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WHAT NEW OPPORTUNITIES THE USE OF DIVERSE POLYMERIC CRYOGELS OPENS FOR THE IMMOBILIZATION OF MOLECULES AND CELLS

It is well known that diverse polymers and polymeric materials are widely used in modern Biotechnology. These are: plastic vessels, flasks and accessories, various chromatographic matrices, different gels for electrophoretic procedures, gels for immune tests, gel bases of solid nutritional media, numerous soluble polymers, carriers of immobilised molecules and cells, etc. In general, one can say that without polymer materials both functioning and progress of Biotechnology is not possible principally.

Present brief overview considers some specific kind of polymer materials, which, so far, are not widely known for biotechnologists. These materials are related to the polymeric gels. A variety of problems of Applied Biochemistry and Biotechnology to be solved using polymer gels, as well as a broad range of biological objects encountered, put new, often contradictory, requirements for the gels. However these new requirements stimulate the development and the commercialisation of new gel materials for biological applications. This is also valid for such direction of Biotechnology, as preparation of biocatalysts based on immobilized molecules or cells. As similar immobilized systems consist of two major components, namely, biological catalyst and its carrier, the properties of these both are of great significance upon the development of high efficient immobilized systems. That is why, the participation of experts in Materials Science in respective R&D is preferable rather than the efforts of the biotechnologists themselves to solve all the tasks to be met.

Such spatially structured polymer systems as gels (or lyogels in terms of colloid chemistry), can be produced either through the limited swelling of non–linked polymers, or by swelling of xerogels, or under the gel–formation of monomer or high–polymer precursors in the medium of a solvent. By this means one may prepare the chemically linked covalent–type gels, ionotropic gels, as well as the non–covalent or physical gels. Lyogels of the more sophisticated morphology are usually formed originating from the colloid sols. One of the new types of polymer gels with considerable potential in Biotechnology, including preparation of polymeric carriers of immobilized molecules and cells, is so–called *cryogels* (from the Greek krios (kryos) meaning frost or ice) [1]. Cryogels are formed as a result of cryogenic treatment (freezing, storage in the frozen state for a definite time and defrosting) of low– or high–molecular–weight precursors, as well as colloid systems, all capable of gelling. The cryogels as immobilization matrices are considered below.

GENERAL FEATURES OF THE PROCESSES RESULTING IN THE FORMATION OF CRYOGELS

Our studies demonstrated that all the above listed types of gel–formation could be realised in the cryogenic variants, carrying out the respective gel–formation process at appropriate regimes of freezing and thawing. Therefore, the cryogels can also be defined as the gels formed under the conditions of frost–bound specimens.

Moderate freezing of initial solution of monomeric or polymeric precursors of a future gel results in the macroscopic solidification of the sample. However, on a microscopic level the system is not totally solid. It contains unfrozen areas, so–called unfrozen liquid microphase, where solutes are concentrated. Such freeze–induced concentrating phenomenon promotes the gel–formation within the volume of unfrozen regions. After defrosting, specific macroporous gels (that is, cryogels) are obtained, where the pores are filled with the thawed liquid. Hence, the polycrystals of frozen solvent play a role of pore–forming agents. Since during crystallization each crystal, the ice in case of aqueous systems, grows until the contact with a facet of another crystal, the macropores in cryogels are interconnected. Just such character of morphology of various cryogels makes them attractive materials for application in many fields, in Biotechnology, in particular [1–4].

Depending on the properties of initial system and conditions of cryogenic treatment one can obtain two types of cryogels:

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Paper presented as a lecture.

(i) Macroporous cryogels with the pores of 0.1–10 micrometers in size.

(ii) Supermacroporous (sponge–like) cryogels with the pores of tens and even hundreds micrometers.

So gross size of pores in these materials is large enough for the unhindered diffusion of solutes of any molecular weight, therefore a true choice of appropriate cryogel matrix as a carrier for the biocatalyst immobilization ensures the absence of additional barriers for mass exchange. Typical representative of the first sort of cryogels is macroporous poly(vinyl alcohol) cryogel (cryoPVAG), and of the second kind – supermacroporous poly(acryl amide) cryogel (cryoPAAG). Both types of the materials were examined as immobilization matrices.

Immobilisation of Molecules and Cells on/in Macroporous Cryogels

Cryogels based on poly(vinyl alcohol) are formed as a result of freeze–thaw treatment of concentrated aqueous solutions of the polymer [1,5,6]. The studies of physicochemical properties and structure of cryoPVAGs showed that their porosity mainly depends on the initial polymer concentration, freezing temperature and, especially, thawing rate. Recently, it was found that additives of various surfactants can exert considerable effect on the porosity characteristics of PVA cryogels [7]. For instance, the addition of sodium dodecyl sulphate in an amount of about 6 millimoles per litre gives rise to increase in the cross–section of pores from about 1 micrometer to 5 and more micrometers. Hence, using different soluble additives we are able to modify the morphology of pores in such macroporous matrices as PVA cryogels.

In spite of high porosity, cryogels of poly(vinyl alcohol) possess very good mechanical properties and low sensitive to the abrasive erosion even in the reactors with very intense stirring. Therefore, these gel materials are well suitable for the use as immobilisation carriers, for the immobilisation of enzymes, in particular.

Principally, there are two ways for the preparation of such immobilised biocatalysts:

(i) Covalent attachment of an enzyme after grafting of appropriate reactive groups to the matrix.

(ii) Physical entrapment of insoluble forms of enzymes, for instance their cross–linked preparations, as fillers in the macroporous matrix of PVA cryogel. In this latter case, mechanical strength of the enzyme–containing matter is not significant, since overall mechanical characteristics of such composites are determined by the cryogel support. This second approach allows reaching considerable increase in loading capacity in respect of an enzyme. The approach is under patenting now [8].

Covalent attachment of enzymes to cryoPVAGs requires preliminary grafting of reactive groups to the OH–containing matrix, and virtually all known methods commonly used for "activation" of cellulose–, agarose, dextran–based matrices are feasible in case of PVA cryogels. Then the enzyme of interest is coupled to the modified macroporous carrier.

Thus, α–chymotrypsin or pancreatic lipase covalently immobilised in PVA cryogel beads were used for the enatiospecific hydrolysis of certain phenylalanin derivative in low water media [9,10]. It was found a very high increase in stability of this enzyme in polar organic solvents like MeCN. Immobilised biocatalysts exhibited good activity for a long time and produced the final product with high yield and optical purity. In contrast, the same enzyme immobilised in Sephadex or controlled–pore glass showed poor results in respect both chemical yield and optical purity of final amino acid.

CryoPVAG–immobilised subtilisin, trypsin and thermolysin were used for the enzymatic synthesis of organosoluble peptides [11–13]. It was observed that free subtilisin completely deactivates in the MeCN media with high content of dimethyl formamide, whereas the enzyme immobilised in cryogel carrier was stable and active for a long time. More than ten p–nitroanilides of three and tetra peptides were synthesised using the only portion of immobilised biocatalyst. It was possible to use certain carboxy– and amino–components without protection of reactive pendant groups, thus demonstrating a very high specificity of such biocatalyst in the reactions of formation of α–peptide bonds. In fact, these data show promising potential of PVA–cryogel–immobilised enzymes for the biocatalysis in non–aqueous media, when the polymeric framework of the carrier supplies the enzyme with such amount of water, which is required for the biocatalysis.

Yet another prospective field of the use of macroporous PVA cryogels is the development of immunoaffinity sorbents for the isolation and purification of such gross sorbates as viruses [14,15]. In a standard way of the process immobilised specific antibodies interact with respective antigen, and non–bound antigens are washed out. Then, the target antigen is liberated upon splitting the complex "antigen–antibody". If we work with small virus particles the capacity of cryogel–based immunosorbent is comparable with the capacity of Sepharose–based sorbent. But with increase in the virus size the merits of macroporous system become more and more pronounced. In the case of large viruses the capacity of cryoPVAG–based sorbent already 8 times higher, because even large viruses can penetrate in to the macroporous cryogel, and in case of Sepharose sorbent only surface of the particles is involved in the process.

At last, PVA cryogels turned out to be the excellent carriers for the entrapment immobilization of whole cells [2,16]. The scheme of such an immobilisation is as follows: cell biomass is mixed with PVA solution, the

obtained suspension is subjected to freeze–thaw treatment, and, after washing, the immobilised microbial biocatalyst is ready for use. The cells entrapped in such macroporous gel turn out in the microenvironment, where no additional diffusion barriers exist for enter of substrates to the cells and rejection of metabolites from the cells.

This carrier was tested for the immobilisation of microbial cells of different systematic groups, the cells capable of exhibiting diverse functionality.

For instance, *Citrobabacter intermedius* bacteria contain active tyrosine phenol lyase, and PVA–cryogel–entrapped cells were able to biotransformation of the mixture of pyruvate, fluoro–phenol and ammonia to fluoro–tyrosine, at least, for two months [17]. Thermophilic yeast *Kleveromyces marxianus* produced higher concentrations of low–molecular–weight metabolite ethanol, when the cells were entrapped in PVA cryogel, than the cells immobilised in frequently used Ca–alginate gel [18]. Micelial fungus *Aspegillus clavatus* after immobilization in cryogel matrix was able to the prolonged biosynthesis of high–molecular weight metabolite, enzyme ribonuclease, whereas free cells could be used only in a single run [19]. Proline–oxidating *Pseudomonas cells* in the immobilised state were used in the flow–through biosensor for L–proline, whereas free cells could be used only one time and only in the batch potentiometric measuring cell [20].

In order to obtain diverse immobilised biocatalysts in a form of beads, we elaborated special cryogranulating set–up [21]. This device installed in the author's Lab allowing the fabrication of cryogel beads of different size.

In the one of the latest developments the immobilised biocatalyst for the production of lactic acid has been elaborated [22]. Nowadays, the manufacture of this substance permanently increases, especially for the preparation of biodegradable plastics. The results obtained on productivity and final concentration of lactic acid are higher than the same parameters known for any other immobilised systems, thus demonstrating high efficiency of cryoPVAGs as carriers of immobilized microorganisms.

Immobilisation of Molecules and Cells on/in Supermacroporous Cryogels

Among the supermacroporous cryogels possessing spongy morphology poly(acrylamide) cryogels are the most studied [1,23–25]. They are formed as a result of copolymerisation of acrylamide and bis–acrylamide in moderately frozen aqueous medium. The porosity characteristics of these cryogels mainly depend on the freezing conditions. The deeper the cooling, the smaller the ice crystals formed and, hence, the smaller the size of macropores in similar sponge–like gels. The studies performed have recognized the major factors influencing the properties, structure and kinetics of cryotropic gel–formation at different negative temperatures. Just these data were the fundamental basis for the development of supermacroporous gel materials of biotechnological interest, for instance, development of immobilization matrices, special sorbents and continuous chromatographic beds.

Thus, if to prepare such polyacrylamide cryogel as a cylindrical block directly in a chromatographic column, the water can freely pass through the super-macroporous cryogel without necessity to produce particles of the gel [24]. The flow rate characteristics of the columns are determined by the gel concentration, ratio of acrylamide to bis–acrylamide in the initial feed and by the freezing temperature. Such hydrodynamic properties of spongy cryogels make them promising candidates for the development of sorbents and chromatographic matrices for the work with biological nano and micro–particles, for instance, organelles or even whole cells.

The latter idea was recently realised for the case of immobilized metal–chelate chromatography of *Escherichia coli* cells [26], whose surface could, as supposed, contain proteins with available histidine residues. Upon passing the cell suspension through the plain column without metal chelate groups all the cells were detected in an effluent. That is, no cell retardation took place, which, in principle, could be caused by physical clogging or by the non–specific sorption. Then the iminodiacetate groups were introduced in to the polyacrylamide cryogel and loaded with copper ions. The metal chelate column has bound the most part of a population, and only small amount of cells has not been bound. Apparently, these non–bound cells did not have available chelating groups. Thereafter, the temporarily immobilised cells were desorbed specifically by the action of EDTA or imidazole. Eluted cells completely retain their viability. Interestingly that the less strong chelating agent, imidazole, gave rise to some additional separation of bound cell population.

Similar supermacroporous chromatographic material, but with other affinity ligand, protein A, was used in the development of systems for separation of animal cells, in this case B and T lymphocytes [27]. It was found that breakthrough liquid contained practically only T–cells. Subsequent elution of adsorbed cells with specific immunoglobulin gave of about 50 per cent recovery of B–cells.

The studies in the field of cell temporary immobilisation and chromatography on supermacro-porous continuous cryogels are only at the early stages, so one may hope that further development will lead to even greater impressive results.

Permanent cell immobilization using supermacro-porous cryogels, analogously to the immobilization of

biopolymers, could be accomplished either via attachment (covalent or adsorption), or via entrapment techniques. The example of the former case is the chemical coupling of *E. coli* cells with aldehyde derivative of cryoPAAG [28]. For cell entrapment in pore walls of spongy cryogels, a biomass (microbes, spores, organelles) is suspended in a solution of gel precursors (monomers for polymerisation gelation [28–31] or reactive polymer and cross–linking agent [32,33]), and the mixture is cryogenically treated. This gives rise to the supermacroporous matrix containing immobilized cells entrapped in the cross–linked gel phase of pore walls. It is noteworthy that such biomass–containing material could be fabricated not only from organic, but also from inorganic polymer, for example, cryo–silica gel (silica gel formed under freeze–thaw conditions) [34].

Supermacroporous spongy cryogels can be obtained as thermoreversible physical ones, as well. Thus, the procedure for the preparation of agarose sponge–like cryogels has been developed [35], and these materials turned out to be efficient scaffolds for the use in cultivation of animal cells, since supermacroporous structure of the material allows exploring whole volume of the scaffold rather than the surface of a support. Introduction of specific ligands improves the cell adhesion. It was shown that insulinoma cells were adhered, propagated, developed and retain normal level of insulin secretion [36].

In other words, these data demonstrate yet another prospective field of biotechnological implementation of polymeric cryogels, namely, creation of efficient cell culture scaffolds with open–pore morphology capable of ensuring high square for the adhesion of anchorage–dependent animal cells.

CONCLUSIONS

1. Cryogels – are promising polymeric materials of biotechnological interest.

2. There are certain possibilities for variation within wide limits of size and architecture of pores (their "smart morphology") in polymeric cryogels.

3. Cryogels can be efficiently used as high–porous carriers of immobilized molecules and cells; cryogels can functioning as chromatographic matrices employed for operation with biological nano and micro particles; cryogels can also be used as scaffolds for the cultivation of diverse cells, etc.

4. All the progress achieved in the development of cryogel–based materials for biotechnology was hardly possible without preliminary long–term basic research of the regularities of cryotropic gel–formation and the properties of respective cryogels.

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