INTRODUCTION

Recent trends in the biomaterial engineering include modifications of biomaterial surfaces with biological compounds to turn bioinert materials into bioactive ones, providing not only favorable physicochemical and mechanical properties, but also biological activity. Surface functionalization can be done with diazonium compounds, which is a fast, simple and efficient method of surface modification and it can be carried out in mild conditions. One approach is based on the functionalization of biologically active compounds [e.g., biotin (Dequaire et al., 1999), mild conditions. One approach is based on the functionalization and it can be carried out in diazonium chemistry. These groups may then react with functional diazonium groups. These groups may then react with functional groups of the compounds to be coated with (Vergnol et al., 2013; Le et al., 2014). Both electrochemically (Dequaire et al., 1999; Corgier et al., 2005; Vergnol et al., 2013) and photochemically-driven (Plewa et al., 2011) reactions of diazonium groups may be applied.

Diazonium chemistry can be used to obtain 2D and 3D cell culture surfaces and scaffolds, e.g., for photocrosslinking the ultrathin films using the layer-by-layer (LbL) technique. The surfaces were functionalized with insulin using diazonium chemistry. Such functionalized surfaces were used to culture human mesenchymal stem cells (hMSCs) to assess their suitability for bone tissue engineering and regeneration. The activity of insulin immobilized on the surfaces (DR/Pec/Ins) was compared to that of insulin dissolved in the culture medium. Human MSC grown on insulin-immobilized DR/Pec surfaces displayed increased proliferation and higher osteogenic activity. The latter was determined by means of alkaline phosphatase (ALP) activity, which increases at early stages of osteoblasts differentiation. Insulin dissolved in the culture medium did not stimulate cell proliferation and its osteogenic activity was significantly lower. Addition of recombinant human bone morphogenetic protein 2 (rhBMP-2) to the culture medium further increased ALP activity in hMSCs indicating additive osteogenic action of immobilized insulin and rhBMP-2.

Keywords: diazoresin, pectin, insulin, bone morphogenetic protein 2, alkaline phosphatase, osteogenesis, human mesenchymal stem cells, cell culture surfaces
EXPERIMENTAL

MATERIALS

Pectin (Pec) (degree of esterification 70.2%, Sigma Aldrich), 4-diazodiphenylamine sulfate (DDS, Sigma Aldrich), paraformaldehyde (POCH, Gliwice), zinc chloride (POCH Gliwice), tetraethyl orthosilicate (TEOS, Fluka) were all reagent grade and used as received. Water was distilled twice and deionized using Simplicity MilliPure Water Purification System. Insulin from bovine pancreas (Sigma-Aldrich), sodium chloride (POCH Gliwice), sodium tetraborate decahydrate (Fluka), boric acid (>99.5%, Sigma-Aldrich), Brij 35 (Sigma-Aldrich) were used as received.

CELL CULTURE REAGENTS

Unless stated otherwise, all cell culture reagents (media and sera) were purchased from Life Technologies. Bovine Serum Albumin (BSA), L-proline and sodium pyruvate used to prepare serum-free media were purchased from Sigma Aldrich. rhBMP-2 was purchased from R&D Systems (USA), dissolved as recommended by the manufacturer and used at the final concentration of 100 ng/ml.

APPARATUS

UV–Vis spectra were measured using a HP 8452A diode-array spectrophotometer. IR spectra of the irradiated DR/Pec films and of the films with photoimmobilized insulin were obtained on a Bruker IFS 48 spectrometer. Atomic force microscope (AFM) (Picoforce, Veeco, USA) working in tapping mode was used to characterize the surfaces without and with immobilized insulin in air. Standard silicon cantilevers (Veeco) with nominal spring constant 40 N/m and the tip radius <10 nm were used for all the measurements. Photocrosslinking of the DR/Pec films and photoimmobilization of insulin on their surface were carried out using Rayonet photoreactor equipped with six 8 W lamps with constant 40 N/m and the tip radius 10 nm were used for all the measurements. Photocrosslinking of the DR/Pec films and photoimmobilization of insulin on their surface were carried out using Rayonet photoreactor equipped with six 8 W lamps with
concentration of 200 mg/ml) and L-proline (final concentration of 100 mg/ml) were used in SFM instead of FBS. The experimental setup was identical as described previously for serum-containing medium. The cells were seeded and cultured in SFM starting at day 0.

**CELL VIABILITY ASSAY**

The number of viable hMSCs was estimated using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). Cells were washed once with PBS and each well of a 24-well plate was covered with 200 μl of a solution prepared as a 1:10 (v/v) dilution of 3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt (MTS) and phenazine ethosulfate in phenol red-free alpha-MEM. Cells were then incubated for 30 min at 37°C in a humidified 5% CO₂ atmosphere. Next, the media from each well were transferred to separate wells in 96-well plate and the absorbance was measured at 490 nm.

**ALKALINE PHOSPHATASE ACTIVITY ASSAY**

ALP activity was assayed with the use of Alkaline Phosphatase Assay Kit, SigmaA86C. At culture day 7 cells were assayed for ALP activity. Briefly, following MTS test, cells were rinsed 3x with PBS. Then, 200 μl of the cell digestion buffer containing Cell Assay Buffer stock solution, composed of 1.5 M Tris, 1 mM ZnCl₂, MgCl₂•6H₂O, diluted 1:10 in dH₂O and 1% of Triton X-100, was added to each well in 24-well plate and the cells were kept at 4°C overnight. The following day cells were incubated for 30 min at 37°C. The cell lysates were transferred into clean centrifuge tubes, vortexed and centrifuged. To assay for ALP activity, 900 μl of ALP substrate solution (i.e., 37.1 mg of pNPP in 20 ml of Cell Assay Buffer, prepared as described above, was combined with 100 μl of the cell lysate. Following gentle mixing and incubation for 10 min at room temperature, changes in A₄₀₅nm were measured over 6 min at 1-min intervals. The obtained values were next normalized against the number of cells obtained from the cell viability assays (MTS test), as described above.

**RESULTS AND DISCUSSION**

**IMMobilization of insulin on DR/Pec films**

The main goals of this study was to examine whether insulin can be covalently immobilized on a surface using a mild photochemical method and to verify whether such a surface would support growth and differentiation of hMSC. For this purpose DR/Pec photocrosslinkable films were prepared using a method we developed previously (Plewa et al., 2011). The films were previously shown to support the growth of hMSCs and stimulate their osteogenic response (Plewa et al., 2011).

To attach insulin to the surface of the DR/Pec cell culture surfaces they were layered on the quartz plates, immersed in insulin solutions of different concentrations (i.e., 1.2 nM–36 μM) in a buffer of pH = 7.5), and irradiated with the lamps emitting light with the maximum intensity at λ = 350 nm. This wavelength is absorbed by DR leading to its decomposition and the formation of the reactive phenyl cations, which could react with insulin molecules with the formation of the covalent bonds. The phenyl cations also react with carboxyl Pec groups which leads to the photocrosslinking of the polymeric layers forming the surface (Plewa et al., 2011). The procedure of insulin immobilization is summarized in Figure 1.

Since UV radiation is known to induce chemical and, consequently, functional changes in proteins (Kerwin and Remmele, 2007; Pattison et al., 2012), it was important to examine whether the UV radiation applied did not affect insulin activity. It has been found that irradiation of insulin at 276 nm leads to photodimerization of tyrosine groups within insulin molecules and thus to the formation of covalent insulin dimers (Correia et al., 2012). It can also lead to breaking of disulfide bridges connecting two polypeptide chains of insulin. These changes are reflected in the increase of insulin absorption around 240–285 nm and 285–320 nm (Correia et al., 2012). However, for inducing the photoreaction between DR layers in the films and insulin we used light with comparatively long wavelength, i.e., with maximum intensity at 350 nm. At this wavelength insulin absorption is negligible (insulin does not contain tryptophan (Correia et al., 2012), which absorbs the strongest and at longest wavelengths among the amino acids). Therefore, we expected insulin molecules not to be transformed by the applied radiation. Indeed, we found no changes in the UV spectra of insulin solutions (data not shown) upon irradiation in our particular experimental conditions.

To examine whether insulin was covalently attached to the surface of the irradiated films they were analyzed using spectroscopic and microscopic techniques. Figure 2 shows the FTIR spectrum of the surface irradiated in the presence or absence of insulin (DR/Pec/Ins or DR/Pec, respectively), and the spectrum of solid insulin (powder).

In the FTIR spectrum of the surface irradiated in the insulin solution (DR/Pec, Ins, c_ins = 1.8 μM) a weak band at 1654 cm⁻¹ and a strong band at 3400–3100 cm⁻¹, characteristic of insulin, are present. These bands are absent in the spectrum of the reference (DR/Pec) support, thus confirming the presence of insulin molecules on the surface of the film.

Bradford method of protein detection and quantitation was used to further confirm the presence of immobilized insulin on
FIGURE 2 | FTIR spectra for the surfaces irradiated in the presence of insulin (DR/Pec/Ins, solid line), in the absence of insulin (DR/Pec, dotted line), and the spectrum of pure solid insulin (dashed line).

FIGURE 3 | UV spectra of DR/Pec films irradiated in the absence and in the presence of different concentrations of insulin and then immersed in 0.01% w/v CBBG solution for 24 h.

We found the intensity of the 595 nm absorption band increased with increasing concentration of insulin solutions in which the films were immersed (Figure 3). This indicated increasing amounts of insulin that were immobilized on the surface of the DR/Pec surfaces.

The presence of insulin on the DR/Pec/Ins surface was also confirmed using AFM microscopy (Figure 4). The AFM images revealed that the surface of the DR/Pec film irradiated in the absence of insulin is rather smooth with the RMS roughness equal to 0.87 nm while the roughness of the film irradiated in the presence of insulin is much higher (1.74 nm). The increased roughness of this film is due to the several structures seen on the film which can be interpreted as covalently attached insulin molecules. We found several molecules of immobilized insulin per 1 μm². The contact angle of the DR/Pec/Ins surfaces ranged from 20 to 30° vs. 14° for the surfaces not functionalized with insulin. However, we found no clear dependence of the contact angle on the amount of immobilized insulin.

FIGURE 4 | AFM images of the surfaces irradiated (A) in the absence of insulin (DR/Pec) and (B) in 36 μM insulin solution (DR/Pec/Ins).

HUMAN MSC MORPHOLOGY ON THE INSULIN-FUNCTIONALIZED SURFACES (DR/Pec/Ins)

As we previously showed that DR/Pec surfaces supported growth and differentiation of hMSC, in this work we have extended these studies to examine whether insulin photochemically attached to the DR/Pec surface is able to influence hMSC proliferation and differentiation. Figure 5 shows phase-contrast microscopic images comparing the morphology of hMSC cells cultured for 24 h on the DR/Pec surfaces functionalized with different amounts of insulin and on a reference DR/Pec film.

The images reveal clear differences in the morphology of the cells grown on DR/Pec films with and without immobilized insulin. A homogeneous layer of elongated hMSC cells covered the surface of the DR/Pec film in the absence of insulin, whereas in the presence of insulin hMSC grew in scattered foci with rounded cells in the center and radially protruding elongated and adhering cells. The fraction of rounded cells increased on the film coated with a higher amount of insulin. We also observed that the presence of insulin immobilized on the surface of the supports promotes hMSC proliferation and this was further confirmed by cell viability assay.

THE EFFECT OF INSULIN ATTACHED TO THE DR/Pec FILM ON hMSC VIABILITY AND ALP ACTIVITY

EFFECT OF IMMobilIZED INSULIN ON hMSC VIABILITY

The effect of insulin immobilized on the DR/Pec/Ins surface on hMSC viability was evaluated quantitatively using MTS test. Cells viability was compared to hMSC cultures where insulin was added to the culture medium. We found that the addition of 36 μM insulin to the culture medium does not change the rate of hMSC...
proliferation (Figure 6). However, the hMSCs proliferation rate was increased by 17% on DR/Pec/Ins surfaces obtained with 36 μM solution of insulin. This suggested the insulin immobilized on the surface can be of higher availability to the cells than insulin simply dissolved in the culture medium. For the sake of comparability, both tests were performed using the same 36 μM insulin solutions.

**EFFECT OF IMMOBILIZED INSULIN ON OSTEOGENIC hMSC RESPONSE**

The osteogenic response of hMSCs grown on insulin-immobilized DR/Pec culture surfaces was assessed by measuring ALP activity in these cells. We found that insulin-immobilized DR/Pec culture surfaces supported osteogenic response of cultured hMSCs (Figure 7) much stronger than if insulin was supplied in the culture medium.

**HUMAN MSC PROLIFERATION AND DIFFERENTIATION UPON CULTURE ON INSULIN-IMMOBILIZED DR/Pec SURFACES IN THE PRESENCE OF rhBMP-2**

Earlier reports indicated that rhBMP-2 osteogenic potential can be enhanced in hMSC cultures by addition of insulin to the chemically-defined serum-free culture medium (Osyczka and Leboy, 2005). Thus, we examined whether surface-immobilized insulin supports the osteogenic effect of rhBMP-2. Since the proteins present in the serum-based medium may shield the interactions between insulin and rhBMP-2, hMSCs were cultured in the serum-free medium.

The results obtained indicated that addition of 100 ng/ml BMP-2 had no cytotoxic effect on hMSC cells cultured on the DR/Pec/Ins surface (data not shown) while it significantly increased ALP activity in hMSCs (Figure 8).

Thus, one can conclude that the osteogenic effects of insulin immobilized at the surface of DR/Pec surfaces and of rhBMP-2 added to the serum-free medium are additive.

The finding, that the osteogenic action of the DR/Pec surfaces (Plewa et al., 2011) is strengthened by the immobilized insulin (Figure 7) and further increased by the addition of rhBMP-2 (Figure 8), not only confirms previous data of additive positive action of insulin and rhBMP-2 in adult human MSC osteogenesis, but it is also very important from the practical point of view since it implies that the dose of BMP-2 required for bone regeneration may be significantly decreased by its application in combination
with DR/Pec/Ins support applied in situ in the form of, e.g., films or microspheres. This would allow limiting the high treatment costs and the adverse effects which are due to high BMP-2 doses usually applied in therapy such as osteolysis (Lewandrowski et al., 2007), immune response (Carragee et al., 2011), swelling (Shields et al., 2006), and even life-threatening complications (Carragee et al., 2011). It is suggested that the risk related to the therapeutic administration of recombinant human BMP is 10–50 times higher than previously estimated based on industry-sponsored studies (Carragee et al., 2011). That is why other systems reducing the required dose of BMP-2 have been already proposed (La et al., 2014). The data we obtained may lead to the development of well-controllable BMP delivery systems for specific bone-targeting therapies. Therefore, our further studies will aim at immobilizing both molecules and examining their osteogenic potential in adult human bone marrow cultures.

Although the molecular mechanisms of cell response to photoimmobilized or soluble insulin have not been a purpose/focus of this study, our results are in line with the earlier reports by Ito et al. who found that insulin immobilized on different supports (polystyrene, poly(methyl methacrylate), poly(oxyethylene), polyacrylic acid) stimulates the growth of mouse fibroblasts, bovine endothelial cells, and mouse sarcoma cells (Chen et al., 1997a,b; Ito et al., 1997; Li et al., 1997) and shows different or stronger effects than insulin in solution. We suppose that insulin photoimmobilized on the DR/Pec films may have a greater mitogenic activity in hMSC cultures compared to its soluble equivalent, just like in the studies described by Ito. This may be due to inhibition of insulin internalization into the cells and thus immobilized insulin is constantly available for hMSCs and stimulates their growth and differentiation for a longer time compared to its free form. This, however, needs further studies to verify the hypothesis.

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