2D Engineering of Protein-Based Nanoparticles for cell guidance

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ABSTRACT

Cells responses, like positioning, morphological changes, proliferation and apoptosis, are the result of complex chemical, topographical and biological stimuli. Here we show the macroscopic responses of cells when nanoscale profiles made with Inclusion Bodies (IBs) are used for the 2D engineering of biological interfaces at the microscale. Novel and deep statistical data obtained with fibroblasts cultivated over supports decorated with GFP-derived IBs using patterns with various shapes and sizes demonstrate that these cells preferentially adhere to the IBs areas and align and elongate according to the IBs pattern proving the success of this novel protein-based nanomaterial in the field of tissue engineering

Keywords: Protein nanoparticles, cell guidance, fibroblasts, microcontact printing, tissue engineering, inclusion bodies.

1 INTRODUCTION

Bacterial inclusion bodies are insoluble protein clusters commonly observed in recombinant bacteria. Since most of the heterologous proteins produced in Escherichia coli tend to aggregate, at more or less extent, as inclusion bodies, the formation of these particles has been observed as a bottleneck for protein production, at both laboratory and industrial scales. Interestingly, the mechanical stability and regularity in size of inclusion bodies combined with the biological activity of their forming polypeptides, has recently prompted researchers to consider these protein nanoparticles as biocompatible functional materials, with unexpected and promising applications in tissue engineering.

In this study we characterize the relevant nanometer-scale properties of IBs and explore the extent to which they can be tailored by simple approaches. Moreover, we will see how IBs-grafted patterned surfaces using the microcontact printing technique not only dramatically stimulate mammalian cell proliferation exclusively on the IBs patterned areas but also stimulate cell guidance playing with the nanopatterned features (dots, stripes of different sizes) proving the potential of IBs nanoparticles in tissue engineering and regenerative medicine among other promising biomedical applications.

2 INCLUSION BODIES AS NANOPARTICULATE BIOMATERIALS

IBs are harvested from bacterial cultures by harsh mechanical procedures to disrupt the cell wall. On purification, IBs are observed as pseudospherical particles (Figure 1) with limited size dispersion, ranging from 50 to 500 nm in diameter. IB average size depends on the particular protein species, genetic background of the cell and harvest time [1-2]. The biological origin, mechanical stability and regulatable size of IBs, together with the increasing demand for fully biocompatible and tunable nanostructured materials, raises the question of to what extent these protein particles can function as particulate biomaterials for biomedical applications. In recent years, numerous studies have supported functionalization and nanostructuring of surfaces in tissue engineering and regenerative medicine. Among the available approaches, the generation of nanostructured and nanopatterned surfaces with either inorganic or organic materials is especially appealing. These coatings not only improve cell adhesion and proliferation, but also influence more complex cellular processes such as cell differentiation and motility [3]. However, the need for complicated coating techniques, low versatility, limited physicochemical characteristics and cytotoxicity have delayed further progress in this area.

2.1 Nanoscale properties of IBs

The biophysical features of these proteinaceus nanoparticles, such as activity and size, have been never engineered and very few was known about their physicochemical properties (wettability, stiffness,…). In this study, we have characterized the relevant nanoscale

properties of IBs as particulate materials using SEM, DLS, AFM and confocal microscopy. Moreover, we have also explored how the produced nanoparticles can be tailored (stiffness, size, shape, etc…) by simple approaches (see Fig. 1).

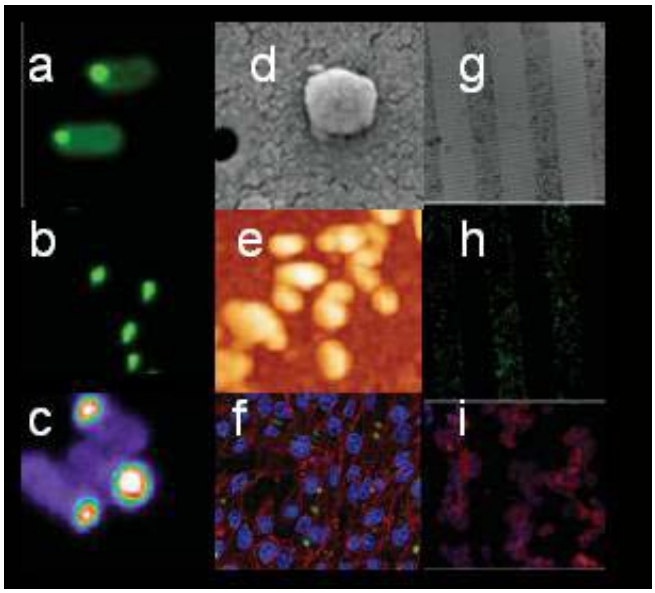

Figure 1. IBs formed by the green fluorescence protein (GFP) when produced in bacteria (a), after purification (b), and under confocal analysis for fluorescence mapping (c). In (d) and (e), purified inclusion bodies observed by SEM and AFM respectively. In (f), BHK21 cells growing on polystyrene plates decorated with GFP IBs. In (g), a silica surface patterned with pure GFP IBs, that still being fluorescent (h), drive the growth of BHK 21 cells under the same lineal pattern (i).

Acting as inert materials, their geometry, stiffness, Z-potential, wettability, size and morphology can be tuned by selective production using defined E. coli genetic backgrounds. In particular, deficiencies in chaperones, DnaK and ClpA, or in the protease ClpP lead to anomalous quality control and protein deposition patterns, which significantly affect the nanoscale properties of IBs produced in these mutants [1,4]. Interestingly, the impact of extracellular matrix hydrophobicity on stem cell adhesion, spread and differentiation was evaluated in a recent study by screening substrate variants with different properties [6].

To determine how the different genetics of IB bioproduction affects their elasticity, force spectroscopic atomic force microscopy (AFM) was used to measure the Young's modulus of IBs in a statistical way. While wild type IBs showed a monomodal elasticity distribution, IBs produced in specific phenotypes (DnaK- and ClpA-) split into two elasticity populations, in agreement with wettability data. The same technique equipped with a closed loop tracking system allowed to obtain spatial distribution of the elasticity regimes over the different IBs and to develop stiffness maps, where each force versus indentation measurement was mapped over the target IB. The Young´s modulus was homogeneously spread over wild type IBs, with mean values of 3.6 ± 0.56 MPa. On the contrary, for DnaK- and ClpA- IBs two elasticity populations were observed with the harder areas segregated from the softer ones. The above data indicated that, the genetics of IB fabrication determines the coexistence of more rigid structures and softer ones, and that they seem to be localised on determined areas of individual IB particles. This fact confirmed the structurally diversity inside the protein aggregates as discussed above.

The contact angle identified as optimal for surface colonization (57º) is within the range of angles exhibited by IBs and perfectly matches that of IB variants produced in DnaK– and ClpA– cells [4], which further supports the use of IBs as convenient substrate materials to favor mammalian cell colonization. [7]

In bottom-up approaches to topographic engineering, IBs formed by irrelevant proteins successfully stimulate surface colonization by mammalian cells without any sign of cytotoxicity (Figure 1f) [4]. Because they are bioadhesive, IBs enhance cell retention on the decorated substrate. In addition, IBs activate filopodia-mediated cell sensing and suitable mechanotransduction circuits that stimulate proliferation via activation of the ERK pathway [5].

2.2 2D engineering of IBs for cell guidance

Cell responses like positioning, morphological changes or proliferation are the result of complex chemical, topographical and biological stimuli. In this context, cells respond positively to 2D engineering of biological interfaces at the microscale using IBs. Data obtained with fibroblasts cultivated on IB-patterned supports demonstrate that these cells preferentially adhere to the IB-covered areas and align and elongate according to IB patterns [11].

We have successfully engineered at the microscale supports by decorating them with a novel protein-based nanomaterial, based on bacterial IBs, using a modification of the μ-CP technique, that allows increasing the mass transfer of IBs from the stamps to the substrates. After a first optimization of the IB density used for the patterning, we have cultured fibroblast cells over these supports and established a protocol to investigate IB-based regulation of cellular functions. One of the contributions of this study relies on the design of a methodology for the statistically analysis of the images of cells cultured at different times over patterns of IBs, with various geometries and densities, to study the behaviour of cells. This method of analysis represents a new useful and generic tool for other researchers in the field. Specifically, in the present work, these analysis enabled to study the influence of different microscale structuring of IBs on the orientation, morphology and positioning of cells, demonstrating the

importance of 2D microscale engineering and the usefulness of the IBs nanoscale profiling for cell guidance. Thus, we have used the microcontact printing (μCP) technique to decorate silicon supports with 2D patterns of the IBs. This technique is recognized as a cheap, fast and versatile tool to control the surface chemistry and address surface properties at the micro-level scale. However, unlike other research on this topic we have not changed the local wettability of the supports, letting cells adhere freely all over the surface. Six different pattern geometries to print the IBs were selected: stripes of 5, 20 and 50 μm width (and the same distance between stripes), dots of 20 μm diameter (spaced correspondingly) and two control surfaces with randomly distributed IBs and without IB´s. We cultured 1BR3.G human skin fibroblasts during 24, 48 and 72 hours.

Fluorescence images of dyed cells (membrane and nuclei) were used as a tool to study the cell´s position, orientation and morphology in respect to the patterned IBs which gives a green fluorescence (Figure 2). The results obtained from the image analysis were treated in a novel, statistical way. Gathered data clearly proves that cells preferentially adhere to IB-rich areas, aligning and elongating according to the IB pattern and choosing the shortest way to reach new adhesion spots on the IBs.

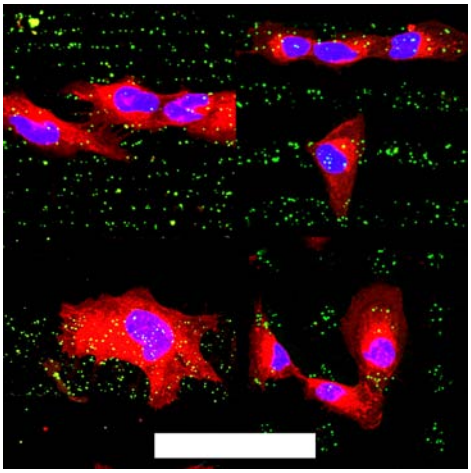

Figure 2: Confocal microscopy images of fibroblasts cultivated over IB´s-decorated supports with various patterns: stripes of 5μm, 20μm, and 50μm wide, and 20μm dots. Green – IB´s (GFP). Red – membrane (CellMask). Blue – nuclei (Hoestch). Bar length indicates 100 μm

3 CONCLUSIONS

This 2D engineering technique based on IBs fills the gap between existing techniques which are based on the local modification of the chemical nature of the surface and those based on the modification of the topography at the nanoscale level [7] by physical methods, since IBs combine at the same time biofunctionalization and topographical modification of the roughness. It has therefore proved that IBs are interesting and useful nanomaterials in the control of cell culture as well as promising biomaterials for regenerative medicine. Success of the applications of IBs as inert nanostructured materials tested so far for tissue engineering cannot be more promising. However, further multidisciplinary research is still needed to fully implement IB-based material platforms in emerging bionanotechonological applications.

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