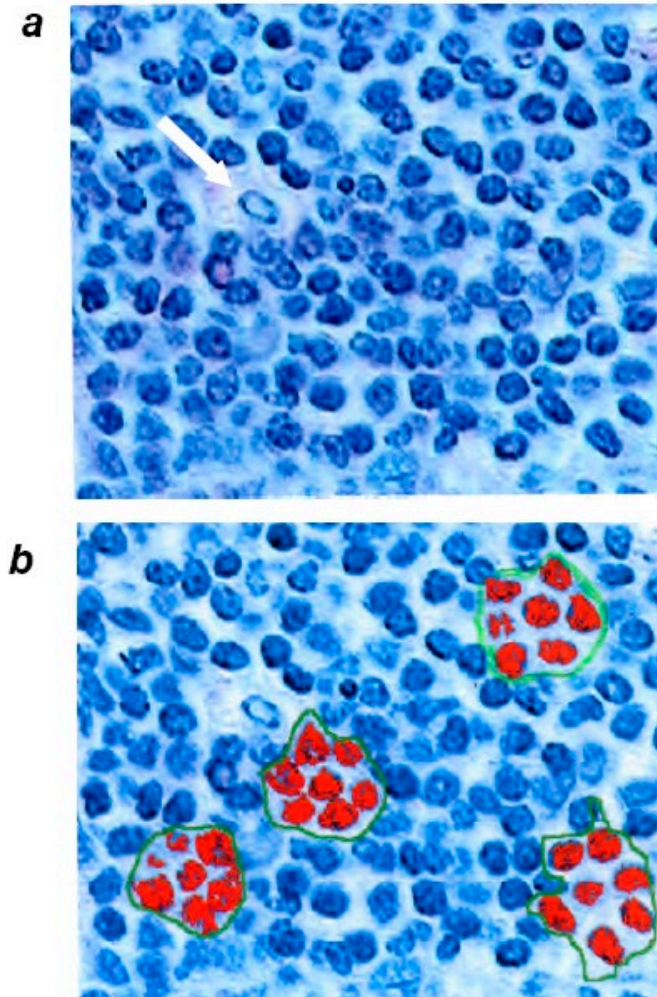


Figure 4 – Lymph node (human). In *a*, the arrow indicates a macrophage. Lymphocytes densely packed are frequently arranged so as to mimic a two-dimensional hexagonal pattern. (*b*) Same microscopic area as *a*, with groups of densely packed lymphocytes evidenced by colour.

by six others (Figure 1.3 in: Conway and Sloane, 1993); likely, they are representative of the *in vivo* dynamic cellular network. Remarkably, relatively dense aggregates are also formed in dense disordered packing of hard spheres (Anikeenko and Medvedev, 2007). Cell clusters were not apparent in a few histological samples of round cell sarcomas. This might be due to altered properties of tumor cell membranes and/or to disproportions between cell size and thickness of tissue slices.

5. Discussion

As stressed in the Introduction, the idea that the basic properties of inanimate packing can spread over living matter was suggested by the considerable size tolerance at the level of single nanoparticle catalysts assembled in structures resembling the fcc lattice.

The starting point of this study was the image of stacked cannonballs (Szpiro, 2003) reminding histological pictures of round-shaped cells closely packed though individually distinct (Ritchie, 1970). The results of this paper are compatible with the straightforward interpretation that, in keeping with computer-based simulations or results with inanimate models, in living tissues lymphocytes and, possibly, other kinds of roundish cells, mimick disk packing (two dimensions) as well as sphere packing (three dimensions).

In the living matter the interstice is fundamental at any level of organization, either normal or pathological (Guiot et al., 2007, and references therein). In this paper, the width of the interstitial space (i.e., VS) of living matter in mammalian tissues has been considered theoretically and tested experimentally by simulations with inanimate models. Likely, the key features of the packing of calibrated spheres depicted in Figures 1 and 2 and developed in Figure 3 also involve living matter. Furthermore, arrangements of seven to eight lymphocytes in dense cell packing (Figure 4b) are quite consistent with "dense packing of disks and spheres inside various geometrical shapes" generated by numerical procedures (Boll et al., 2000, and references therein). Likely, loose packing of round cells in living tissues, either at random and/or disordered, may greatly facilitate physiological changes concerning cell volume and shape, contacts with other cells and, in general, cell trafficking owing to the favorable ratio of the interstitial space (VS) to total cell volume depicted in Table 2 and Figure 2. In addition, loose-fitting packings facilitate changes in microcirculation and hydration of the extracellular matrix. These concepts best fit in with some cell populations of mesenchymal origin, first of all lymphocytes whose specific actions are based on homeostatic trafficking between lymphoid organs and tissues via the blood and lymph. Scanning electron microscopy combined with immunological methods shows that lymphocytes in spleen and nodes, although crowded, are well separated (Farber et al., 1978; Kunkel, 2001) and are free to contact each other and other kinds of cells (Dustin et al., 2001; Friedl and Gunzer, 2001). The model of lymph node shown here deserves to be compared with haematopoietic and connective tissue tumours, whose cells "lie singly, each separated from the next by the intercellular substance" as well as by the stromal vasculature (Ritchie, 1970). However, large interstitial spaces are not the rule. Indeed, as Table 1 shows, many tissues and organs of different embryological origin whose organ-specific cells are elongated or polyhedral, display much smaller VS values, in keeping with the idea that supracellular structures evolved and assembled together so as to maximize metabolic power and to minimize internal transport distances (Levin, 2003). Remarkably, increases in tissue packing density from the developmental age up to adulthood, perhaps best shown in the rat diaphragm (Gosselin et al., 1993), are macroscopic examples of the inherent variability of biological systems in which the turnover of living matter plays a role. Anyhow, a decreased VS % in a given tissue might be pathological. It is perhaps not merely coincidental that increased cell packing density has been described in specific

cerebral cortical areas in some neurological diseases, most importantly schizophrenia (Selemon et al., 1998). Tumour cells have a number of mitochondria lower than normal (Aisenberg, 1961; Melli, 1962; Hoshino, 1963), therefore they offer clear-cut examples of increased VS in the cytoplasm (Melli, 1962) although exceptionally the opposite has been described (see below). As to the cytoplasmic compartment of cells, in humans a marked increase of packing density of mitochondria referred to a pathological multiplication of these organelles is characteristically observed in some tumours of the salivary glands, the so called "mitochondriomas" (Majno and Yoris, 1996). An anomalous replication of tumour mitochondria is worthy of attention. Also cellular swelling of high amplitude deserves further attention, because it hinders the extracellular space and dilutes intracellular structures. In this way, both macromolecular crowding and intracellular ionic equilibrium are modified, thus interfering with biochemical reactions (Del Monte, 2005, and references therein) possibly involved in the molecular structure and function of nanosystems (Nussinov and Aleman, 2006; Lu et al., 2007) typically inherent to system biology and to scaling relationships inherent to biological complexity (Brown and West, 2000; Huang and Wikswo, 2006).

6. Conclusions

The interconnection between morphometric data and integrative pathophysiology deserves to be further discussed and explored. For instance, the thesis that a clinically palpable tumour (about 1 gram of tumour tissue) contains 10^9 cells has been widely accepted since 1975. It can be found in valuable textbooks of general pathology and molecular cell biology and even in still recent journal articles referring to clinically detectable tumours (see, for instance: Majno and Yoris, 1996; Alberts et al., 2008; Tuckwell et al., 2008). Then, in 2009 the consistency of the "magic" number 10^9 has been questioned and discussed in connection with the general issue of the metastatic efficiency of primary tumors (Del Monte, 2009), moreover the underlying implications have been pinpointed by prestigious reviews on metastases (Talmadge and Fidler, 2010; Lydon and O'Malley, 2011). The present study on crowding in living tissues can be considered a "holistic approach in cellular and cancer biology" (Waliszewski et al., 1998).

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